The actions of the anthelmintics pyrantel and levamisole, at the single channel level, on somatic muscle of the nematode parasite *Ascaris suum*.

Susan J. Robertson

Dissertation submitted to the University of Edinburgh for the degree of Doctor of Philosophy. 1993
Preface:

I, hereby, declare that the experimental work is entirely my own composition and it has not previously been submitted in whole, or in part, to this or any other University. The substance of Chapter 5 and 6 have been accepted for publication, the substance of Chapter 7 has been submitted for publication.

Acknowledgements:

Firstly, I would also like to thank my parents who provided finance throughout my undergraduate degree. I would also like to thank family and friends for providing support over the last few years.

I would like to thank Dr. Richard Martin for advice and helpful discussions during the three years I worked in his lab. I would also like to thank Dr. Anne Pennington and Dr. Diane Dixon for their help and advice.

I would also like to thank Dr. John Kusel for reading the manuscript and his helpful comments.

This work was made possible by Merck Sharp & Dohme (U. S. A.), who provided financial support throughout the duration of my studies. I would finally like to thank Colin Warwick for his help with the graphic work.
Chapter 1  GENERAL INTRODUCTION

Chapter 2  ABSTRACT

Chapter 3  INTRODUCTION

3.1 Anatomy of Ascaris body muscle
  3.1.1 Structure of Ascaris muscle
    3.1.1.1 General cross section of muscle
    3.1.1.2 Belly
    3.1.1.3 Arm
    3.1.1.4 Spindle
  3.1.2 History
  3.1.3 Muscle contraction
  3.1.4 Energy required for muscle contraction
  3.1.5 Electrical activity of the somatic muscle cells
    3.1.5.1 Resting membrane potential
    3.1.5.2 Spontaneous activity of the somatic muscle cell
    3.1.5.3 Ionic mechanisms of the wave forms
  3.1.6 Conclusion

3.2 Nervous system of Ascaris suum.
  3.2.1 History
  3.2.2 Anatomical structure
  3.2.3 Reciprocal inhibition.
  3.2.4 Indirect Responses.
  3.2.5 Propagation of contractile waves.
  3.2.6 Graded responses.
  3.2.7 Passive signal transmission in Ascaris nerves.
3.2.8 Tonically active synapses
3.2.9 Physiology of motor neurones
  3.2.9.1 Excitatory motor neurones.
  3.2.9.2 Inhibitory motor neurones
3.2.10 Physiology of interneurones
3.2.11 GABA associated neurones

3.3. Neurotransmitters and Pharmacology of the neuromuscular system.
  3.3.1 Acetylcholine
  3.3.2 GABA
  3.3.3 Neuropeptides
  3.3.4 Histamine
  3.3.5 Dopamine
  3.3.6 Serotonin
  3.3.7 Receptor types located on the Nerves of Ascaris suum.
  3.3.8 Summary

3.4 ANTHELMINTICS
  3.4.1. Levamisole.
    3.4.1.1 Vertebrates
    3.4.1.2 Invertebrates
      3.4.1.2.1 Insects
      3.4.1.2.2 Nematodes
        Ascaris suum
        Caenorhabditis elegans.
        Heamorchus contortus.
        Litomosoides carinii.
        Dipetalonema viteae
    3.4.2 Pyrantel and morantel
      3.4.2.1 Vertebrates
3.4 2.2 Invertebrates

3.4.2.2.1 Insects
3.4.2.2.2 Nematodes

Ascaris suum
Caenorhabditis elegans.
Nippostrongylus brasiliensis
Angiostrongylus cantonensis
Haemonchus contortus

3.4.3. Bephenium
3.4.4. Methyridine
3.4.4.1 vertebrates
3.4.4.2 Invertebrates

3.4.5 General summary of the reported differences of the actions of anthelmintics thought to act a ACh receptors

3.4.6. Recovery

3.5 Aims of thesis

CHAPTER 4 METHODS

4.1. Collection and maintenance of the parasite Ascaris suum.

4.2. The vesicle preparation.

4.3. Solutions

4.4 Patch clamp technique
4.4.1 Patch-electrodes
4.4.2 Application of pyrantel or levamisole
4.4.3 Composition of pipette and bath solutions.
4.4.4 Bath electrodes.
4.4.5 Mechanical set-up.
4.4.6 Measuring the pipette resistance.
4.4.7 Obtaining a seal
4.4.8 Comparing the vesicle preparation with intact muscle cell
preparation.
4.4.9 Sources of background noise.

4.5 Determination of channel conductance and current / voltage
relationships.

4.6 Data processing.
4.6.1 Setting the threshold level and transition detection.
4.6.2 Measurement of rise time and dead time of the filter.
4.6.3 Definition of a burst
4.6.4 Fitting exponential components to the open, closed and burst
durations.
4.6.5 Correction for missing events
4.6.6 Nachschalag phenomenon
4.6.7 The simple Channel-block model.
4.6.8 Calculation of the blocking site within the channel pore.

4.7 Determination of the pKa of levamisole

4.8 Statistical methods
4.8.1 Mean and standard error
4.8.2 Student's T-tests
4.8.3 Linear regression
4.8.4 Exponential distribution
4.9. Drugs

CHAPTER 5 RESULTS SECTION 1: "Levamisole activated single channel currents from muscle of the nematode parasite *Ascaris suum*.

5.1 Introduction

5.2 Results

5.2.1 Channel conductance and Current / Voltage relationships
5.2.2 Exponential curve-fitting
5.2.3 Voltage-sensitivity of open- and burst-time durations
5.2.4 Probability of channel opening.
5.2.5 Evidence of Channel-Block
5.2.6 Estimation of blocked-times
5.2.7 Estimation of $k_B$
5.2.8 Determination of $K_B$
5.2.9 Burst durations
5.2.10 Desensitisation

5.3 Discussion

5.3.1 The simple block model.
5.3.2 Calculating the blocking site within the pore.
5.3.3 Comparison with acetylcholine-activated channels in *Ascaris*.
5.3.4 Comparison with previous voltage-clamp experiments in *Ascaris*
5.3.5 Possible therapeutic significance

CHAPTER 6 RESULTS SECTION 2: "Application of levamisole to the cytoplasmic surface of the muscle membrane leads to activation of nicotinic acetylcholine receptors in the nematode parasite *Ascaris suum*.

6.1 Introduction
6.2 Results

6.2.1 Application of levamisole to the cytoplasmic surface of the membrane results in an increase in channel activity.

6.2.2 Current-voltage relationships before and after bath application of levamisole.

6.2.3 High cytoplasmic concentrations of levamisole lead to desensitisation and open channel-block.

6.2.3.1 Desensitisation

6.2.3.2 Channel-block

6.2.4 Does levamisole pass through the membrane?

6.3. Discussion

CHAPTER 7 RESULTS SECTION 3: "The action of pyrantel as an agonist and an open channel blocker at nicotinic receptors in isolated Ascaris suum muscle vesicles."

7.1 Introduction

7.2 Results

7.2.1 Single-channel currents

7.2.2 Current-Voltage relationships and channel conductance

7.2.3 Exponential curve fitting

7.2.4 Open-time distributions show a degree of voltage sensitivity.

7.2.5 Burst-time distribution exhibit a degree of voltage-sensitivity.

7.2.6 Closed-time distribution

7.2.7 $P_{\text{open}}$

7.2.8 Channel-block

7.2.9 Burst durations
7.2.10 Desensitisation

7.3 Discussion

7.3.1 channel-block

7.3.2 Calculating the blocking site within the pore.

7.3.3 General comparison of currents activated by ACh, pyrantel and levamisole from Ascaris suum

CHAPTER 8 GENERAL DISCUSSION

8.1 Channel-block model

8.1.1 The simple channel-block model

8.1.2 Can the channel enter a closed state directly from the blocked state?

8.1.3 An alternative scheme for block.

8.1.4 Model used for examining data collected when ACh activated the channel.

8.1.5 Comparison of rate constants estimated using the simple channel-block model for levamisole and pyrantel with those estimated for ACh agonists.

8.2. Desensitisation

8.3 Analysis of closed-times

8.4 Future work

CHAPTER 9 REFERENCES
LIST OF FIGURES

Chapter 3

Figure 3.1 Transverse section of the nematode parasite *Ascaris suum*.

Figure 3.2 Diagram of myoneural junctions (syncytium) in transverse sections

Figure 3.3 Diagram of the muscle fibre

Figure 3.4 Spontaneous electrical activity from *Ascaris* muscle cell.

Figure 3.5 The location of the dendritic and neuromuscular output regions for each of the seven neuronal types.

Figure 3.6 Diagram of the ventral nerve cord of a female *Ascaris* showing the position, length, and handedness of motorneuron commissures.

Figure 3.7 Locations of major and minor nerve cords.

Figure 3.8 Diagram of the nervous system located in the head region of *Ascaris suum*.

Figure 3.9 Chemical structure of ACh and the anthelmintics thought to act at ACh receptors

Chapter 4

Figure 4.1 Photograph of an *Ascaris suum* nematode, a coin was placed next to the parasite to indicate size.

Figure 4.2 Illustration of the muscle flap preparation of *Ascaris suum*.

Figure 4.3 Schematic diagram of vesicle formation of the muscle vesicles and recording techniques using the cell-attached and isolated inside-out patch clamp configurations.

Figure 4.4 Photograph of the experimental set-up.

Figure 4.5 Schematic diagram showing the electrical set up of the experimental equipment.
Chapter 5

Figure 5.1 Individual channel openings recorded from an isolated inside-out patch.

*1 / V* plot of channel currents activated by *3 μM* levamisole in an isolated inside-out patch.

Figure 5.2 Levamisole (3 μM) activated channels from a cell-attached patch illustrating the voltage sensitivity of open-time durations.

Figure 5.3 Histogram of open, closed and burst-times from the experiment illustrated in figure 5.2.

Figure 5.4 Graph showing *P_{open}* versus levamisole concentration

Figure 5.5 Levamisole-(30 μM) activated channels recorded from an isolated inside-out patch. A: membrane potential -75 mV

Figure 5.6 Histogram of the open-time, burst-time and closed-time distributions from the experiment illustrated in figure 5.5.

Figure 5.7 Illustration of channel activity with 30 μM levamisole illustrating fast flickering channel-block and semi-log plot of *k_B* against membrane potential, all values are mean ± s.e..

Figure 5.8 Reciprocal plot of open-time constant T2 against concentration.

Figure 5.9 Semi-log plot of *K_B* versus membrane potential.

Figure 5.10 Channel currents showing long (seconds) closed-times between clusters of openings.

Figure 5.11 Blockage frequency plots versus concentration

Chapter 6

Figure 6.1 Channel records from an isolated inside-out patch at membrane potential of +75 mV, showing the effect of the addition of 71.4 μM levamisole to the bath solution.
Figure 6.2. The current voltage relationship for an experiment with 2 μM levamisole in the pipette (O) compared to the current-voltage relationships after the addition of 142 μM levamisole to the bath solution (O).

Figure 6.3. Channel records from an isolated inside out patch with 2 μM levamisole in the pipette solution and 807 μM levamisole in the bath solution +75 mV.

Figure 6.4 Channel records from an isolated inside-out patch with 2 μM levamisole in the pipette and 926 μM levamisole in the bath solution.

Figure 6.5 Graph showing titration of levamisole with molar NaOH. The pKa of levamisole is 8.0.

Chapter 7

Figure 7.1 Current / Voltage plots for pyrantel-activated cation channels

Figure 7.2 Illustration of a single channel opening and an all-points histogram showing distribution of channel amplitude.

Figure 7.3 Illustration of possible sub-conductance levels from channel records with 100 μM pyrantel in the patch pipette.

Figure 7.4 Pyrantel-activated channels from a cell-attached patch illustrating the voltage sensitivity of open-time durations.

Figure 7.5 Histograms of the open, burst and closed durations at ±75 mV with 0.1 μM pyrantel in the patch pipette.

Figure 7.6 Pyrantel-(100 μM) activated channels.

Figure 7.7 Semi-log plot of k_B against membrane potential.

Figure 7.8 Reciprocal plot of open-time constants T2 against concentration.

Figure 7.9 Histograms of the open, burst and closed durations at ±75 mV with 100 μM pyrantel in the patch pipette.

Figure 7.10 The plot of blockage frequency versus concentration at a membrane potential of -75 mV.
LIST OF TABLES

Chapter 3

Table 3.1 The ratio of the three forms of myosin light chains found when either saline, saline & GABA or saline & ACh are used to perfuse the muscle.

Table 3.2 Relative concentrations of ions in the muscle cell and haemolymph of *Ascaris*.

Table 3.3 Values for the factors X and Y, calculated using the constant field equation (1), when GABA, or ACh added to the saline solution.

Table 3.4 Agonist profile of ACh receptors in *Ascaris*.

Table 3.5 Antagonists at the *Ascaris* ACh receptor.

Chapter 5

Table 5.1 Conductance and reversal potentials for levamisole activated channels

Table 5.2. Open-time kinetics for levamisole activated channels.

Table 5.3. Closed time kinetics for levamisole activated channels.

Table 5.4 Burst-time kinetics for levamisole activated channels.

Table 5.5 Corrected mean open-time

Table 5.6 Corrected mean burst duration.

Table 5.7 $P_{open}$ values.

Table 5.8 The effect of membrane potential on mean block time

Chapter 6

Table 6.1 Open time kinetics for levamisole activated channels.
Table 6.2  % increase in $P_{\text{open}}$ after the addition of levamisole to the extracellular solution.

Table 6.3  Closed-time kinetics for levamisole activated channels

Chapter 7

Table 7.1 Individual values for channel conductance for pyrantel activated channels.
Table 7.2 Open-time kinetics for pyrantel activated channels.
Table 7.3 Closed time kinetics.
Table 7.4 Burst-time kinetics for pyrantel activated channels.
Table 7.5 Corrected mean open-times (ms)
Table 7.6 Corrected mean burst durations (ms)
Table 7.7 $P_{\text{open}}$ values
Table 7.8 Mean block durations (ms)

Chapter 8

Table 8.1 Comparison of values obtained for the rate constants $k_{+B}$, $k_{-B}$ and $K_B$ obtained in this study, with those obtained in other studies of block of nicotinic channels.
Abbreviations

Units:

A  Ampere
Å  Ångström
C  Coulomb
°C  degree Celsius
Hz  Hertz
J  joule
K  Kelvin
M  molar
m  metre
mol  mole
min  minute
Ω  Ohm
S  Siemens
s  second
V  Volt

Unit prefix:
k  kilo
M  mega
m  milli
μ  micro
n  nano
p  pico

Mathematical abbreviations:  log  logarithm base 10
                              ln  logarithm base e or natural logarithm
Constants:

\[ R \quad \text{gas constant} \quad 8.314 \text{ J K}^{-1} \text{ mol}^{-1} \]
\[ F \quad \text{Faraday’s constant} \quad 9.648 \times 10^4 \text{ C mol}^{-1} \]
\[ k \quad \text{Boltzmann constant} \quad 1.38 \times 10^{-23} \text{ J K}^{-1} \]
\[ N \quad \text{Avogadro constant} \quad 6.022 \times 10^{26} \text{ mol}^{-1} \]

ACh \quad \text{acetylcholine}

GABA \quad \gamma\text{-aminobutyric acid}

TEA \quad \text{Tetraethyl ammonium}

c-AMP \quad \text{cyclic adenosine-mono-phosphate}

ATP \quad \text{adenosine-tri-phosphate}

FMRFamide \quad \text{phe-met-arg-phe-amide}
Nematode parasites inflict much suffering on human and animal hosts. Approximately one in five of the world's human population is infected with intestinal nematodes (STANDEN, 1975), while infestation in domestic animals is a major source of economic loss. Anthelmintic drugs, such as levamisole and pyrantel are used to control these infestations. One such target species is the parasitic nematode *Ascaris suum*, which lives in the intestine of the pig. The adult female is about 30 cm long and 0.6 cm in diameter. The nematode has 50,000 muscle cells and 250 nerve cells. This parasite makes a suitable model for the investigation of the mode of action of anthelmintic compounds as it has large muscle cells which facilitate electrophysiological techniques (JARMAN, 1959). The thick shelled eggs, which are resistant to extreme environmental conditions, remain viable for more than four years and facilitate infection (URQUHART et al., 1987). *Ascaris* infestation of pigs can cause indigestion, diarrhoea, jaundice, obstruction of the intestines due to severe infestation, and poor growth rate. The most significant effect is economic loss due to poor feed conversion and slower weight gains which leads to an extension of the fattening period by 6-8 weeks (URQUHART et al., 1987). A close relative of *Ascaris suum* is *Ascaris lumbricoides*, one of the most common parasites of man, rare in Britain but very common in Europe and third world countries. The only way to distinguish between the two parasites is by the row of small teeth on the lips of *Ascaris suum* (LAPAGE, 1963). The nematodes although very similar are species specific and infection of man with *A. suum* or pig with *A. lumbricoides* is rare (URQUHART et al., 1987).

The anthelmintic action of compounds were tested as far back as 1926 when TOSCANO-RICO employed segments of *Ascaris*, suspended in a chamber and attached to a kymograph, to record the effects of anthelmintic drugs on rhythmic contractions exhibited by the worms. Later BALDWIN (1943) and BALDWIN & MOYLE (1947,
1949) worked on *Ascaris* strips (with intact nerve cords), initially tied off in "sausages" and later cut along the lateral line to allow the drugs direct access to the muscle cells. The use of electrophysiological techniques such as current-clamp, voltage clamp and more recently patch clamp techniques has lead to a greater understanding of the mode of action of certain anthelmintics. Studies on the nematode, *Ascaris suum*, have reported drug induced spastic paralysis of *Ascaris suum* associated with muscle depolarisation and subsequent contraction, when ACh, pyrantel or levamisole were applied to whole worms or to a flap preparation (DEL CASTILLO et al., 1963; ACEVES et al., 1970; AUBRY et al., 1970; VAN NEUTEN, 1972; COLES et al., 1975; MARTIN, 1982; HARROW & GRATION, 1985; COLQUHOUN et al., 1991). Levamisole and pyrantel are thought to act as selective agonists at ACh receptors present on the muscle cells of parasitic nematodes. Other classical anthelmintics, such as piperazine and avermectin, have been studied and their mode of action in *Ascaris* reported. It appears that receptors for ACh, GABA and possibly other neurotransmitters are a site of action of anthelmintic compounds. The aim of the present study was to investigate the action of the anthelmintics levamisole and pyrantel at the single-channel level in *Ascaris*, to determine if they activated the extrasynaptic nicotinic ACh receptors present in isolated muscle vesicles (MARTIN et al., 1990; PENNINGTON & MARTIN, 1990). The results reported in this thesis show that levamisole and pyrantel activate currents with similar properties to those activated by ACh (PENNINGTON & MARTIN, 1990) confirming that their mode of action as that of ACh agonists. In addition both compounds produced open channel-block and desensitisation.
The patch-clamp technique was used to examine single channel currents activated by the anthelmintics levamisole and pyrantel in muscle vesicles from *Ascaris suum*. Cell-attached and isolated inside-out patches were used. Levamisole (1-90 μM), applied to the extracellular surface, activated channels which had apparent mean open-times in the range 0.12-2.23 ms and linear current / voltage relationships with conductances in the range 19-46 pS. Pyrantel (0.03-100 μM) activated cation selective channels with linear current / voltage plots and mean open times in the range 0.22-4.14 ms. With pyrantel there were at least two conductance levels: main conductance 41± 2.04 pS (mean± s.e., n=28), smaller conductance 22.4 ± 0.34 pS (mean±s.e., n=8). Ion-replacement experiments for both agonists showed the channels to be cation selective. The kinetics of the channels were analysed. Generally open- and closed-time distributions were best fitted by two- and three exponentials respectively, indicating the presence of at least two open states and at least three closed states. The distributions of burst-times were best-fitted by two exponentials. Channel open- and burst-times were voltage-sensitive: at low levamisole (1-10 μM) or pyrantel concentrations (0.1-10 μM), they increased with hyperpolarisation. At higher concentrations of levamisole (30 μM & 90 μM) and pyrantel (100 μM), flickering channel-block was observed at hyperpolarised potentials. Using a simple channel-block model, values for the blocking dissociation constant, K_b were determined as: 123 μM at -50 mV, 46 μM at -75 mV and 9.4 μM at -100 mV for levamisole; and 37 μM at -50 mV and 20 μM at -75 mV for pyrantel. At the higher concentrations of levamisole (30 μM & 90 μM) and pyrantel (100 μM) long closed-times separating "clusters" of bursts were observed, at both hyperpolarised and depolarised membrane potentials and this was interpreted as desensitisation.

Although the simple channel-block model was used to describe the data, there were limitations in the use of this model: for example, the burst durations did not increase with concentration as forecast by the simple channel-block model. Limitations of this model are discussed.
It was concluded that levamisole and pyrantel act in *Ascaris suum* by opening nicotinic ACh channels but in addition they produced open channel-block.

The patch-clamp technique was also used to examine the effect of intracellular levamisole on nicotinic ACh receptors in muscle vesicle preparations from *Ascaris suum*. Initial experiments were performed where channels were activated with levamisole (2 μM) applied in the patch-pipette, isolated inside-out patches were used. In addition, levamisole (30-926 μM) was added to the cytoplasmic membrane surface via the bath solution. In 8 out of 9 experiments, addition of levamisole to the bath solution resulted in an increase in channel activity; a voltage-sensitive open channel-block and desensitisation. The open channel-block occurred at hyperpolarised potentials, an observation consistent with levamisole (a cationic substance) blocking the channel from the extracellular surface. Thus it was concluded that levamisole crossed from the cytoplasmic side of the membrane, via the lipid phase, to the extracellular surface of the patch. In the presence of high cytoplasmic concentrations of levamisole open channel-block was not observed at depolarised potentials suggesting channel asymmetry.
CHAPTER 3
INTRODUCTION

The first sections of the introduction describes the basic structure of the muscle and nervous system of the nematode Ascaris suum. Subsequent sections describe the neurotransmitters present in this nematode. The neurotransmitters and their receptor systems are of particular interest because the receptors represent target sites for anthelmintic compounds.

3.1 Anatomy of Ascaris body musculature.

3.1.1 Structure of Ascaris muscle.

3.1.1.1 General cross section of muscle

Figure 3.1 shows a transverse section of the nematode Ascaris suum. The outer layers of the nematode consist of the cuticle, the hypodermis and then the muscle layer. The musculature of Ascaris consists of entirely longitudinal muscles except for the muscles of the reproductive system (HARRIS & CROFTON, 1957). The nematode has several hypodermal intrusions into the muscle layers: the two lateral lines, and the region of hypodermis which surrounds the dorsal and ventral nerve cords.

The Ascaris muscle cells are classified as obliquely striated (ROSENBLUTH, 1963, 1965a,b, 1967). The muscle cells consist of three regions: the spindle or contractile region, the bag or belly which contains the nucleus and the arm (Figure 3.2).
Transverse section of the nematode parasite *Ascaris suum*, and schematic diagram of the muscle cell.
Diagram of myoneural junctions (syncytium) in transverse sections. The belly contains the nucleus of the cell and is continuous with the striated fibre. The arm region originates from the belly and subdivides into fine processes as it approaches the nerve. The individual axons are embedded in a trough like extension of the hypodermis which directly underlies the nematodes cuticle (ROSENBLUTH, 1965b).
3.1.1.2 Belly.

The belly region of the *Ascaris* muscle cell balloons in the body cavity or pseudocoele of the nematode and contains the nucleus of the cell (DeBell et al., 1963). Previous reports have suggested the belly's function was to aid in the movement of digestive products within the gut and that it acted as an endoskeleton (Harris & Crofton, 1957). Rosenbluth (1965b) reported that the belly was a large sac which, except for the nucleus, has no discrete internal structures. The cortical region of the belly contains fibrillar bundles of cytoskeleton and a row of small mitochondria (Rosenbluth, 1965b). The fact that the belly is filled, almost up to the plasma membrane with glycogen particles, which are depleted during starvation or exercise (Rosenbluth 1963, 1965b) led to the belief that the main function of the belly was that of a store providing glycogen when food sources are low.

3.1.1.3 Arm.

The muscle belly gives rise to the muscle arm which may originate from any part of the belly (Rosenbluth, 1965b). Each cell may have up to 5 arms (average 2.7) raising the possibility that each arm may integrate different combinations of neuronal inputs (Stretton, 1976). The arms break up into several finger like processes, which interdigitate with processes from other arms forming a cap of muscle fingers before making synaptic contact (Rosenbluth, 1965b). As the arms approach the nerve cord they become thinner and spaces between them smaller (Rosenbluth, 1965b). The arms reach the nerve cord in discrete bundles separated by gaps 400-500 μm wide (Del Castillo et al., 1989). Muscle arms do not cross the nerve cord, so innervation is exclusively dorsal or ventral. Cytoplasmic specialisation only occurs at the presynaptic or neuronal side of the myoneural junction where: giant mitochondria (Rosenbluth, 1963, 1965b); microtubules; and two sizes of vesicle (200-600 Å and 600-1200 Å in diameter) (Reger, 1965) were observed. Electron dense patches were observed in pre- and post-synaptic membrane, these patches were thought to represent structures.
responsible for presynaptic transmitter release and post synaptic receptor localisation (REGER, 1965). Mitochondria and vesicles occurred together, with vesicles clustering against the axolemma, some were found as a pockets continuous with the axolemma (ROSENBLUTH, 1965b). If the synaptic vesicles function as neurohumoral agents, then the presence of two types of vesicles raised the question of whether there are two types of transmitter substance (REGER, 1965).

The neuromuscular gap was reported to be 500 Å in width (ROSENBLUTH, 1963, 1965b), so the muscle fingers and nerves come close but don't merge as originally thought by ROHDE (1892) and GOLDSCHMIDT (1910). The nerve cord is surrounded by a trough like hypodermis with bare areas where the arm processes meet the nerve fibres (ROSENBLUTH, 1965b). Several areas of close sarcolemmal contacts between muscle cells seen at or near neuromuscular junctions (REGER, 1965) have been observed, which were thought to represent several types of tight junctions between neighbouring muscle arms (ROSENBLUTH 1963, 1965b). These junctions appear in circumscribed bands rather than "zonulae occludens", so do not function to isolate the nerve cord from the rest of the Ascaris body, instead gap junctions appear to have two functions: synchronisation of electrical activity of entire blocks of muscle cells (ionic currents to flow readily from one muscle cell to the other forming a functional syncytium (ROSENBLUTH, 1965b)); and to control muscle cells which fail to establish direct chemical synaptic connections (DEL CASTILLO et al., 1989). The syncytium consists of $10^5$ muscle cells processes coupled to each other and connected to the motor nervous system by chemical synapses. The syncytium has high electrical excitability which enables amplification of signals generated under the influence of nervous activity, making sure that they reach the muscle spindle (DEL CASTILLO et al., 1989).
3.1.1.4  Spindle.

The spindle region contains the contractile apparatus of the muscle cell. The contractile apparatus of *Ascaris* muscle in cross section is made up of U shaped fibres, attached in the middle to the hypodermis (Figure 3.3) (ROSENBLUTH, 1965a). Each fibre has two sections: the sarcoplasmic core, which occupies the hollow of the U and is continuous with the muscle belly; and the cortex or wall of the U, which contains the contractile apparatus (ROSENBLUTH, 1965a). The muscle fibres are connected to each other by means of a network of connective tissue (ROSENBLUTH, 1967).

The contractile apparatus is made up of a double array of thick and thin filaments which interdigitate with each other to give the classic A,H and I bands (Figure 3.3) (ROSENBLUTH, 1963, 1965a). However, unlike vertebrate muscle there is no Z line, instead there is a less structured dense band. This is a region containing Z bundles (sheaf like aggregates of thin filaments) and dense bodies (ROSENBLUTH, 1963, 1965a). Dense bodies can be resolved into tightly packed thin filaments and are often closely associated with the plasma membrane. Several dense bodies occur in the centre of each dense band and are surrounded by Z bundles from the adjacent I bands on both sides (ROSENBLUTH, 1965a). The Z bundles, which serve to link small groups of thin filaments in the same sacromere together in parallel and to adjacent sacromeres in series, are closely associated to the dense bodies. Dense bodies are closely associated to the plasma membrane and to its invaginations all along the length of the fibre, presumably to link the force of contraction of myofibrils to the sarcolemma (ROSENBLUTH, 1965a). In the middle of I zone, deep invaginations of the plasma membrane occur, associated with flattened cisternae and dense bodies (analogous to the dyad and triad systems of vertebrates). These invaginations, which are finger like projections of the plasma membrane, extend into the striated cortex almost to the core of the fibre. Invaginations of plasma membrane together with dyads and triads are thought to constitute low impedance patches, through which local currents are
channelled selectively (ROSENBLUTH, 1965a). The dyads and triads serve to couple the sarcoplasmic reticulum and the t-system, allowing signals to be transmitted from the plasma membrane (ROSENBLUTH, 1967). *Ascaris* muscle is distinctive in that the sarcoplasmic reticulum is virtually nonexistent, this probably correlates with the slow speed of *Ascaris* muscle (ROSENBLUTH, 1967).
Diagram of the muscle fibre. The muscle fibre can be seen as a U shaped filament. The enlarged section shows the pattern of striation in three planes. In the XZ plane the myofilaments are staggered with the result that the striations are oblique (hence the classification, obliquely striated muscle) rather than transverse. The arrangement of thick and thin filaments into I, A, and H zones is illustrated.
3.1.2 History.

The history of research into the musculature of *Ascaris* was reviewed by DE BELL (1965). A brief outline of this review is given below.

The study of the musculature of the nematode *Ascaris* dates back as far as 1808 when RUDOLPHI described all dermal and medullary substances including the lateral lines as muscle. OTTA in 1816 then suggested that the median lines (now called nerve cords) were in fact a chain of nerve ganglia. In 1821 BOJANUS and in 1824 CLOQUET incorrectly proposed that the vesicles (muscle bag) and transverse strands (arms) were vessels and not musculature. Then in 1845 DUJARDIN concluded that nematodes had no nervous system. The views of BOJANUS (1821) and CLOQUET (1824) were disputed by SIEBOLD in 1848 who classified cross strands as transverse muscles. MEISSNER (1853) doubted the existence of transverse muscle. He noted that if transverse muscles contracted, the skin of the worm should wrinkle in longitudinal folds. This was not observed, which he believed was reason to doubt the existence of these muscles. However he noted from an internal study of the anatomy of *Ascaris*, that tissue strands which occupy the position expected of transverse muscle were present. He incorrectly concluded that these transverse strands were in fact nerves which have two insertions points, one on the longitudinal muscle and one at the nerve. He did not consider that the transverse muscles could be non contractile. He thought that muscle innervation was via a peripheral nervous system with the neuromuscular junction between the muscle and the transverse fibre. We now know that the transverse fibre and the vesicle are part of the muscle cell. WEDL (1985) and WALTER (1856) incorrectly believed that transverse fibres were nerves. WEDL believed the nuclei located near the edge of the longitudinal ribbon were nuclei of ganglion cells.

It was not until 1860 with the observations of SCHNEIDER that some order was obtained. He noted that all nematodes had the same basic structure. The muscle cells
were described as having two distinct regions, a striated contractile region and a non-contractile vesicle and transverse strand region. At this time other investigators could not conceive a non-contractile muscle. In 1863 Schneider went on to describe the circumpharyngeal ring and median lines. He concluded that the ring was a nerve organ and the median lines were nerves connected to the nerve ring. Proof of this suggestion was obtained later. However, he noted that muscle fibres made connections to the nerve ring as well as the median lines (nerve cords). Thus the rule of muscle innervation was established by SCHNEIDER in 1866:

"In the nematodes, the nerves do not branch out to the muscles, instead branches of muscle cells reach the nerves."

The views of SCHNEIDER were not immediately accepted. BASTIAN (1866) agreed that the longitudinal contractile region, the vesicle and the transverse strand were all part of the same cell, but he believed the transverse strand was contractile, aiding in movement of the food in the digestive tract of the nematode. LEUCKART (1876) incorrectly rejected the idea of a non-contractile strand and suggested that the median lines consisted of a chitinous substance not nerves. JOSEPH (1882), DEINEKA (1908) and PLENK (1924) also wrongly ascribed a contractile function to the transverse strands. BUTSCHLI (1874) was not sure whether the transverse strands were nerve or muscle but observed connections between the circumpharyngeal ring and longitudinal nerves in the median lines. This was regarded as the proof needed to validate SCHNEIDER'S theory on the nematode nervous system. ROHDE (1885) made similar observations. ROHDE (1892) described the longitudinal nerves, which he thought made multiple interconnections with other nerves within the nerve cord. He also noted that when the muscle fibre made contact with the nerves, the hypodermal tissue of the median lines was missing. These contact points between nerve and musculature occurred only in certain places and not along whole width. ROHDE also noted:
"the muscle processes split up before insertion through repeated divisions into smaller branches which connect directly with the uppermost nerve fibres".

ROHDE believed that the muscle was innervated through its vesicular material and replied to BUTSCHLI's doubts on whether the transverse strands were muscle or nerve by citing the example in the protozoa Vorticella, where a stimulus is transmitted by the cell protoplasm to the contractile threads of the protozoa. ROHDE thus concluded:

"In the Ascaris the medullary substance by a similar process transmits the stimulus from the nerve fibre by it processes."

APATHY (1894) suggested another theory of muscle innervation, where the longitudinal nerve fibres entered into the transverse muscle processes with no break, through the cell to the longitudinal contractile ribbon. RHODE and APATHY exchanged bitter words on this point. The role of the fibres continued to be debated. Most views sided with ROHDE (BILEK, 1910; GOLDSCHMIDT, 1908, 1909, 1910; HIRSCHLER, 1910; CAPPE DE BAILLON, 1911; ROSKIN, 1925; MUELLER, 1929). PLENK (1924) however still regarded the fibres as neither conductive or supportive, he thought of them as contractile. Modern view is in support of RHODE.

Another matter of controversy was whether the nerve and muscle processes merged at the neuromuscular junction, BILEK (1910) thought they did not whereas GOLDSCHMIDT (1910) thought they did. It is now known that there is a gap 500 Å wide (ROSENBLUTH, 1963, 1965a). Another type of innervation was described by DIENEKA (1908): "all cytoplasmic processes...form together a kind of second muscle layer whose fibres run circularly". GOLDSCHMIDT dismissed DEINKA's interpretation
concluding that DEINEKA "has fallen pray to a quite unbelievable series of errors" and suggested that DEINEKA "interpreted his manifestly beautiful preparation erroneously owing to his curious idea that he had to find the same in Ascaris as in vertebrates".

GOLDSCHMIDT’s own interpretation followed that of ROHDE: the muscle cells coalesce with the nerve fibres in order to receive their innervation. Each muscle cell can send out a large number of processes to the nerves. The nerve fibre can be covered with processes along their whole length. In 1911, CAPPE DE BALLION named the contractile part of the cell the "Spindle", the vesicle the "belly" and the transverse strand or innervation process the "arm". The subdivisions of the arm he called "digitations". In 1963 with the aid of new technology ROSENBLUTH eventually revealed the mode of muscle innervation in nematodes.

3.1.3 Muscle contraction

Muscle contraction in Ascaris occurs by two methods. There is the classic sliding mechanism of the thick and thin filaments as well as shear. The shear involves the staggering of the thick filaments with respect to each other. The shearing movement allows both synchronous and asynchronous contraction of individual sacromeres (ROSENBLUTH, 1967). During contraction the degree of overlap of thick filaments increases and the stagger decreases. However on extension of the muscle, the thick filaments shear apart. During shear, the separation of thick filaments is controlled by connective tissues which connect to the dense bodies. Under maximum shear the connective tissues are pulled taut preventing further movement of the thick fibres (ROSENBLUTH, 1967). The difference between the relaxed and contracted muscle is that the contracted muscle can maintain tension, whereas the relaxed muscle is very plastic and loses tension rapidly after stretch. Therefore it would seem that in contracted muscle the shear and sliding mechanisms are impeded, probably through actin myosin interactions (ROSENBLUTH, 1970). The Z lines of vertebrates usually
prevent shearing, the lack of these structures in Ascaris permits the great extensibility of this muscle, which is useful in animals that have no exo- or endo-skeleton to amplify their range of muscle movements through levers. The force of the contraction is transmitted to the connective tissue and cuticle by means of the intracellular skeleton and the dense bodies (ROSENBLUTH, 1967). The cuticle, hypodermis and lateral lines which run in parallel with the muscle, are relatively inelastic and restrict the range of motion of the muscle (ROSENBLUTH, 1967).

3.1.4 Energy required for muscle contraction.

Glycogenolysis provides the principle source of substrates for ATP synthesis during non feeding in Ascaris (HARPUR, 1963; ROSENBLUTH 1965b; DONAHUE et al., 1982). During starvation glycogen from the muscle belly is metabolized (FAIRBAIRN, 1970). Studies of glycogen metabolism in whole worms and in isolated muscle tissue (DONAHUE et al., 1981a, b) showed that the enzymes glycogen synthase and glycogen phosphorylase are present in the muscle tissue. These enzymes act to regulate the synthesis and degradation of glycogen. Due to the unique muscle structure in Ascaris muscle, synthesis and mobilisation of glycogen can occur simultaneously. This allows utilisation of carbohydrates from the intestine during host feeding at the same time permitting muscle movement to maintain the position of the parasite in the gut.

Glycogen metabolism is regulated by both c-AMP dependent and c-AMP independent pathways (DONAHUE et al. 1981a, b).

Serotonin has been identified as the primary messenger which promotes a dose dependent increase in intramuscular c-AMP and subsequent activation of protein kinase in the Ascaris muscle (DONAHUE, et al. 1981b). This appears to be analogous to that described for mammalian systems.
The c-AMP independent pathway is less well understood but is thought to be regulated by calcium. Experiments in Ca free solutions showed that some divalent cations could partially substitute for calcium in the activation of the contractile system. The order of activation was Ca$^{2+} >= $Cd$^{2+} > $Sr$^{2+} > $Ba$^{2+}$ (KUNITOMO & NAGAKURA, 1974). Perfusion of the muscle with ACh (1-50 µM) promoted muscle contraction. The energy source appeared to be endogenous glycogen. Muscle contraction was correlated with increasing glycogen phosphorlyase activity and decreasing glycogen synthase activity and was inhibited by GABA (DONAHUE et al., 1982). Neither perfusion of ACh or GABA altered c-AMP levels, thus activation / inactivation of enzymes in glycogen metabolism can occur via a c-AMP independent pathway (DONAHUE et al., 1982). LEHMAN & SZENT-GYORGYI (1975) showed that regulation of muscle contraction in all systems studied is by calcium dependent mechanisms. Vertebrate skeletal muscle contraction is characterised by an actin mediated mechanism in which calcium binds to the troponin complex. Vertebrate smooth muscle contraction is thought to be regulated by calcium dependent phosphorylation of regulatory myosin light chains.

Ascaris muscle appears to have systems similar to both vertebrate smooth and skeletal muscle. LEHMAN & SZENT-GYORGYI (1975) identified actin-mediated and myosin-linked calcium dependent contractile mechanisms in the closely related human intestinal parasite Ascaris lumbricoides.

The regulation of fast twitch responses in Ascaris suum muscle appears to function via an actin-mediated, troponin-dependent contractile mechanism (DONAHUE et al., 1985). The regulatory proteins for this mechanism (tropomyosin, 3 troponin subunits, 3 myosin light chains and a calcium / calmodulin dependent myosin light chain kinase) have been identified (DONAHUE et al., 1985). MARTIN & DONAHUE (1987) indicated that Ascaris suum muscle also contained the myosin mediated contractile mechanism.
Perfusion of the muscle with ACh resulted in a dose dependent increase in contraction without significant change in the phosphorylation of the regulatory myosin light chain. In *Ascaris* the regulatory light chain (LC2) exists in three forms depending on the degree of phosphorylation. Table 3.1 below shows the ratios of the three forms found in *Ascaris* muscle perfused with saline, saline & GABA and saline & ACh (MARTIN & DONAHUE, 1987). In contrast to ACh, GABA induced a dose dependent relaxation of *Ascaris* muscle associated with a decrease in phosphorylation of the myosin regulatory light chain (MARTIN & DONAHUE, 1987). Certain anthelmintics (e.g. avermectin and piperazine and chlorpromazine) have also been shown to alter the ratios of the three types of myosin light chain in *Ascaris* muscle.

3.1.5. Electrical activity of the somatic muscle cells.

**3.1.5.1 Resting Membrane potential**

The ionic mechanisms maintaining the resting membrane potential of vertebrate smooth and skeletal muscle are different. It is therefore of interest to determine what mechanisms are involved in the obliquely striated muscle of *Ascaris*. One of the most striking features of the *Ascaris* muscle is the relative insensitivity of the membrane potential to change in the composition of extracellular potassium, & sodium (Caldwell & Ellory, 1968; Del Castillo et al., 1964c, Brading & Caldwell, 1971). The resting membrane of the *Ascaris* somatic muscle cell is around -30mV (Brading & Caldwell, 1964, 1971; De Bell et al., 1963; Del Castillo et al., 1964a, b, c; Caldwell & Ellory, 1968; Weisblatt & Ruscel, 1976; Weisblatt et al. 1976; Martin, 1980; Wann 1987; Holden-Dye et al., 1989).

The relative concentrations of sodium, potassium and chloride in the muscle cell are compared with that of the hemolymph in Table 3.2.
Table 3.1. The ratio of the three forms of myosin light chains found when either saline, saline & GABA or saline & ACh are used to prefuse the muscle.

<table>
<thead>
<tr>
<th></th>
<th>LC_2</th>
<th>LC_2P</th>
<th>LC_2PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.42</td>
<td>0.48</td>
<td>0.12</td>
</tr>
<tr>
<td>Saline &amp; GABA</td>
<td>0.82</td>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>Saline &amp; ACh</td>
<td>0.41</td>
<td>0.49</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 3.2 Relative concentrations of ions in the muscle cell and haemolymph of *Ascaris*.

<table>
<thead>
<tr>
<th></th>
<th>Na</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Cl</th>
<th>Fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascaris</em> muscle cell</td>
<td>53</td>
<td>89</td>
<td>----</td>
<td>----</td>
<td>16.5</td>
<td>----</td>
</tr>
<tr>
<td><em>Ascaris</em> haemolymph</td>
<td>129</td>
<td>24.6</td>
<td>5.9</td>
<td>5.9</td>
<td>52.7</td>
<td>51</td>
</tr>
</tbody>
</table>

Concentrations are in mM, (Hobson et al., 1952; Brading & Caldwell, 1964).
BRADING and CALDWELL (1964) found that the membrane potential was relatively insensitive to potassium and chloride changes in external medium. They suggested that some sort of stabilising mechanism occurred, possibly the relative ion permeabilities of the membrane altered. However in 1968, CALDWELL & ELLORY showed that the stability of the membrane potential in media of different ionic composition does not appear to be related to any obvious change in permeability of the membrane to these ions.

DEL CASTILLO et al. (1964c) proposed that there was a high resistance potassium battery, and a low resistance chloride battery present in the muscle cell membrane. These batteries were proposed to be shunted by a relatively large conductance to sodium ions. Sodium transport occurs across the membrane but this was found to be different from the normal sodium shunt found in cells, since the removal of external potassium or the presence of the inhibitor ouabain do not have any effect (Caldwell & Ellory, 1968). Since chloride appeared to play such an important role in the maintenance of the membrane potential of Ascaris muscle, inorganic anions were used to replace chloride in the extracellular solutions. The membrane potential decreased only when chloride was substituted by SO₄²⁻, showing instead an increase in the presence of Br⁻, NO₃⁻, or I⁻. These results suggest that these anions permeate the membrane more readily than chloride giving a permeability sequence of NO₃⁻ > I⁻ > Br⁻ > Cl⁻ > SO₄²⁻. Later a similar permeability sequence was also determined for Ascaris somatic muscle SCN⁻ > NO₃⁻ > I⁻ > Br⁻ > CLO₃⁻ > Cl⁻ (PARRI et al., 1991). The order of permeability of mammalian smooth muscle is Cl⁻ > SO₄²⁻ > Br⁻ > NO₃⁻ > I⁻. DEL CASTILLO et al. (1964c) also noted that the membrane potential of the muscle cell did not alter significantly when bathed in Ascaris perienteric fluid compared to 30% (V/V) artificial sea water. The main difference between the two solutions was the presence of organic anions (~2/3 of the extracellular anions) which can be replaced by
Cl\(^{-}\) without changing the membrane potential. This lead them to suggest that these organic anions may play a role in maintaining the polarisation of the cell.

The resting membrane potential being relatively insensitive to external concentrations of sodium, chloride and potassium defies the predictions of the Goldman constant field equation, suggesting that a large factor other than these ions are responsible for this unusual behaviour (BRADING & CALDWELL, 1971). The results obtained were interpreted in the form of the constant field equation containing additional terms for the contribution of ions and charged groups other than potassium, sodium and chloride.

\[
E = \frac{RT}{F} \log_e \left( \frac{p_K[K]_0 + p_{Na}[Na]_0 + p_{Cl}[Cl]_0 + x}{p_K[K]_1 + p_{Na}[Na]_1 + p_{Cl}[Cl]_0 + y} \right)
\]

The contributions of the additional terms (x & y) were found to be large and to outweigh the contributions of potassium, sodium and chloride. Since equation (1) takes into account the effect of these ions BRADING and CALDWELL (1971) proposed that it was unlikely that the unusual behaviour of the membrane could be ascribed to a low resistance chloride battery or a high sodium conductance as had DEL CASTILLO et.al. (1964c). They proposed instead, that the unusual properties were due to the effects of an electrogenic transport mechanism (BRADING 1965, quoted by BRADING & CALDWELL, 1971). Large fluxes of carboxylic acids were also know to occur across this membrane, some of which were larger than that of sodium, potassium and chloride fluxes (ELLORY, 1967, quoted by BRADING & CALDWELL, 1971). CALDWELL (1972, 1973) suggested that the unusual behaviour of the Ascaris muscle membrane is related to an electrogenic sodium pump in which the outward movement of sodium was not linked to the movement of another ion. WANN (1987) indicated that if an electrogenic pump was present, the pump was not ATP-dependent, because of the lack of temperature dependence on membrane potential and input conductance.
γ-aminobutyric acid (GABA) was shown to cause a reversible hyperpolarisation when applied in normal media whereas ACh induced a reversible depolarisation of the membrane (BRADING & CALDWELL, 1971). They also noted that GABA and ACh could suppress each other's effects. Nicotine also depolarised the membrane but this was irreversible (BRADING & CALDWELL, 1971). Previous experiments have suggested that the site of action of ACh and piperazine was at the syncytium and that the muscle bellies were insensitive to these compounds (DEL CASTILLO, 1963, 1964a, b). However BRADING & CALDWELL (1971) using muscle cells with most of the arms severed, observed that GABA could exert an effect on the potential of the muscle belly, suggesting the presence of receptors on the belly region of the muscle.

Using the modified Goldman equation (1) the results obtained when GABA and ACh were added, were used to calculate values of X and Y (Table 3.3.) (BRADING & CALDWELL, 1971).
Table 3.3  Values for the factors X and Y, calculated using the constant field equation (1), when GABA, or ACh added to the saline solution.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Value of X</th>
<th>Value of Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline, different Ca²⁺ Conc., low Cl⁻, high K⁺,</td>
<td>290</td>
<td>1300</td>
</tr>
<tr>
<td>Na⁺ free saline</td>
<td>45</td>
<td>224</td>
</tr>
<tr>
<td>normal saline &amp; GABA</td>
<td>55</td>
<td>245</td>
</tr>
<tr>
<td>normal saline &amp; ACh</td>
<td>141</td>
<td>260</td>
</tr>
<tr>
<td>normal saline &amp; Adrenaline</td>
<td>146</td>
<td>648</td>
</tr>
</tbody>
</table>
ELLORY (quoted BRADING & CALDWELL, 1971) reported that GABA did not affect the movement of radioactive potassium or chloride across the muscle membrane. This led BRADING & CALDWELL (1971) to suggest that the change in membrane potential induced by application of GABA, was a result in a decrease in factors X and Y, see equation (1). The effect on sodium permeability was less clear, ELLORY (quoted by BRADING & CALDWELL, 1971) found that sodium movements were virtually unaffected by GABA, whereas BRADING & CALDWELL (1971) suggested that the sodium permeability was reduced relative to the potassium and chloride permeabilities. They explained these results by suggesting that GABA blocked an electrogenic carrier mechanism. They thought GABA acted by a dual mechanism, reducing the passive movement of sodium and increasing a carrier mediated exchange diffusion of sodium. They also suggested that ACh may interact with this carrier mechanism (BRADING & CALDWELL, 1971). However it has also been reported that GABA application results in membrane hyperpolarisation associated with an increase in the permeability to Cl or K (JARMAN, 1964). GABA receptors have been identified on the muscle belly membrane (MARTIN, 1980), which when activated induce a chloride conductance. ACh receptors have also been located on the belly membrane which when activated induce a cationic conductance (MARTIN 1982). THORN & MARTIN (1987) have shown the presence of a large calcium-activated chloride channel in the muscle belly region. Recent work (Dr. D. Dixon, personal communication) suggests that this chloride channel conducts most anions and is active at the resting membrane potential of the muscle cells. Although it may function to eliminate metabolic waste products from the muscle cell, it is possible that this channel is involved in maintaining the resting potential of the cell.

It can be seen that a satisfactory mechanism to explain the unusual properties of the membrane potential of the Ascaris muscle cell have not yet been found. The role that the ACh, GABA and calcium-activated chloride channels play in maintaining resting
membrane potential has not been determined, although it appears that the calcium activated channel may have an important function.

3.1.5.2 Spontaneous activity of the somatic muscle cell.

The somatic musculature of Ascaris has at least three types of electrical activity: slow waves, spikes and modulations (Figure 3.4) (WEISBLATT & RUSSEL, 1976).

A series of simple or complex spikes superimposed on the normal resting potential of the muscle cell were originally recorded by JARMAN (1959) using large potassium chloride filled micropipettes inserted into the bag region of the Ascaris muscle cell. The active depolarisations between muscle cells in the same region were correlated, i.e. between cells in the dorsal musculature or between cells in the ventral musculature. JARMAN (1959) noted that this correlation could only be removed if the region between the muscle cells and the nerve was cut. He concluded that the connections between the muscle cells and nerves were essential for the correlation to exist. No correlation was found between depolarisations from cells in the dorsal musculature compared to those in the ventral musculature JARMAN (1959).

Later, DE BELL et al. (1963) also noted the occurrence of spontaneous depolarisations (1.5-7 per second) superimposed on the resting membrane potential of the muscle cell. They showed that each spontaneous potential consisted of a slow wave (~ 0.2 seconds in duration) with a superimposed spike potential. Although the two types of depolarisations occurred together, the phase relations between both events were far from constant. Electrical stimulation of the nerve, but not the muscle, resulted in the induction of similar depolarisations. The wave of excitation travelled at a velocity of 6cm / seconds. DE BELL et al. (1963) thus suggested, that the spontaneous potentials originated at the syncytium. The syncytium is located at the end of the muscle arms, where the presence of gap junctions allows electrical coupling between
neighbouring muscle cells (DE BELL et al., 1963). Further evidence was obtained which suggested that the spikes were initiated at the syncytium under direct control of the nervous system by DEL CASTILLO et al. (1967), who showed that direct stimulation of the syncytium altered the frequency of the spontaneous depolarisation.

WEISBLATT & RUSSEL (1976) as well as studying the spikes and slow waves, noticed a third type of activity, modulation. Modulation appeared as long lasting depolarising potentials (up to 20s) which modulated the spontaneous activity of the cell. The length of the modulation waves corresponded with the motions in intact worms. Slow waves were classified as depolarisations of 100-400 ms in duration with amplitudes of 2-20 mV; graded spikes superimposed on the slow waves had durations of 10-50 ms with amplitudes of up to 16 mV. WEISBLATT & RUSSEL (1976) confirmed the suggestions of DE BELL et al. (1963) and DEL CASTILLO et al. (1967) who indicated that the slow waves were myogenic, originating at the syncytium. They also suggested that calcium may be directly involved in the generation of the myogenic activity.
Spontaneous electrical activity from *Ascaris* muscle cell (WEISBLATT & RUSSEL (1976). A & B slow waves and spikes from two muscle cells, time scale 10 mV x 1s. C. modulating spontaneous activity with superimposed slow waves and spikes, time scale 10 mV x 12s.
3.1.5.3 Ionic mechanisms of the wave forms.

JARMAN & ELLORY (1969), WANN (1987), and WEISBLATT et al., 1976 noted that in Ascaris muscle, tetrodotoxin, lanthanum, and cobalt did not have any significant effect on the spike potentials, suggesting that the ionic mechanisms of the spike activity were more akin to smooth muscle, with calcium spikes, than to skeletal muscle, with sodium spikes. The amplitude of the spike potentials of Ascaris were shown to vary with calcium concentration and to persist in sodium-free media (WEISBLATT et al., 1976). These findings confirmed the report of JARMAN & ELLORY (1969). However, high doses of the calcium channel blockers, verapamil and cinnarizine, had no clear effect on the spikes (WANN, 1987). Variations in potassium concentration and application of TEA did not affect the spike potentials, this suggested that potassium was not involved in the termination of the spike potentials in Ascaris muscle (WEISBLATT et al., 1976). However, further evidence is required to confirm this, as TEA only blocks some types of potassium channels.

Ion replacement experiments showed that the slow waves appeared to be mediated by both calcium and sodium. Slow waves also persisted in the presence of ouabain, this suggested that they do not result from an electrogenic pump (Na/K). Application of TEA had no effect upon the amplitude or the duration of individual slow waves suggesting that a potassium conductance was not involved. The mechanism of generation and termination of slow waves is not known (WEISBLATT et al., 1976).

Under conditions where calcium influx was reduced, long duration square waves were observed. These waves resembled slow waves in their ionic dependence but differed in their sensitivity to TEA and to variation in the external potassium concentration. It was thought that square waves and modulation waves involved the same channels (WEISBLAT & RUSSEL, 1976b). The rapid rise and fall of square waves suggested the presence of voltage-sensitive "calcium" channels. A mechanism where
internal calcium accumulation regulates calcium conductance was proposed where the interval between square waves may represent pumping out of calcium (WEISBLATT et al., 1976).

Summary
Although the ionic mechanisms of the spontaneous depolarisations are not fully understood it is known that three types of spontaneous activity occurs in the muscle of Ascaris suum. These potentials appear to be myogenic in nature, with calcium playing an important role. It is thought that the myogenic modulations are associated with rhythmic contractions of the nematode.

3.1.6 Conclusion
Although there are similarities between Ascaris muscle and both vertebrate smooth and striated muscle, the Ascaris muscle appears to form a new type of muscle classified as obliquely striated. The muscle cell is structurally different from vertebrate muscle in that it is segregated into three regions (spindle, belly and arm). The most striking difference occurs in the way muscle cells send processes to the nerve cord.
3.2. Nervous system of *Ascaris suum*.

3.2.1 History

The nervous system is relatively simple and consists of 298 neurones. There are three major regions: the anterior nerve ring and associated ganglia; the longitudinal nerve cords; and the smaller caudal ganglia.

In 1816, OTTA (quoted by JOHNSON & STRETTON, 1980) claimed to show a chain of ganglia cells located in the ventral cord. We now consider these to be nuclei of somatic motor neurones. The gross anatomy of the nervous system and the presence of commissures were described in 1866 by BASTAIN and HESSE (1892) (quoted by JOHNSON & STRETTON, 1980). They concluded that the commissures had a function, coordinating the activity of the somatic muscles.

GOLDSHMIDT (1908, 1909, 1910) made major advances in the understanding of the nervous system, when he established that the nervous system was very small with about 250 neurones, reproducible from animal to animal. At the time of these investigations, techniques were not available for physiological studies, so only anatomical observations were made. GOLDSHMIDT (1908, 1909, 1910) suggested that the musculature was divided in half by the two major nerve cords (named the dorsal and the ventral nerve cord), each half was innervated exclusively by one of the major nerve cords. Later, STRETTON (1976) observed that muscle cells in the most anterior region were also innervated by the nerve ring and the anterior third of the body was also innervated by sub-dorsal and sub-ventral cords, known collectively as sub-lateral cords. GOLDSHMIDT (1908, 1909, 1910) also observed cell bodies of the dorsal and ventral axons in the circumesophageal ganglia which he thought to be motor. GOLDSCHMIDT (1908) separated neuronal cell bodies of the head into sensory
neurones, motorneurones and interneurones. He based his conclusions solely on anatomical criteria.

Further investigations were undertaken over fifty years later when GOODWIN et al. (1963) showed the presence of both inhibitory and excitatory nerve fibres in *Ascaris*. He concluded that these fibres passed from the nerve ring down the length of the *Ascaris* body. Eighty percent of the inhibitory fibres were thought to be located in the ventral nerve cord with the remaining twenty percent in the dorsal nerve cord. GOODWIN et al. (1963) also showed that stimulation of the head region sometimes led to inhibition of the spontaneous contractions of the body, as did high doses of piperazine.

STRETTON (1976) began to investigate the nervous system of *Ascaris*, which in conjunction with his co-workers led to a detailed understanding of the nervous system of this parasite.

3.2.2 Anatomical structure

Anatomically and physiologically, the motorneurones of *Ascaris* were divided into seven classes: V-1; V-2; DE1; DE2; DE3; DI and VI (Figure 3.5). The D cells innervated the dorsal musculature and the V cells the ventral musculature. E cells were thought to be excitatory while I cells were inhibitory. V-1 and V-2 were thought to be excitatory although at that time there was no direct evidence (STRETTON et al., 1978). The geometry of each neurone was simple, each neurone sent out a straight fibre with at most two branch points, some were completely unbranched. The neurones had short (1-2 mm) spines where synaptic contact was made with the muscle cells (STRETTON et al., 1978). Ventral stimulation of DE1, DE2 or DE3 neurones resulted in depolarising responses from the dorsal musculature, hence these neurones were classified as dorsal excitators (STRETTON et al., 1978). Hyperpolarising responses were recorded in the
dorsal musculature when the type DI motorneurones were stimulated ventrally, hence this neuronal type was classified as dorsal inhibitors (STRETTON et al., 1978). Ventral stimulation of VI resulted in no dorsal response. Similar experiments were performed by the stimulation of the dorsal processes of the neurones while recording from the ventral musculature. Hyperpolarising responses were produced by VI cells, hence this neurone type was classified as a ventral inhibitor. No responses were obtained from dorsal stimulation of the neurone types DE1, DE2, DE3 or DI. All these responses were abolished if the commissure of the particular neurone was cut. Depolarising responses gave rise to action potentials in muscles so the motorneurones eliciting these responses were classified as excitatory (STRETTON et al., 1978; JOHNSON & STRETTON, 1980; WALROND & STRETTON, 1985a). Stimulation of either DI or VI induced muscle hyperpolarisation, associated with a reduction of spontaneous activity, so these motorneurones were classified as inhibitory (STRETTON et al., 1978; JOHNSON & STRETTON, 1980). Direct stimulation of the V-1 and V-2 was not possible, stimulation of the entire ventral cord lead to an excitatory response in the ventral muscle. Therefore at least one of these neurone types appears to be excitatory.
The location of the dendritic and neuromuscular output regions for each of the seven neuronal types. Forked projections represent the neuromuscular output regions. Cell bodies (black circles) are in the ventral nerve cord. Thickened lines represent dendritic regions (Stretton et al., 1978; Johnson & Stretton, 1985).
The motor neurones of *Ascaris* are arranged in repeating patterns called segments. Motor neurones located in the ventral cord send out branches (called commissures) which transverse the circumference of the nematode, before joining the dorsal cord (STRETTON, 1976). In *Ascaris*, commissures are single nerve fibres buried in a layer of hypodermal tissue underlying the cuticle. The *Ascaris* nervous system was found to contain a basic repeating unit of seven fibres, six right handed commissures to every left handed commissure (Figure 3.6). Females have five copies of the repeating segment; plus a triplet of left handed commissures, which are located anterior to the first segment; and a single left handed commissure, located posterior to the fifth segment. Males are basically the same except for the four extra left handed commissures located at the posterior end (STRETTON, 1976). These nerves are thought to be excitatory and did not show the presence of GABA using immunocytochemical methods (GUASTELLA et. al., 1991).

Cell bodies were observed to be located in the ventral cord or in the head and tail ganglia. All fibres (except two dorsal nerve fibres) arose from commissures with processes in the ventral cord where their cell bodies were located (STRETTON, 1976). The repeating segments contain one copy of the motorneurons DI, DE2 and DE3, two copies of the motorneurons VI, DE1, V-1 and V-2. V-1 and V-2 have no commissures only processes in the ventral cord (STRETTON, 1976).
Diagram of the ventral nerve cord of a female *Ascaris* showing the position, length, and handedness of motoneuron commissures. A. vertical line represents the entire length of the nerve cord; horizontal lines represent the commissures. B. Anteriormost 5% of the ventral nerve cord expanded 8-fold. There are also two commissures (one left and one right) which occur posterior to the one left which follows the fifth repeating unit are not included in the diagram. Inset, diagram of the repeating segment of *Ascaris* commissures. Vertical line represents the ventral nerve cord, horizontal lines represent the commissures. circles in the nerve cord represent positions of neuronal cell bodies (O, excitatory motoneurons; ●, inhibitory motoneurons). (JOHNSON & STRETTON, 1985).
The six large intersegmental axons are confined to the ventral cord with cell bodies located in the nerve ring at the head or tail. These neurones are located deep in the nerve cord with no neuromuscular synapses. In the ventral cord, the interneurones make synapses with the dendritic regions of the DE1, DE2, DE3, V-1 and V-2 motorneurones but not onto the DI or VI cells. Their function seems to be to control the motorneurones (STRETTON et al., 1978). Thirty smaller interneurones were identified in the ventral cord. These interneurones synapse onto each other, or onto the large interneurones (STRETTON et al., 1992).

The nervous system of Ascaris was observed to contain several nerve cords (Figure 3.7). As well as the major nerve cords (dorsal and ventral), lateral nerve cords (dorsal lateral and ventral lateral) and sublateral nerve cords (Dorsal sublateral and ventral sublateral) were identified. The sublateral cords were only observed in the anterior region of the nematode and the ventral lateral cord was only found in the posterior region. Although the precise function is not known, the sublateral cords have several intrinsic fibres, some of which are thought to be motorneurones and may be involved in movement on the head region of the nematode. VI and DI motorneurones have been shown to have sublateral branches at the dorsal sublateral and ventral sublateral nerve cords respectively (JOHNSON & STRETTON, 1987).
Locations of major and minor nerve cords. Transverse section of adult 
Ascaris 
showing only the cuticle (stippled), hypodermis (black) and nerve cords. The inner cross section is from the anterior region of the animal which is narrower and is drawn inside the more thicker posterior cross section. The major ventral (V) and dorsal (D) nerve cords are located at the inner edge of medial swellings of the hypodermis. The ventral sublateral (V subL) and dorsal sublateral (D subL) nerve cords are located in small hypodermal swellings adjacent to the large hypodermal swellings known as the lateral lines. The sublateral cords are located only in the anterior region of the nematode. The ventral lateral (VL) and dorsal lateral (DL) nerve cords are located at the margins of the lateral lines. The dorsal lateral extends through the entire animal; the ventral lateral cord is only present in the posterior region. The small lateral cord located centrally at the medial edge of the lateral line is not shown on this diagram. (JOHNSON & STRETTON, 1987)
The head region of *Ascaris* (Figure 3.8) contains 162 of the 298 neurones in the animal and consisted of the nerve ring associated with loosely organised ganglia: named dorsal ganglion, ventral ganglion and lateral ganglia according to their respective positions.

The retrovesicular ganglion (RVG) is located in the ventral cord 2 mm posterior to the nerve ring. Anterior to the RVG is a triplet bundle of commissures. A larger bundle of commissures know as amphid commissures are located just posterior to the nerve ring and two smaller bundles of commissures (derid commissures) are located posterior to the lateral ganglia (ANGSTADT et al., 1989; GUASTELLA et al., 1991).

The caudal region of the nervous system consisted of 30 neuronal somata arranged in four small ganglion: preanal; two small lumbar; and a dorsal rectal (SITHIGORNGUL, 1987 quoted GUASTELLA et al., 1991).

The first recordings directly from the nerve cord (ANGSTAD et al., 1989) showed that an interneurone in the RVG exhibited spontaneous plateau potentials. They also suggested that in the head the synaptic connectivity between DE2 and DI interneurones was different than that found in posterior regions.
Diagram of the anterior ganglia in a preparation slit near the dorsal axis and opened flat. NR, nerve ring; DC, dorsal nerve cord; VC, ventral nerve cord; LLL, left lateral line; RLL, right lateral line; VG, ventral ganglion; DG, dorsal ganglion; LG, lateral ganglion; RVG, retrovesicular ganglion. There is a triplet of left ventrodorsal commissures just anterior to the RVG, and a pair of right ventrodorsal commissures just behind the VG. Two larger bundles of ventrolateral fibres, located posterior to the NR, are called the amphidial commissures (AC), and two smaller bundles of ventrolateral fibres, located at the posterior margin of the LG are the derid commissures (DeC) (SITHIGONGUL et al., 1990).

Figure 3.8
3.2.3 Reciprocal inhibition.

The inhibitory motorneurones (VI & DI) have dendrites in one nerve cord and neuromuscular synapses in the other. The dendritic regions receive their synaptic input from other motorneurones. The motorneurones DE1, DE2 & DE3 were shown to have synaptic contacts with the VI neurones and the V-1 and V-2 motorneurones were shown to have synaptic contacts with the dorsal inhibitor DI. Electrophysiological experiments confirmed the connection between the VI and DE1, DE2 & DE3. However, physiological studies are still needed to confirm the DI connections (STRETTON et al., 1978). The synapses between the DE neurones and the VI neurones are excitatory (WALROND & STRETTON, 1985b). Each DE neurone excited two VI dendrites in the neuromuscular output region of the DE motorneuron; the VI neurone which had its commissure located nearest to the DE neurone's commissure and the next anterior VI neurone (WALROND et al., 1985c). A circuit between the dorsal and ventral nerve cords was postulated which allowed reciprocal inhibition. This enabled dorsal or ventral bends in Ascaris at discrete sites along the body of the nematode (STRETTON et al., 1978; JOHNSTON & STRETTON, 1980).

3.2.4 Indirect Responses.

Responses in muscles that did not receive synaptic input from a particular neurone were also observed (WALROND et al., 1978; WALROND, 1979: quoted by JOHNSTON & STRETTON, 1980). These results were interpreted as impulses travelling via the "syncytium" rather than via neurones. The rate of transmission appeared to be slower from syncitial propagation (11 cm s\(^{-1}\)) than for neuronal propagation (21 cm s\(^{-1}\)) (JOHNSTON & STRETTON, 1980; WALROND & STRETTON, 1985b). The rates of transmission contrast with the findings of WEISBLATT & RUSSEL (1976), who concluded that fast conduction was mediated by electrical coupled muscle cells and the slower conductance was neuronal. However at the time of the WEISBLATT & RUSSEL (1976) study, details of synaptic interactions between motorneurones and interactions
between interneurones and excitatory motorneurones were not known in any great
detail. Also the method used by WEISBLATT & RUSSEL (1976), of plucking muscle
arms from the region of the nerve cord, has been shown to damage the nerve (DAVIES,
quoted by WALROND & STRETTON, 1985b). Therefore their deductions, that any
inhibition of impulses which occurred due to removal of the muscle arms from the
region over the nerve cord must be due to synctial propagation, now seems to be
inaccurate. Finally in the WEISBLATT & RUSSEL (1976) preparation, commissures
connecting the dorsal and ventral cords would have been present, which would have
served as neuronal pathways by-passing the region where the muscle cells were
plucked.

Thus muscle cells can be activated directly via chemical synapses, or indirectly via
electrical synapses (WALROND & STRETTON, 1985b).

3.2.5 Propagation of contractile waves.

Ventral activation of DE motorneurones evoked both depolarising and
hyperpolarising responses in the dorsal musculature. The inhibitory responses
recorded beyond the zones of slow and fast conductance were thought to result from
synaptic activation of the DI motorneurone (WALROND & STRETTON, 1985b). These
flanking zones of inhibition blocked the propagation of excitation from one neurone,
spreading along the whole length of the nematode (WALROND & STRETTON, 1985b).
This is necessary for the production of a waveform in which the dorsal and ventral
muscle cells are contracted in different regions of the body at the same time. Taken
together with the system of reciprocal inhibition, activation of the DE motorneurons
should produce an omega (Ω) shape in the body. This is formed by a zone of
excitation, flanked by regions of inhibition in the dorsal musculature and a
complementary zone of inhibition in the ventral musculature. These Ω's were thought
to be elementary postures of which the body waveform was composed (Walrond & Stretton, 1985b).

3.2.6 Graded responses.
Stimulation of the excitatory and inhibitory motorneurones with varying extracellular current pulses, evoked graded potentials in the muscles (Walrond et al., 1985a). The synaptic transmission between motorneurone and motorneurone was also graded (Davies & Stretton, 1989b).

3.2.7 Passive signal transmission in Ascaris nerves.
Although motorneurones transmit information over long distances they do so by passive signalling, no all or nothing spikes were observed. Studies on the commissures (which are more easily accessible) showed that the nerves had a membrane potential of -30 mV to -40 mV (Davies & Stretton, 1989a). The membrane capacitance was like that of most nerves (0.4-0.9 μF cm⁻²), however the membrane resistance was exceptionally high (61-251 kΩ cm⁻²). This allows passage of the current, along the length of the commissure with little decrement. Steady state current voltage plots were linear which indicated the presence of few voltage-sensitive channels. However strong depolarising currents produced graded "onset transients" which were reversibly blocked by Co²⁺. The blockage by Co²⁺ did not however influence the input resistance of the commissure. Davies & Stretton (1989a) therefore concluded that the propagation of signals was graded with the voltage sensitive channels having little significant amplifying effect. Higher membrane resistances were found in inhibitory fibres which appeared to compensate for the smaller diameter of these fibres, as both the excitatory and inhibitory commissures have similar space constants (Davies & Stretton, 1989a). In any nerve fibre, if a current is injected, the fraction of the current that appears at a distant site depends on how much leaks across the membrane. In neurones with action potentials, the membranes may be
quite leaky, but there are voltage-sensitive channels that act as amplifiers. In *Ascaris* this amplification mechanism is lacking, the input signal invariably decays as it propagates along the fibre, the high membrane resistance ensures that it decays relatively little with distance.

Although motoneurones in *Ascaris* do not conduct action potentials, recently extracellular recordings from the nerve cords have shown that there are a few neurones in the ventral nerve cord which propagate action potentials (DAVIES & STRETTON, 1990). These action potentials were calcium-dependent and correlated one to one with postsynaptic potentials in motoneurones.

Excitators rely on passive signalling, whereas inhibitors can also generate oscillatory potentials, which may also play a role in signalling (DAVIES & STRETTON, 1989b). The oscillatory signals were thought to generated by calcium conductances which appeared to be generated in the nerve cord process or cell body, and then spread passively over the commissural membrane with a conduction velocity of 20-25 cm s⁻¹. The potentials showed clear thresholds and K⁺ currents were thought to limit their amplitude and duration (ANGSTADT & STRETTON, 1989). The function of these slow potentials in control of motor behaviour in *Ascaris* remains to be established. However, the muscles in the output region of inhibitory neurone were observed to contract and relax in phase with the slow potentials, suggesting that their effects on muscle membrane potentials could be translated into actual movement. This idea still requires further investigation as there was a large discrepancy between the cycle length of the slow potentials (300-1400 ms) and forward locomotory waves (6-18 s) (ANGSTADT & STRETTON, 1989).
3.2.8 Tonically active synapses

Hyperpolarisation of excitatory motorneurones resulted in the hyperpolarisation of post synaptic cells (DAVIES & STRETTON, 1989b). Hyperpolarisation of inhibitory motorneurones resulted in the depolarisation of post synaptic cells (DAVIES & STRETTON, 1989b). They suggested a plausible explanation for these results was that motorneurones released neurotransmitters tonically at resting membrane potentials, and that hyperpolarisation of these motorneurones decreased this release.

3.2.9 Physiology of motorneurones

3.2.9.1 Excitatory motorneurones.

DEL CASTILLO et al. (1963) used ionophoresis of ACh on Ascaris muscle and suggested that ACh was the excitatory transmitter. Choline acetyl transferase (the enzyme which synthesizes ACh) was localised in the dorsal commissures of the excitatory motorneurones DE1, DE2 and DE3 (JOHNSON & STRETTON, 1985). JOHNSON & KASS (1978) and KASS et al. (1980) showed that the cholinergic antagonists, d-tubocurarine blocked the response to stimulation of DE1 motorneurones. These results suggested that the excitatory transmitter in Ascaris excitatory motorneurones was ACh.

3.2.9.2 Inhibitory motorneurones

DEL CASTILLO et al. (1964b) first suggested that GABA was the inhibitory transmitter in Ascaris. Subsequently, GABA specific antibodies were shown to stain the commissures of all VI and DI motorneurones (JOHNSON & STRETTON, 1987). GABA immunoreactivity was also detected in the nerve cord in the VI and DI nerve cells (GUASTELLA et al., 1991). This confirmed the work of JOHNSON & STRETTON (1987) who originally suggested that labelling of commissures could identify inhibitory motorneurones.
3.2.10 Physiology of interneurones

Interneurones mediate direct stimulation of excitatory motorneurones in the ventral cord; stimulation of the ventral cord posterior to the ventral processes of these neurones leads to indirect stimulation by the interneurones. Application of various drugs have been used to investigate the motorneurone-interneurone synapses. Avermectin Bla and curare blocked these indirect responses (KASS et al. 1980, 1984). He suggested that cholinergic synapses were present and that avermectin mimicked an inhibitory input. Piperazine and muscimol also blocked the interneurone-excitary neurone interactions (KASS et al., 1984). The actions of curare, avermectin, piperazine and muscimol were compatible with the interpretation that this pathway contained sites sensitive to ACh and GABA (KASS et al., 1984). GABA immunocytochemistry could not detect the presence of GABA in the interneurones in the ventral cord (GUASTELLA et al., 1991). GUASTELLA et al. (1991) thus suggested that, if inhibitory interneurones were present in the ventral cord they did not use GABA as a neurotransmitter. This result is relevant to the action of avermectin, which was thought to mimic the actions of GABA agonists. Speculation that the action of avermectin was due to the release of GABA from a putative GABA interneurone must be now be abandoned (GUASTELLA et al., 1991).

3.2.11 GABA associated neurones

As well as the inhibitory motorneurones GUASTELLA & STRETTON (1991) suggested that ten cephalic neurones and a single dorsal rectal ganglion neurone use GABA as a neurotransmitter.

The use of both $^3$H-GABA uptake (GUASTELLA & STRETTON, 1991) and immunocytochemistry (GUASTELLA et al., 1991) has lead to the classification of three types of GABA associated neurones in Ascaris: (1) neurones that contain endogenous GABA and possess a GABA uptake system; (2) neurones that contain endogenous GABA but either lack GABA uptake mechanisms or possess a system of low activity.
(somatic inhibitory motorneurones); (3) neurones that lack endogenous GABA but possess GABA uptake mechanisms (e.g. some excitatory cholinergic neurones in the RVG). These findings suggest that in the somatic inhibitory neurones, GABA uptake was not essential for the termination of the effects of GABA at these synapses.

The presence of GABA uptake mechanisms in cholinergic neurones would be of interest for studies of co-localisation of neurotransmitters in neurones. In *Ascaris* there are examples of neurones which showed co-localisation of different peptide-like immunoreactivities in the same cell and the anterior deirid neurones contained four peptide-like immunoreactivities as well as dopamine (*Sulston et al.*, 1975; *Sithigorgulg et al.*, 1990).
3.3. Neurotransmitters and Pharmacology of the neuromuscular system.

Preview

Two common transmitters, acetylcholine (ACh) and γ-aminobutyric acid (GABA) modulate electrical activity in the somatic muscle cells of *Ascaris suum*. There is also evidence that serotonin, histamine, dopamine and neuropeptides may play an important role. GABA is thought to be the inhibitory transmitter and ACh is thought to be the excitatory transmitter in the nervous system (section 3.2.9).

It was originally proposed (NORTON & DE BEER, 1957; DEL CASTILLO et al., 1964a) that the locations of both ACh and GABA receptors were exclusively at the neuromuscular junction. However BRADING and CALDWELL (1971) recorded both ACh and GABA responses from *Ascaris* muscle bags after they had severed the arms. MARTIN (1980, 1982) using ionophoresis, confirmed that extrasynaptic GABA and ACh receptors are located in the membrane of the bag region of *Ascaris* muscle. The physiological role of these extrasynaptic receptors is unknown. However they appear to be one of the sites of action of several anthelmintic compounds.

3.3.1 Acetylcholine

A stimulating action of ACh on *Ascaris* muscle was demonstrated by BALDWIN & MOYLE (1949) and confirmed by NORTON & DE BEER (1957) and ASH & TUCKER (1967). MELLANBY (1955) showed that ACh or a similar choline ester is a constituent of *Ascaris* tissues. BALDWIN & MOYLE (1949) incorrectly concluded from experiments, which showed that ACh induced muscle stimulation did not fall off with time, that there was little or no esterase activity. Later specific cholinesterases were identified in *Ascaris* tissue (BUEDING, 1952; LEE, 1962). NORTON & DE BEER (1957) showed that the anticholinesterase, physostigmine, enhanced the response to ACh by 50%. This was not confirmed by ONUAGULUCHI (1989) or COLQUHOUN et al. (1990) who observed that physostigmine antagonised the effect of ACh in *Ascaris*. These same authors reported that another anticholinesterase, neostigmine, augmented
the effect of ACh in *Ascaris* tissue. Neostigmine (0.3 mg ie. 1mM) was shown to shorten the length of *Ascaris* when injected into the whole worm, similar results were observed with ACh (0.1 mg ie. 1.8 μM) and levamisole (1 μg ie. 0.2 μM) (Kass et al. 1980).

Del Castillo et al. (1964a) showed that bath application of ACh induced muscle depolarisation accompanied by changes in frequency and amplitude of the spike potentials. Direct application of ACh by ionophoresis to various regions of the muscle cell indicated that ACh acted at the syncytial region but not at the belly, spindle or arm regions close to the belly. Brading & Caldwell (1971) observed that ACh induced muscle depolarisations were obtained from muscle cells which had their arm severed. They concluded that these experiments indicated the presence of extrasynaptic ACh receptors. These authors did not consider the connections from muscle cells to the sublateral nerve cords located in the lateral lines. Thus direct proof of the presence of ACh receptors on the bag membrane was not obtained until 1982 when Martin (1982), using ionophoresis applied ACh to the bag membrane. Application of ACh to *Ascaris* muscle cells induces contraction of the muscle cell associated with membrane depolarisation and an increase in cation conductance (Baldwin & Moyle, 1949; Norton & De Beer, 1957; Del Castillo et al., 1964a; Brading & Caldwell, 1971; Hayashi et al., 1980; Martin, 1982; Harrow & Gratian, 1985; Donahue et al., 1982; Martin & Donahue, 1987; Onuaguluchi, 1989).

The ACh receptor of *Ascaris* somatic muscle may be classified as nicotinic, although nicotine is less potent on this receptor than might be expected if it was acting at a true nicotinic receptor. Nicotinic agonists produce contractions which are antagonised by d-tubocurarine (Norton & De Beer, 1957; Baldwin & Moyle, 1949; Del Castillo et al., 1964a; Martin, 1982; Natoff, 1969; Hayashi et al., 1980; Rozkova et al., 1980; Colquhoun et al., 1990, 1991; Walker et al., 1991) whereas muscarinic
agonists had little effect on this preparation (NATOFF, 1969; ROZKOVA et al., 1980; COLQUHOUN et al., 1990, 1991; WALKER et al., 1991) (Table 3.4 + 3.5). Pilocarpine and muscarine have a weak hyperpolarisation effect (COLQUHOUN et al., 1991), this might indicate the presence of muscarinic receptors of lower sensitivity than the nicotinic ACh receptors.

NATOFF (1969), ROZKOVA et al., (1980), COLQUHOUN et al. (1990, 1991) and WALKER et al. (1991) have attempted to classify this receptor. The agonist profile of this receptor suggests a similarity to the vertebrate ganglionic nicotinic receptor: the ganglionic nicotinic agonists, dimethylphenylpiperazinum (DMPP) and metahydroxyphenylpropyl-trimethylammonium (HPPT), were by far the most potent agonists; but another ganglionic agonist, tetramethyl ammonium, was less potent; and some neuronal agonists, e.g. decamethonium and anatoxin, had a slight agonist activity. There are some discrepancies in the potency sequences of the agonists: NATOFF (1969) found nicotine to be the second most potent agonist with a potency between that of DMPP and ACh; COLQUHOUN et al. (1990, 1991) found that the activity of nicotine was less than both ACh and DMPP.

The actions of antagonists on this receptor make classification more complex, no single class of antagonist acts at the receptor consistently. Mecamylamine, a ganglionic antagonist, and benzoquinonium, a neuromuscular junction blocker, are the two most potent antagonists. The ganglionic blocker hexamethonium in contrast, has only a weak antagonistic activity and the neuromuscular junction blockers, decamethonium, d-tubocurarine and pancurium were not potent.

Overall, the nicotinic receptor in *Ascaris* muscle cannot readily be classified in regard to the mammalian system, although the action of ganglionic agonists supports the view that they may be similar to the vertebrate neuronal receptor rather than muscle nicotinic
receptor. The actions of two toxins, α-bungarotoxin and neosurugatoxin, which discriminate between vertebrate muscle and some neuronal nicotinic receptors (CHIAPPINELLI, 1985 quoted WALKER et al., 1991) support this view: α-bungarotoxin is a highly selective and potent antagonist of vertebrate muscle nicotinic receptors but only blocks the response in Ascaris by 50% at 1μM, neosurugatoxin on the other hand is a selective and potent antagonist of the vertebrate neuronal nicotinic receptors and blocks the extrasynaptic Ascaris nicotinic receptor with an IC50 of 0.17 μM.

It should be pointed out that the previous studies involve application of compounds to muscle flap preparations which may still contain nerves. The pharmacology of receptors on Ascaris nerves have still to be determined. The affect of compounds at the receptors located on the nerves has not been studied. It remains unclear whether this site of action will effect the results determined above to classify the muscle receptor.

The ACh receptor appears to be associated with a ligand gated cation channel. PENNINGTON & MARTIN (1990) analysed single channel currents recorded from the belly membrane of Ascaris muscle cells. They showed that ACh (1-100 μM) activated cation selective channels of two amplitudes, the larger 40-50 pS and the smaller 25-35 pS. Both channels had linear current / voltage relationships, and a mean open times in the millisecond range, independent of voltage. Analysis of the larger conductance, revealed information on the open-, closed- and burst-time kinetics of the ACh channel. At high concentrations (25-100 μM) desensitisation was thought to occur. The extrasynaptic ACh receptor appears to be one of the sites of action for the anthelmintic compounds levamisole, pyrantel and morantel (HARROW & GRATTON, 1985).
### Table 3.4: Agonist profile of ACh receptors in *Ascaris*.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>6-55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>5-55</td>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrantel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4-1</td>
<td>0.5</td>
</tr>
<tr>
<td>Moranite</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4-1</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>nicotinic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metahydroxyphenylpropyltrimethylammonium (HPTT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.5</td>
<td>9</td>
</tr>
<tr>
<td>Dimethylphenylpiperazinum (DMPP)</td>
<td>0.2-0.6</td>
<td>0.7</td>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Nicotine</td>
<td>2-38</td>
<td>35</td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Anatoxin neuronal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Tetramethyl ammonium (TMA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.5</td>
</tr>
<tr>
<td>Suberyldicholine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Trimethylammonium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.5</td>
</tr>
<tr>
<td>Decamethonium</td>
<td>73-366</td>
<td>130</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

68
<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
<th>Concentration</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinylcholine</td>
<td></td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>Pyridine-2-aldoxime methiodide (2PAM)</td>
<td></td>
<td>757-3029</td>
<td></td>
</tr>
<tr>
<td>Cytisine</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2(nitromethylene)tetrahydro 1,3-thiazine (NMTHT)</td>
<td>no effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentytrimethylammonium</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Muscarinic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>muscarone</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furterthionium</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arecoline</td>
<td>ineffective up to 1022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-furmethide</td>
<td>170</td>
<td>weak depol</td>
<td></td>
</tr>
<tr>
<td>McN-A-343</td>
<td></td>
<td>weak hyperpol.</td>
<td></td>
</tr>
<tr>
<td>Muscarine</td>
<td></td>
<td>weak hyperpol.</td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td></td>
<td>no effect</td>
<td></td>
</tr>
<tr>
<td>Bethanechol</td>
<td></td>
<td>no effect</td>
<td></td>
</tr>
<tr>
<td>Oxatremorline</td>
<td>ineffective up to 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methacholine</td>
<td>ineffective up to 511</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

69
<table>
<thead>
<tr>
<th>Drug</th>
<th>Publication</th>
<th>IC50 (μM)</th>
<th>A2 (μM)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecamylamine</td>
<td>Natoff (1969)</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ganglion &gt; neuromuscular blocker</td>
<td>Rozhkova (1980)</td>
<td>0.33</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>Histronicotoxin</td>
<td>Colquhoun <em>et al.</em> (1990,1991)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blocks nicotinic channel</td>
<td>Walker <em>et al.</em>, 1991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-methyl-lycaconitine</td>
<td>Baldwin &amp; Moyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blocks neuromuscular blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strychnine</td>
<td></td>
<td></td>
<td>1.29</td>
<td>100</td>
</tr>
<tr>
<td>Chlorisondamine</td>
<td></td>
<td>1.74</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>Ganglion blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraphenylphosphonium</td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>nicotinic channel blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinacrine</td>
<td></td>
<td></td>
<td></td>
<td>2.05</td>
</tr>
<tr>
<td>blocks nicotinic channel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Bungarotoxin</td>
<td></td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>potent neuromuscular blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Tubocurarine</td>
<td></td>
<td>2.34</td>
<td>0.44</td>
<td>3.10</td>
</tr>
<tr>
<td>neuromuscular &gt; ganglionic</td>
<td></td>
<td></td>
<td></td>
<td>5.26</td>
</tr>
<tr>
<td>Pancuronium</td>
<td></td>
<td>3.20</td>
<td></td>
<td>4.49</td>
</tr>
<tr>
<td>neuromuscular &gt;&gt; ganglion block</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethaphan</td>
<td></td>
<td>4.27</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Ganglion blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td></td>
<td></td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td></td>
<td>71</td>
<td>72</td>
<td>6.7</td>
</tr>
<tr>
<td>Muscarinic &gt; ganglionic</td>
<td></td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Decamethonium</td>
<td></td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Neuromuscular blocker</td>
<td></td>
<td></td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td></td>
<td>1.12</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>Ganglionic blocker at channel site</td>
<td></td>
<td></td>
<td></td>
<td>329</td>
</tr>
<tr>
<td>Dihydró-β-erythroidine</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>neuronal nicotinic blocker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.2 GABA

STANDEN (1955) demonstrated the gradual paralysing action of piperazine, on the parasite Ascaris suum., which was reversible if the nematode was placed in piperazine free solution. It was originally proposed that the action of piperazine was due to a curare like action at the neuromuscular junction which blocked the stimulatory action of ACh (NORTON & DE BEER, 1957). Piperazine was shown to induce flaccid paralysis in Ascaris which was antagonised by ACh (BROOME, 1961). GOODWIN et al. (1963) later suggested that the piperazine block of ACh induced contraction, was a result of piperazine acting as a mimic of the inhibitory neurotransmitter. Later still it was shown that application of GABA produced muscle hyperpolarisation, associated with an increase in Cl⁻ conductance (BRADING & CALDWELL, 1964, 1971; ASH & TUCKER, 1966,1967; MARTIN, 1980, 1982, 1987; DONAHUE, 1982; WANN, 1987; ONAGULUCHI, 1989). Piperazine like GABA causes hyperpolarisation associated with an increase in Cl⁻ conductance (ASH & TUCKER, 66; DEL CASTILLO et al., 1964a,b; MARTIN 1982). SAZ & BUEDING (1966) thought that piperazine acted by altering the permeability of muscle membrane to the ions responsible for the maintenance of the resting potential, similar to the effects of GABA. Piperazine acts as a GABA agonist but is 100 times less potent (DEL CASTILLO et al., 1964a,b; MARTIN, 1987; ONAGULUCHI, 1989). Muscimol and piperazine have been reported to induce flaccid paralysis similar to that observed with GABA, however high doses of piperazine were needed to obtain the same effect as GABA suggesting that piperazine was less potent (KASS et al., 1982). Both piperazine and GABA induce currents, under voltage clamp, with reversal potentials around that predicted by the Nernst equation for Cl⁻, confirming that these compounds open Cl⁻ channels in the membrane (MARTIN, 1982). Piperazine induced muscle hyperpolarisation and a reduction of spike discharge frequency, muscle relaxation and antagonised the effects of pyrantel (AUBRY et al., 1970). JOHNSON & STRETTON (1987) using GABA immunocytochemistry suggested that
GABA was present throughout the muscle cells and was not solely located at the region of the cell containing synapses.

Del Castillo et al. (1964a,b) only observed effects when either GABA or piperazine were ionophoretically applied to the syncytial region, suggesting that the syncytium was the sole location of GABA receptors. However Brading & Caldwell (1971) using their severed arm method, reported earlier for ACh, concluded that extrasynaptic GABA receptors were present on the muscle bag membrane. This was later confirmed by Martin (1980; 1985) using ionophoresis experiments on the bag membrane.

Single-channel recordings using the patch-clamp technique (Martin, 1985; Duittoz & Martin, 1991b) showed that both application of GABA and piperazine to muscle bag membrane activated a Cl⁻ conductance (22 pS) with linear current / voltage relationships. Two smaller subconductance levels were also observed. The mean open-time of GABA activated channels was 32 ms compared to 14 ms for piperazine activated channels. Increasing the agonist concentration increased the mean open-time as well as the frequency of channel openings. The observed difference in potency of GABA and piperazine was reflected in both the minimum concentration needed to activate the channel at the single channel level (piperazine > 200 µM, GABA > 1 µM) and the shorter mean open-time of piperazine activated channels.

The Ascaris GABA receptor, like vertebrate GABAₐ receptors, is linked to a Cl⁻ channel. Most of the vertebrate GABAₐ agonists are active at the Ascaris receptor (Holden-Dye, et al., 1988, 1989) suggesting that in terms of agonist properties, the Ascaris receptor is closely related to vertebrate GABAₐ receptors. However this is where the similarity ends: none of the classical GABAₐ antagonists are potent in Ascaris. Bicuculline, picrotoxin, securinine, pitrazepine,
tertbutylbicyclophosphothionate, dieldrin and SR95531 are either weak or inactive GABA antagonists on *Ascaris* (Holden-Dye *et al.* 1988; Colquhoun *et al.*, 1989; Duittoz & Martin, 1991a). SR95103 which is a weaker antagonist than SR95531 at the vertebrate GABA<sub>a</sub> receptor was shown to be more potent than SR95531 in *Ascaris* (Duittoz & Martin, 1991a). Duittoz & Martin (1991a) suggested that the difference in antagonist activity between compounds on the vertebrate GABA<sub>a</sub> and *Ascaris* GABA receptors was due to different accessory binding sites. These differences are of great interest in the design of new anthelmintic compounds.

### 3.3.3 Neuropeptides

In *Ascaris* a FMRFamide-like peptide was detected in the anterior nerve ring, the cephalic papillary ganglia, the lateral ganglia and the dorsal-rectal ganglion, using immunocytochemistry. Immunoreactivity was also detected along the length of the main nerve cords, and to a lesser extent in the pharyngeal nerve cords (Davenport *et al.*, 1988). The first identified peptide (AF1) was isolated and sequenced by Cowden *et al.* (1989) from the head region of *Ascaris*. This heptapeptide was shown to be a FMRFamide-like neuropeptide with the sequence Lys-Asn-Glu-Phe-Ile-Arg-Phe. A second peptide (AF2) was identified by Cowden & Stretton (1990) which has the sequence Lys-His-Glu-Tyr-Leu-Arg-Phe. Injection of either the isolated peptides or the synthesised AF1 and AF2 peptides blocked locomotory waves in *Ascaris* (Cowden *et al.*, 1989; Cowden & Stretton, 1990). Both AF1 and AF2 produce multiple effects on muscle tension including relaxation, contraction and rhythmic activity (Cowden & Stretton, 1990). Intracellular recording techniques showed AF1 exerted a dramatic effect on the electrical properties of VI and DI inhibitory motorneurones: slow oscillatory potentials (either spontaneous or electrically induced) were rapidly and reversibly abolished. The AF1 reversibly reduced the steady state input resistance of these neurones but did not affect synaptic transmission or input resistances of excitatory neurones (Cowden *et al.*, 1989). Since signal transmission in
Ascaris is passive, changes in the membrane input resistance would produce dramatic effects on the functioning of neuronal circuits. In the case of AFI even though synaptic transmission is still intact, a decrease in input resistance would reduce the space constant ($\lambda$) of the neurone, effectively removing the neurone from the locomotory circuit.

It now appears that multiple peptidergic systems, including the family of FMRFamide-like peptides, exist in Ascaris. Sithigornngul et al. (1990) using different antipeptide antisera demonstrated that twelve antisera had immunoreactivity in different subsets of neurones in Ascaris. These twelve antisera were raised against: LHRH; Aplysia peptide L11; Aplysia peptide 12B; small cardioactive peptide B; neuropeptide Y, FMRFamide; gastrin-17; cytolecystokininoctapeptide; $\alpha$-melanocyte stimulating hormone; calcitonin gene related peptide; corticotropin releasing factor; and vasoactive intestinal peptide. Little is known about their function but it is probable that further investigation will shed light on this. The understanding of neuropeptide systems in Ascaris might provide another area for the development of anthelmintic compounds.

3.3.4 Histamine

Bath application of histamine (1 mM) to muscle flap preparations of Ascaris had no effect on the membrane potential or input resistance (Martin, 1982). Onuaguluchi (1989) reported that (10 $\mu$M) failed to induce contraction in Ascaris. High concentrations of histamine have been localised in the tissues near the nerve ring and in the neuromuscular system (MiYAGAWI, 1961; Phillips et al., 1975, 1976). Piperazine was found to reduce the histamine content of Ascaris suum, but it also greatly enhanced the uptake of histamine from the incubation fluid (Phillips et al., 1976). Phillips et al. (1976) noted however that the relative content of histamine in the head and body wall increased and under these conditions they also noted, that the
paralysing action of piperazine was enhanced. Loss of motility of *Ascaris* was observed with high concentrations of histamine in the external media (PHILLIPS, 1978). This loss of mobility was inhibited by the H₂ antagonist, metiamide but was unaffected by diphenhydramine an H₁ antagonist. In muscle flap preparations histamine relaxed the muscle when the muscle tone was high (PHILLIPS, 1978). MARTIN (1982) reported that histamine had no effect on the membrane potential or input conductance of the somatic muscle. Histamine, however, inhibited the stimulating effect of ACh on this muscle, this action was unaffected by diphenhydramine but was further suppressed by metiamide (PHILLIPS, 1978). The actions of histamine on ACh muscle contraction were mimicked by the H₂ agonist 4-methyl-histamine but were unaffected by the H₁ agonist 2-methyl-histamine. These preliminary experiments suggested the presence of histamine H₂ receptor type in *Ascaris* tissue (PHILLIPS, 1978). Pharmacological experiments using more agonists and antagonists are needed, before classification should be attempted.

3.3.5 Dopamine

Some evidence for the presence of a catecholamine in parts of the *Ascaris* nervous system (nerve ring, ventral ganglia, 4 cephalic cell bodies, two deirid cell bodies and two posterior lateral cell bodies) has been shown by SULSTON et al. (1975). They also located eight neurones from the nematode *C. elegans* that use dopamine as the putative transmitter. If the nervous system of both nematodes are similar as frequently suggested (eg. WALROND et al., 1985a; ANGSHYTADEM et al., 1989) then further investigation may reveal dopamine as a neurotransmitter in *Ascaris*.

3.3.6 Serotonin

Perfusion of saturating levels of serotonin (5-HT) through isolated *Ascaris* muscle segments resulted in inactivation of glycogen synthase and activation of glycogen phosphorylase associated with a concomitant rise in cyclic AMP levels (DONAHUE
et.al., 1981c). Unlike the neurotransmitters GABA and ACh, serotonin does not induce muscle contraction or relaxation but appears to regulate the glycogen metabolism in Ascaris muscle (DONAHUE et al., 1981c). Ascaris muscle and intestinal tissues (like mammalian tissue) appears to synthesize 5-HT from tryptophan and molecular oxygen (MARTIN et al., 1988). Tryptophan is hydroxylated to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase, the enzyme aromatic l-amino acid decarboxylase then converts 5-hydroxytryptophan to 5-HT. The 5-HT metabolites and the two enzymes tryptophan hydroxylase and aromatic l-amino acid decarboxylase have been identified in muscle and intestinal tissues of Ascaris suum (CHAUDHURI et al., 1988a,b). None of the precursors or degradation products were found in the cuticle, perienteric fluid or reproductive tract, suggesting that 5-HT was probably not synthesised in these tissues (CHAUDHURI et al., 1988b). Both histofluorescence studies and indirect immunocytochemical labelling of 5-HT immunoreactive sites, indicated that the muscle, hypodermis and intestine of Ascaris contained levels of 5-HT higher than that found in the cuticle, reproductive tract or nerve (MARTIN & DONAHUE, 1989). From these results MARTIN & DONAHUE (1989) suggested that 5-HT can be absorbed in the gastrointestinal cells of A. suum and be transported by the perienteric fluid of the parasite. The intracellular 5-HT appeared to be sequestered in an iron associated serotonin binding protein localised in amorphous patches within the intestinal cells (MARTIN & DONAHUE, 1989).

Binding studies have been performed with Ascaris muscle tissue and intestinal tissue to try and identify the serotonin receptors (CHAUDHURI & DONAHUE, 1989; ALBRECHT & WALKER, 1991). The experiments of CHAUDHURI & DONAHUE (1989) revealed specific binding for $[^3H]LSD$ in the nanomolar range and specific binding for $[^3H]5-HT$ in the micromolar range. They also found that serotonin (10 μM) and various 5-HT$_2$ agonists and antagonists could inhibit the specific binding of $[^3H]LSD$. Their results suggested that the muscle and intestinal tissues had pharmacologically
distinct serotonergic binding sites; ketanserin had a high specific binding in muscle tissue but not to intestinal tissue. They following potency series for effectors on the *Ascaris* tissue was constructed: LSD[5-HT\textsubscript{1/2}, Dopamine agonist] > mianserin [5-HT\textsubscript{2 agonist}] > quizapine [5-HT\textsubscript{2 antagonist}] > 5-methoxytryptamine [5-HT\textsubscript{2 antagonist}] > 5-methoxy-N,N-dimethyltryptamine [5-HT\textsubscript{2 antagonist}] > DPAT [5-HT\textsubscript{1 antagonist}] > methoxytripiperazine [5-HT\textsubscript{1 antagonist}] > pinadol [5-HT\textsubscript{1 agonist}] > histamine = isoproteronol = epinephrine = norepinephrine = dopamine = propanolol. This series is similar to that obtained for the mammalian serotonin type two receptor, so CHAUDHURI & DONAHUE (1989) concluded that the *Ascaris* serotonin receptors were pharmacologically similar to the mammalian 5-HT\textsubscript{2} receptor. Subsequently the work of ALBRECHT & WALTER (1991) could only yield $K_D$ values in the micromolar range for the agonists, $[^3\text{H}]$mianserin, $[^3\text{H}]$haloperidol [D\textsubscript{2}] and $[^3\text{H}]$spiperone [5-HT\textsubscript{2}/D\textsubscript{2}]. They also obtained values for saturation binding with $[^3\text{H}]$LSD in the nanomolar range with a $K_D$ of 94 nM. The most potent effectors in their study were the D2/5-HT\textsubscript{2} antagonist (+) butaclamol. They also found that neither dopamine or serotonin, up to concentrations of 200 $\mu$M, could displace the specific binding of $[^3\text{H}]$LSD, $[^3\text{H}]$mianserin or $[^3\text{H}]$haloperidol. Thus they concluded that no classification of the *Ascaris* binding sites could be made according to D\textsubscript{1/2}, 5-HT\textsubscript{1/2} and H\textsubscript{1} receptors. For several reasons they concluded that the receptors they describe are clearly distinguished from those CHAUDHURI & DONAHUE had reported: (1) they could not confirm reports of saturation in the low mM range; (2) serotonin was not able to displace $[^3\text{H}]$LSD in their experiments; (3) they obtained differences in specificities for the 5-HT\textsubscript{2} antagonists mianserin, cyproheptadine and quipazine; (4) finally they also found high specificities for several D\textsubscript{2} antagonists, taken with the fact that LSD is also known to bind to dopaminergic receptors they concluded that they could not rule out dopaminergic properties.
3.3.7 Receptor types located on the Nerves of *Ascaris suum*.

Studies performed on the nerves of *Ascaris* have revealed the presence of several types of receptor. SERBERG & STRETTON (1986) suggested that muscarinic receptors may be present in *Ascaris*. Intracellular recordings from cells revealed that the synaptic output of DE1, DE2, and DE3 motorneurones was blocked by d-tubocurarine whereas the muscarinic antagonist, N-methyl scopolamine, blocked responses from DE1 and DE3 neurones. Serotonin was localised in two neurones in the pharynx in female *Ascaris* and in five neurones in the ventral cord of male *Ascaris* (STRETTON & JOHNSON, 1985) which may indicate 5-HT receptors located on *Ascaris* nerves. Glutamate and kainate were shown to depolarise the DE2 motorneurones, whereas NMDA had little effect. This suggested the presence of glutamate receptors (STRETTON, 1991).

3.3.8 Summary

In *Ascaris suum* strong evidence exists to prove that the inhibitory transmitter is GABA and excitatory transmitter is ACh. Unlike GABA and ACh, 5-HT does not cause contraction or relaxation of the muscle but appears to be involved in glycogen metabolism. A detailed pharmacological profile is available for both the somatic muscle GABA and ACh receptors but has still to be obtained for 5-HT muscle receptors. Neuropeptides also seem to play a role in physiological signalling mechanisms. Octopamine has also been shown to effect the locomotory behaviour of *Ascaris* (BUCHANAN & STRETTON, 1991). However the evidence for histamine, dopamine, octopamine and glutamate acting as neurotransmitters is still poor.
3.4 ANTHELMINTICS

The anthelmintics I am interested in are the group of compounds that act at receptors located on the muscle cell and/or the neuromuscular system. These drugs include the compounds, bephenium, methyridine, pyrantel, morantel and levamisole, which appear to act as agonists at the nicotinic ACh receptor (Figure 3.9). This next section outlines the effect of these compounds on both mammalian and invertebrate systems.

3.4.1. Levamisole

Levamisole, a potent anthelmintic used to treat nematode infestations, is the l-isomer of the compound tetramisole. Tetramisole contains a mixture of both the more active l-isomer (levamisole) and the less active d-isomer (dexamisole). Levamisole is (-)-(5)-2,3,5,6-tetrahydro-6-phenylimadaz[2,1-b]thiazole]. Following oral administration in sheep and goats, tetramisole (15 mg Kg\(^{-1}\)) gave excellent results against all stages of most nematodes except Trichuris ovis (WALLEY, 1966; FORSYTH 1966). It is rapidly absorbed from the gastrointestinal tract and has a L.D.\(_{50}\) value of around 300 mg Kg\(^{-1}\) when given orally in mice (RENOUX, 1980).

3.4.1.1 Vertebrates

Although levamisole is widely used for the treatment of nematode infestations, toxic responses such as lacrimation, salivation and muscle tremors have been reported (FORSYTH, 1966; WALLY, 1966). It has been suggested that levamisole may act on cholinergic receptors of the host. Therapeutic doses of tetramisole, levamisole have been shown to be devoid of measurable effects on the autonomous nervous system, thermoregulation, reflexes and gross behaviour in rats and mice (SYMOENS et al., 1979).
Chemical structure of ACh and the anthelmintic thought to act at ACh receptors.
Tetramisole (5-10 µg ml\(^{-1}\) ie. 21 - 24 µM) produced more pronounced irregular spontaneous contractions of guinea pig ileum preparations, which are abolished by atropine (0.1-0.2 µg ml\(^{-1}\) ie. 0.1 µM) (EYRE, 1970). The antagonistic action of atropine suggested that levamisole stimulates muscarinic receptors in the guinea pig ileum either directly or indirectly to induce contraction.

Tetramisole (2 mg Kg\(^{-1}\)) when injected i.v. induced contraction of the cat nictitating membrane and an initial fall in blood pressure followed by a more prolonged (up to 20 minutes) rise in blood pressure (EYRE, 1970). These actions were similar but more sustained than the actions of adrenaline (2-5 µg Kg\(^{-1}\)). Hexamethonium (5-10 mg Kg\(^{-1}\)) did not affect the actions of tetramisole whereas dibenamine (5 mg Kg\(^{-1}\)) inhibited the actions of tetramisole (EYRE, 1970). EYRE (1970) suggested that tetramisole may have been acting, in some way, on the adrenoreceptors and not on nicotinic receptors.

In the rat phrenic nerve diaphragm, tetramisole (1 - 100 µM) augmentes the twitch response of both directly and indirectly stimulated muscle (EYRE, 1970). At a concentration of 100 µM, tetramisole produces a partial neuromuscular block. Tetramisole (10 - 100 µM) partly relieves the partial neuromuscular block induced by d-tubocurarine (300 µ M) (EYRE, 1970).

EYRE (1970) also suggested that tetramisole (10- 100 µM) increases the twitch response in chick isolated biventer nerve muscle preparation and reverses the blocking action of d-tubocurarine (3 µM) on this preparation. Tetramisole (6 µM) also produces contraction of chick isolated semispinalis muscle preparations, which is inhibited by tubocurarine (1.5 x 10\(^7\) M) (EYRE, 1970).
In vivo experiments in rabbits show that tetramisole, at low doses (5-10 mg Kg\(^{-1}\)), induces a sharp transient fall in blood pressure, which is partially antagonised by atropine and in addition enhances neuromuscular transmission. At therapeutic doses (10-25 mg Kg\(^{-1}\)), tetramisole also causes a prolonged fall in blood pressure and enhances neuromuscular transmission. At doses higher than 30 mg Kg\(^{-1}\), sustained hypotension and apnoea together with neuromuscular block are observed (EYRE, 1970).

EYRE (1970) concluded from the above observations that tetramisole induces a neuromuscular paralysis which is non-competitive and which is accompanied by depolarisation like nicotine, succinylcholine and decamethonium.

Experiments performed on rabbit duodenum and guinea-pig ileum (VAN NEUTEN, 1972) suggested that levamisole contracted mammalian intestinal tissue as a result of ganglionic stimulation: levamisole (0.63 mg l\(^{-1}\) ie. 3.1 \(\mu\)M) contracts rabbit duodenum but does not enhance ACh induced contractions. This observation also suggested that levamisole does not appear to act as a cholinesterase inhibitor. The E.C.\(_{50}\) for contracture of the guinea-pig ileum with levamisole is 9 mg l\(^{-1}\) ie. 44.1 \(\mu\)M. This contracture is inhibited in a non-competitive manner by atropine (0.02 mg l\(^{-1}\) ie. 6.9 nM), morphine (0.4 mg l\(^{-1}\) ie. 1.4 \(\mu\)M), and hexamethonium (0.63 mg l\(^{-1}\) ie. 3.1 \(\mu\)M). Application of dexamisole (10 mg l\(^{-1}\), ie. 49 \(\mu\)M or more) to these preparations, induced relaxations which were slightly inhibited by compounds known to be \(\alpha\) blockers. This suggests that the dexamisole induced relaxations may have resulted from stimulation of adrenergic receptors (VAN NEUTEN, 1972). Thus care must be taken when interpreting experiments where tetramisole was used, since this compound contains a mixture of levamisole and dexamisole. This may explain the observation of EYRE (1970) who suggested that tetramisole has some adrenergic actions.
Both levamisole (0.63 mg l⁻¹ ie. 3.1 µM) and tetramisole (higher doses) potentiate contractions induced by sympathetic stimulation of the guinea-pig vas deferens. This potentiation is antagonised by hexamethonium which suggests that levamisole and tetramisole stimulate sympathetic ganglia (VAN NEUTEN, 1972).

Levamisole induced contractions of chick rectal caecum nerve muscle preparation are not inhibited by the application of by cholinesterases. This suggests that levamisole does not induce the release of ACh. The neuromuscular inhibition is typical of drugs (eg. succinylcholine) which produce depolarisation of the motor end-plate (VAN NEUTEN, 1972).

Thus VAN NEUTEN (1972) concluded from the experiments on various tissues (rat duodenum, guinea-pig ileum, rat stomach rabbit spleen, chick rectal caecum and vas deferens of rat or guinea-pig) that levamisole could produce a reversible ganglionic stimulation of mammalian parasympathetic and sympathetic ganglia.

ATCHISON et al. (1992) confirmed the results of EYRE (1970) using isolated rat hemidiaphragm preparations. They showed that levamisole (1-100 µM) blocked nerve evoked contractions of the rat hemidiaphragm in a concentration and time dependent manner. Unlike the experiments of EYRE (1970), ATCHISON et al. (1992) tested the affect of d-tubocurarine using direct stimulation studies. They found that levamisole did not affect twitches evoked in preparations blocked with d-tubocurarine (1 µM) after direct stimulation of the diaphragm. They also showed that the levamisole induced twitch responses were reversed on washing but not by application of physostigimine (5 µM). This confirmed the earlier report of VAN NEUTEN (1972) who suggested that levamisole did not induce a release of ACh since the levamisole induced responses were not inhibited by cholinesterases.
ATCHISON et al., (1992) also tested structural analogues of levamisole on the isolated rat hemidiaphragm preparation. They found that both 3-amino-levamisole and 3-bromo-levamisole, at concentrations between 1 & 100 μM, reduced neuromuscular transmission in a concentration and time dependent manner. The rank order of potency was: 3-bromo-levamisole > levamisole > 3-amino-levamisole. Microelectrode studies on the rat diaphragm muscle have revealed that end-plate regions are depolarised by 3-amino-levamisole (50 μM), but not by 3-bromo-levamisole (50 μM) or levamisole (50 μM) (ATCHISON et al., 1992), and that d-tubocurarine (1 μM) completely blocks the depolarising action of 3-amino-levamisole. ATCHISON et al. (1992) suggested that levamisole may act by mechanisms other than depolarising block in the rat, since pyrantel and 3-amino-levamisole but not levamisole depolarise the rat diahgragm muscle at concentrations sufficient to block phrenic nerve-elicited contractions.

Summary

Although the side effects of levamisole in vertebrates have yet to be fully explained, it appears that one effect of levamisole is that of ganglionic stimulation. This may explain the toxicity produced by high doses of levamisole.

3.4.1.2 Invertebrates

3.4.1.2.1 Insects

In the cockroach (*Periplaneta americana*), levamisole (10^{-5} to 10^{-3} M) applied in the bath solution, results in a dose dependent change in conductance and a corresponding depolarisation of the membrane of the fast coxal depressor motoneurone (PINNOCK et al., 1988). High doses (above 10^{-3} M) induced irreversible changes. Morantel, pyrantel and ACh had similar effects (PINNOCK et al., 1988). A rank order of potency of carbachol >> levamisole > pyrantel > morantel was determined. The acetylcholinesterase inhibitor, neostigmine (0.1 μM), potentiated the actions of ACh but
not levamisole (PINNOCK et al., 1988). This suggested that levamisole did not induce ACh release or was broken down by cholinesterase. The reversal potential for ACh and levamisole activated currents were always the same for any given cell (PINNOCK et al., 1988), and this suggests a similar mode of action. The response to ACh was not attenuated by threshold doses of levamisole (PINNOCK et al., 1988). The ganglionic antagonist, mecamylamine (0.1 μM), abolished the depolarising response to levamisole (100 μM) and ACh (PINNOCK et al., 1988). PINNOCK et al. (1988) thus concluded that the weak insecticidal activity of levamisole was due to its weak cholinergic activity on insect neurones.

ACh has actions on two types of cell in the snail (Helix aspersa): "H" cells, which are inhibited by ACh; and "D" cells, which are excited by ACh (HASSONI et al., 1988). Levamisole is 10 times less active on "H" cells and 100 times less active on "D" cells than ACh (HASSONI et al., 1988). The hyperpolarising action of levamisole on "H" cells was accompanied by increase in conductance. D-tubocurarine (20μM) blocks the inhibitory response of levamisole (1 mM) and ACh (100 μM) (HASSONI et al., 1988). These results are in contrast to experiments performed in Ascaris suum where levamisole is twice as potent as ACh (HARROW & GRATION, 1985). Thus the weak insecticidal activity of levamisole in insects compared to its potent effect in nematodes (HARROW & GRATION, 1985) may reflect a difference in ACh receptors between insects and nematodes.

3.4.1.2.2 Nematodes

Tetramisole has been shown to be active against adult and immature gastrointestinal and pulmonary nematodes but to have no activity against cestodes, trematodes, fungi or bacteria (THIENPONT et al., 1966).
It was first thought that the antiparasitic action of levamisole was a result of its inhibitory action on the fumarate reductase system in nematodes (Van Den Bossche & Janssen, 1968, 1969; Van Den Bossche, 1972, 1980; Pritchard, 1973; Romanowski et al., 1975; Kohler & Bachmann, 1977). Levamisole was more potent than dexamisole at inhibiting the fumarate reductase system in nematodes, a fact consistent with the more potent action of levamisole as an anthelmintic (Van Den Bossche & Janssen, 1969; Van Den Bossche, 1972). Most of the biochemical effects were observed at concentrations higher (> 10^{-3} \text{M}), or incubation periods longer than those concentrations found to induce muscle contraction (Van Den Bossche, 1972; Kohler & Bachmann, 1977) which suggests that the fumarate reductase system was not the primary site of action of levamisole. Also subsequent studies have shown that Ascaris suum and Nematospirodes dubius do not concentrate levamisole in their tissue (Verhoeven & Van Den Bossche, 1972; Coles, 1977). Wang & SAZ (1974) showed that levamisole paralysed the adult filarial nematodes Litomosoides carinii, Dipetalonema vitea and Brugia pahangi, which indicated that the fumarate reductase system was not the primary site of action since these nematodes do not use fumarate reductase for energy metabolism. In vitro, the trematodes Hymenolepis diminuata and Fasciola hepatica accumulate levamisole (Coles unpublished, quoted Coles, 1977), but as previously mentioned levamisole is ineffective against trematodes (Thienpont et al., 1966). This suggests that important differences in the acetylcholine receptors exist between nematodes and trematodes.

**Ascaris suum**

Tetramisole paralyses live Ascaris within 5 minutes of application of 100 \mu g \text{ml}^{-1} \text{ie.} 0.5 \mu M tetramisole to the external bathing media. This paralysis is reversible if the worms are placed in fresh solution containing no tetramisole (Aceves et al., 1970). The rapid effect of levamisole is consistent with the observation that rapid transcuticular uptake of levamisole in Ascaris (Verhoeven & Van Den Bossche, 1972).
Levamisole appears to have a biphasic mode of action: there is an initial contraction followed by a flaccid paralysis (ACEVES et al., 1970; VAN DEN BOSSCHE, 1972; COLES et al., 1974, 1975; DONAHUE et al., 1983).

Using a section of Ascaris muscle, ACEVES et al. (1970) showed that tetramisole (50 
\mu g \text{ ml}^{-1} \text{ ie. } 208 \mu \text{M}) initiates a sustained contracture of the muscle which is similar in amplitude to the maximal contracture induced by ACh (30 
\mu g \text{ ml}^{-1} \text{ ie. } 205 \mu \text{M}). The tetramisole induced contraction is slower in reaching the maximal amplitude than that of ACh, and continues for up to 30 minutes if the drug was not washed out of the bath solution. Relaxation after washing is slower for tetramisole (10 minutes to reach half amplitude) than for ACh (the muscle completely relaxed after 3 minutes washing).

After treatment of the preparation with tetramisole and a subsequent wash, ACEVES et al. (1970) reported that further application of either a second dose of tetramisole or ACh resulted in a 50% reduction in the response.

The initial contracture with levamisole (10 \mu g \text{ ml}^{-1} \text{ ie. } 41.5 \mu \text{M}) was not blocked by d-tubocurarine (1 mg \text{ ml}^{-1} \text{ ie. } 1.5 \mu \text{M}), which suggests that levamisole does not induce contracture by the initiation of ACh release, since at this concentration d-tubocurarine blocks the ACh response (ACEVES et al., 1970). Piperazine at concentrations (300 \mu g \text{ ml}^{-1} \text{ ie. } 3.5 \text{ mM}) known to inhibit spontaneous contractions, did not block the tetramisole induced contraction (ACEVES et al., 1970).

COLES et al. (1974,1975) tested the affect of levamisole on whole Ascaris. The Ascaris were placed horizontally in an open bath and drug solutions were injected into the body cavity. Levamisole (100 ppm \text{ ie. } 415 \mu \text{M}) induced an irreversible contraction (measured up to 30 minutes) which could not be inhibited by prior injection of atropine (20 \mu g \text{ g}^{-1} \text{ of worm}), tubocurarine (20 \mu g \text{ g}^{-1}), hexamethonium (20 \mu g \text{ g}^{-1}) or
piperazine (20 µg g⁻¹) (COLES et al., 1974, 1975). ACh (0.3 µg g⁻¹) induced a reversible contraction which was blocked by prior injection of atropine (20 µg g⁻¹), tubocurarine (20 µg g⁻¹), hexamethonium (20 µg g⁻¹) or piperazine (20 µg g⁻¹) (COLES et al., 1974, 1975). Prior injection of the ganglionic antagonists mecamylamine (5 µg g⁻¹) and pempidine (5 µg g⁻¹) blocked both the levamisole and ACh induced contractions (COLES et al., 1974, 1975). These observations suggested that levamisole acted at receptors similar to ganglionic ACh receptors in vertebrates. Although COLES et al. (1974, 1975) reported no effect of hexamethonium on levamisole contraction, VAN NEUTEN (1972) had earlier reported that hexamethonium reduced but did not prevent the increase in muscle tension produced by levamisole in muscle strip preparations, which again suggests that levamisole acts at receptors similar to ganglionic ACh receptors of vertebrates.

In the presence of hexamethonium, the levamisole induced contractions were less pronounced in whole Ascaris, whereas there was no affect on paralysis (VAN NEUTEN, 1972). VAN NEUTEN (1972) also noted that the paralysis occurred later in worms left lying in a petri dish than in those held in traction forced to use energy. He thus suggested that paralysis may be due to levamisole interfering with the metabolic energy supply. DONAHUE et al. (1983) also noted two distinct phases in response to levamisole: the contractile phase and the relaxation or flaccid paralysis phase. The contractile phase, which DONAHUE et al. (1983) reported to occur at concentrations greater than 4 µM and to reach a maximum at 20 µM, was not associated with changes in energy metabolism but was due to direct stimulation of the somatic muscle. Flaccid paralysis occurred after contraction. The time of onset of the flaccid paralysis was dependent on levamisole concentrations, and was not always observed at lower concentrations. During the relaxation phase or flaccid paralysis the enzyme glycogen synthase was activated and glycogen phosphorylase was inactivated. Concomitant with this, there was an increased synthesis of glycogen. In Ascaris
glycogen metabolism is regulated by both c-AMP dependent and c-AMP independent pathways (DONAHUE et al., 1981a,b). Serotonin has been identified as the primary messenger which promotes a dose dependent increase in intramuscular c-AMP and subsequent activation of protein kinase in the Ascaris muscle (DONAHUE et al., 1981b). Levamisole appeared to inhibit both the serotonin induced c-AMP accumulation and the activation of c-AMP dependent protein kinase (DONAHUE et al., 1983). The c-AMP independent pathway may be controlled together with muscle contraction by the neurotransmitter ACh (DONAHUE et al., 1982). DONAHUE et al. (1982) reported that ACh muscle contraction was correlated with increasing glycogen phosphorlyase activity and decreasing glycogen synthase activity.

DONAHUE et al. (1983) concluded that: inhibition of c-AMP accumulation in response to serotonin; inhibition of the glycogenolytic cascade; and the concomitant rise in glycogen synthesis, which all occurred in Ascaris during with the flaccid paralysis stage, were consistent with levamisole inhibiting the adenylate cyclase of the muscle membrane.

Application of levamisole to the muscle cell depolarised the membrane (ACEVES et al., 1970; HARROW & GRATTON, 1985) and produces an associated with an increase in membrane input conductance (HARROW & GRATTON, 1985).

ACEVES et al. (1970) noted that during the gradual depolarisation of the muscle cell when tetramisole was added to the bath solution, the frequency of the spontaneous spike potentials increased. However when the gradual depolarisation reached 10 mV, the cells were silent. ACEVES et al. (1970) tested the preparation to see if the contracture produced by tetramisole was the result of membrane depolarisation or whether the drug could induce contraction independent of membrane potential. To do this they used high K+ solutions (50 mM) instead of the normal (3 mM) solutions. Ascaris muscle preparations bathed in the 50 mM K+ solution had resting membrane potentials of muscle cells around 3 mV (DEL CASTILLO et al., 1964c; ACEVES et
Tetramisole (30 μg ml⁻¹ ie. 124 μM) produced a contracture with the same characteristics of the tetramisole induced contracture which occurred in low K⁺ solutions. ACh also produced contracture in muscle segments bathed in high K⁺ solutions but the concentrations required were 100 times larger than those needed for ACh in low K⁺ solutions (ACEVES et al., 1970). ACEVES et al. (1970) thus showed that: (1) tetramisole could contract Ascaris muscle without changing the membrane potential; and (2) depolarisation and increased spike frequency initiate the contractions with lower ACh concentrations (2 - 200 μg ml⁻¹ ie. 14 μM - 1.4 mM), while higher doses of ACh produced the permeability changes that lead to contraction independent of any membrane potential changes.

Using two microelectrode voltage-clamp and current-clamp techniques, HARROW & GRATTON (1985) suggested that levamisole acted at extrasynaptic nicotinic ACh receptors present on the somatic muscle cells. Using the current-clamp techniques they showed that levamisole increased the input conductance of the membrane, and that a plot of input conductance against concentration gave a sigmoid dose response curve with an EC₅₀ value of 20 μM. They also tested the agonists morantel, pyrantel and ACh and suggested that on Ascaris the rank order of potency was: morantel = pyrantel > levamisole > ACh. Using the voltage-clamp technique they suggested that levamisole induced currents had current / voltage relationships which were linear between 0 to -30 mV but were non linear at potentials more negative than -30 mV. HARROW & GRATTON (1985) proposed that the non linear section was due to voltage-sensitive desensitisation or channel-block. The reversal potential of the current- voltage plots indicated that the channel current was carried by cations mainly sodium but possibly potassium ions as well.
**Caenorhabditis elegans.**

*C. elegans* is a small free living nematode which is thought to have a similar muscle cell anatomy and nervous system to that of *Ascaris* (White et al., 1976; Walrond et al., 1985; Angstadt et al., 1989).

Lewis et al. (1980) attempted to classify the nematode receptor, they tested the ability of various agonists to induce contraction of preparations of cut *C. elegans*. They found that ACh (10 mM), choline (10 mM), carbachol (0.2 mM), dimethylphenylpiperazium (0.1 mM), nicotine (0.1 mM) and phenyltrimethylammonium (1 mM) all contracted the somatic muscle. Benzyltrimethylammonium (1 mM), decamethonium (10 mM) and succinylcholine (1 mM) were all weak agonists. The compounds benzyltriethylammonium (1 mM), hexamethonium (10 mM), muscarine (10 mM), methacholine (10 mM) and bethanechol (10 mM) were all ineffective. Values given in parenthesis represent the minimum effective concentration required to induce contraction. Thus Lewis et al. (1980) concluded that, based on the agonist profile, the *C. elegans* cholinergic receptor was most like that of the vertebrate nicotinic receptor. It should be noted however that concentrations required to induce contraction were very high.

Lewis et al. (1980) also tested the activity of three ganglionic nicotinic antagonist against the response induced by the agonists levamisole (1 μM), nicotine (200 μM) and carbachol (70 μM). They found that mecamylamine (0.01 mM) antagonised the contraction induced by nicotine and showed a weak antagonistic action at a concentration of 0.1 mM against carbachol and levamisole. Hexamethonium (1 mM) antagonised the contraction induced by levamisole and nicotine but is ineffective against carbachol. Trimethophan camsylate (1 mM) antagonised the affect of levamisole, carbachol and nicotine. Lewis et al. (1980) also tested the potency of three nicotinic antagonists that act at vertebrate skeletal nicotinic receptors against the responses.
induced by the agonists levamisole (1 μM), nicotine (200 μM) and carbachol (70 μM) on C. elegans. They found that d-tubocurarine (0.01 mM) acted as a weak antagonist against the contraction produced by all three compounds; decamethonium (1 mM) was ineffective against levamisole and carbachol induced contractions, but antagonised the nicotine induced responses. Finally gallamine triethiodide (1 mM) antagonised the affect of all three agonists. LEWIS et al. (1980) also tested the affects of three muscarinic antagonists against the effects of levamisole (1 μM), carbachol (70 μM) and nicotine (200 μM) on C. elegans. They found that both 3-quinudidinyl benzilate and 3-quinudidinol at concentrations up to 1 mM were ineffective against the responses of all three agonists whereas atropine (1 mM) antagonised the response to all three agonists. Thus LEWIS et al. (1980) concluded that classification of the C. elegans cholinergic receptor based on the antagonist profile was not possible. Again the concentrations required for an antagonistic affect were very high, which may put some doubt on how selective these compounds are at this nematode receptor. LEWIS et al. (1980) proposed that the inactivity of other agents tested at high concentrations on this preparation (for example honey bee venom (Apis mellifera) and snake venoms (Bungarus multicinctus, Vipera russelli, Naja Naja kaouthia and Dendroaspis polyepsis)) argued for the specificity of the cholinergic compounds obtained in their study.

Lewis et al (1980) concluded that the cholinergic receptor of C. elegans, like Ascaris suum, is most like the vertebrate ganglionic receptor. The Ascaris receptor, however showed a greater sensitivity to both agonists and antagonists, agonist concentrations required in C. elegans were some 10-100 times those needed in Ascaris. Binding studies (LEWIS et al., 1987b) confirmed the conclusions drawn from these pharmacological experiments.

Levamisole (0.5 μM), dexamisole (10 μM) and the levamisole derivative meta-amino levamisole (0.06 μM) were the most potent agonists tested on wild type strains of C.
in terms of the minimum effective concentration required for contraction (LEWIS et al., 1980). However levamisole, dexamisole and meta-amo levamisole caused a more rapid contraction which was less severe and could be blocked more easily by mecamylamine than the carbachol induced contraction (LEWIS et al., 1980). Ortha and para substitutions on the levamsiole molecule resulted in the reduction of activity of the compound whereas meta substitutions had no apparent affects (LEWIS et al., 1980). The stereospecificity of levamisole, its low effective concentration and the effects of phenyl ring substitutions suggested that levamisole acted by binding specifically to a receptor protein (LEWIS et al., 1980).

Binding studies with $[^3\text{H}]$ meta-aminolevamisole, revealed three binding components in *C. elegans*: a high and low affinity saturable binding activity, thought to result from specific binding to receptor; and a third non-levamisole receptor binding activity (LEWIS et al., 1987a). Properties characterising $[^3\text{H}]$ meta-aminolevamisole binding on *C. elegans*, including its stereospecificity and preference in binding levamisole derivatives and cholinergic agonists, were similar to what might be expected from the pharmacological affects of these drugs and support a cholinergic receptor function (LEWIS et al., 1987a). The known vertebrate antagonists, d-tubocurarine, atropine and hexamethonium, did not inhibit $[^3\text{H}]$ meta-aminolevamisole binding, however several antagonists, mecamylamine, α-bungarotoxin and venom of the cobra (*Naja Naja Kaouthia*), allosterically activated $[^3\text{H}]$ meta-aminolevamisole binding (LEWIS et al., 1987a). No synergistic effects between the snake toxin and mecamylamine on the specific $[^3\text{H}]$ meta-aminolevamisole binding components were observed which suggested that both compounds act in a similar manner (LEWIS et al., 1987a). LEWIS et al. (1987a) suggested that the physiological blocking effect of levamisole was explained, if the higher affinity of $[^3\text{H}]$ meta-aminolevamisole observed in vitro represented a physiologically inactive state of the receptor.
LEWIS et al. (1980) also noted that the esterase inhibitors eserine (0.1 mM), aldicarp (2 mM), trichlorfon (1 mM) and neostigmine (1 mM) induced contraction in wild type C. elegans after around 1 hour and killed the nematode after several hours. It is possible that levamisole induced contraction by acting as a cholinesterase inhibitor. To test this LEWIS et al. (1980) pharmacologically blocked nerve action and demonstrated that the contractile effect of levamisole was a result of a direct cholinergic agonism by levamisole on nematodes muscle receptors and not as a result of levamisole acting as a cholinesterase inhibitor on this nematode. Criteria for effective block of nerve action was that the block should eliminate the response to cholinesterase inhibitors without disturbing the response to direct agonists like carbachol (ie. block prevents ACh release but does not affect the receptors directly). Tricaine was the closest LEWIS et al. (1980) could find. Treatment with tricaine (2 mM) blocked the response to the esterase inhibitors for at least 1 hour. When cut worms were removed from the tricaine plus cholinesterase inhibitors to plain salt solutions, the worms contracted powerfully after several minutes recovery from the tricaine treatment. Contraction upon removal from the tricaine solution illustrated that the tricaine induced block was caused by lack of nervous activity and not by death of the worm or of the inaffectiveness of esterase inhibitors in the presence of tricaine. The response to similarly tricaine incubated worms to 0.2 mM carbachol, 0.1 mM levamisole or 0.1 mM ACh was still strong. LEWIS et al. (1980) suggested that the continued levamisole response with tricaine present and the failure of levamisole to potentiate cholinesterase inhibition, indicated that levamisole acts directly at cholinergic muscle receptors.

Levamisole could also act as a cholinergic antagonist (LEWIS et al., 1980), a blocking action following the initial agonism occurred with 1 mM levamisole. This appeared as an initial strong contraction due to the agonistic properties of levamisole, followed after several minutes by a continued but milder contraction due to the antagonistic
properties of levamisole. 1 mM nicotine, had a similar affect whereas 1 mM
dimethylphenylpiperazinium and 1 mM carbachol exerted only agonistic effects.

In 1974, BRENNER proposed that levamisole acted as a cholinergic agonist in C.
elegans, and proposed a method for isolation of mutant strains of C. elegans. He
isolated mutants at 5 different genetic loci which commonly conferred resistance to
levamisole. He found that these levamisole resistant strains were also resistant to the
anthelmintic pyrantel. The isolated mutants fell in to one of three categories (BRENNER,
1974; LEWIS et al., 1980): levamisole resistant uncs (uncoordinated worms),
pseudowild types and twitchers.

LEWIS et al. (1980) identified levamisole resistant strains of C. elegans, known as
uncs, which had mutations at one of seven genetic loci. These mutant strains shared
the same uncoordinated motor behaviour and were shown to respond poorly to
cholinergic agonists at all postembryonic stages. Adult mutants had a similarly mildly
uncoordinated visible phenotype and moved forward in a slowly decaying sine wave,
having lost most of the wild type ability to move sinusoidally in the anterior part of the
body. The backward movement of these mutant strains was much better than their
forward movement, but was still poorer that that of the wild type strains. Mutants at all
seven loci (as newly hatched larvae) could not move backwards at all and moved in a
forward direction in an extremely uncoordinated way. The larvae gained forward
motion similar to that found in adult mutants at the first larval stage, and gained
backward motion similar to that found in adult mutants in the mid second larval stage.
The mutants were also sensitive to hyposmotic shock and were resistant to contraction
by the esterase inhibitors aldicarp and trichlorofon (LEWIS et al., 1980). The sensitivity
to hyposmotic shock may result from cholinergic involvement in osmoregulation or
alternatively from some structural deficiency in the mutants. Both wild type and the
mutant strains of C.elegans contracted in response to the non cholinergic muscle
agonist ouabain and were paralysed in response to the neurotransmitter GABA, suggesting that mutants do not have impaired muscle contraction (LEWIS et al., 1980).

Another class of mutants were also identified, called pseudowild types, they behaved pharmacologically and genetically like mutants moderately impaired in levamisole sensitive function, i.e. it was thought that these mutants had a reduced number of levamisole receptors (LEWIS et al., 1980). Pseudowild type lev1(x22) showed relief from the levamisole induced contraction within 10 minutes after exposure to 1 mM levamisole, but remained contracted for over 90 minutes in a solution of 0.1 mM levamisole. When grown on 1 mM levamisole they have uncoordinated phenotypes of the levamisole resistant uncs, ostensibly because of the blocking action of levamisole. Grown on regular medium their movement was close to that of the wild type. In cut worm assays the pseudowild types contract in response to levamisole and cholinergic agonists, but less vigorously than the wild type. Ouabain had similar contractile effects on both the pseudowild types and the wild type. Pseudowild types treated with 1 mM levamisole recovered considerably over several hours (LEWIS et al., 1980). They had an uncoordinated phenotype similar to the putative receptor deficient mutants. LEWIS et al. (1980) proposed that partial receptor function in pseudowild types could explain why cholinergic blockade induced by levamisole or nicotine in pseudowild type and wild type, resulted in uncoordinated pseudowild types worms but only resulted in milder contraction in wild types. He suggested that levamisole initially induced cholinergic excitation, with increasing drug concentrations or prolonged exposure to levamisole a secondary cholinergic blockade occurred. In the wild type, he suggested that the absolute amount of receptor left in the agonistic state, after cholinergic block occured, would still be sufficient to induce contraction. In pseudowild types however, the absolute amount of receptor left in the agonistic state fell below the threshold for contraction, due to the reduced number of receptors in these strains, and only the blockade of the receptors could be observed which appeared as uncoordinated.
movements. Levamisole (1 mM) treated wild type C. elegans, also showed an uncoordinated phenotype similar to that of the putative receptor deficient mutants or wild type treated with mecamylamine (LEWIS et al., 1980). In the wild type complete block was never achieved, surviving worms recovered considerably and were able to live as hypercontracted, uncoordinated worms. Nicotine (10 mM) also induces a blocked phenotype after initial agonism, however, with nicotine, the wild type recovered with virtually no uncoordinated phenotype (LEWIS et al., 1980).

The third type were called twitchers and were a distinctly different sort of mutant from the pseudowild types or uncs in both visible and pharmacological phenotype (LEWIS et al., 1980). The twitchers showed sporadic muscle twitching superimposed on normal movement. They partially contracted and twitched more violently in levamisole, cholinergic agonists and ouabain. In view of their partial resistance to levamisole, cholinergic agonists and to ouabain, twitchers were thought to result from defects in the muscle contraction / relaxation cycle (LEWIS et al., 1980).

The uncoordinated motor behaviour of the levamisole resistant uncs, the resistance to levamisole and other cholinergic agonists, and the hypotonic osmotic sensitivity could be copied by exposing the wild type, for 5-10 minutes, to 0.1 M of the antagonist mecamylamine (LEWIS et al., 1980) and pseudowild types to levamisole and nicotine. This further supported the idea of some sort of cholinergic blockade occurred in the mutants.

The similarity in phenotype between levamisole blocked mutants, nicotine blocked mutants and mecamylamine blocked wild types, suggested that levamisole, nicotine and mecamylamine, as blocking agents, interacted with the same molecule (LEWIS et al., 1980).
LEWIS et al. (1980) concluded that the levamisole resistant strains lack functional muscle ACh receptors and that most of the wild type function of this molecule was not essential to the life of C.elegans. However it should be noted that in intestinal parasites, eg. *Ascaris suum*, uncoordinated motor behaviour would make it difficult, if not impossible, for the nematode to maintain its position in the intestine and would probably result in the parasite being expelled from its host.

WHITE (quoted LEWIS et al. (1980)) using laser microbeams on *C. elegans* larvae, destroyed either dorsal A motorneurones (equivalent to DE3 in *Ascaris*) or dorsal B motorneurones (equivalent to DE2 in *Ascaris*). Destroying dorsal A motorneurones created nematodes which move forward with difficulty but moved backwards easily; destroying dorsal B motorneurones created nematodes which moved forward easily but moved backwards with difficulty. This partial resemblance to the mutants isolated by BRENNER (1974) and LEWIS et al. (1980) suggested to LEWIS et al. (1980) that their mutants may result from dysfunction of the cholinergic neuromuscular junction. They proposed a two receptor hypothesis which suggested that there were two types of ACh receptors. The predominant form, present on the muscle cell, was sensitive to levamisole and nicotine and affected by levamisole resistant mutations. The other form was resistant to agonist and blocking effects of levamisole and nicotine (LEWIS et al, 1980). The existence of two types of ACh receptor would explain the residual unc motor activity and susceptibility to carbachol and esterase inhibitors (LEWIS et al, 1980).

The mutants *unc-38* and *lev-8* showed differences between the head and body responses (LEWIS et al, 1980). The head region showed more resistance to levamisole and cholinergic agonists than the body region (LEWIS et al., 1980), suggesting that the levamisole sensitive function (ie. nicotinic ACh receptors) were also regionally differentiated.
Mutants of 3 of the 7 genes associated with resistance to levamisole by *C. elegans* were deficient in saturable specific binding to [³H] meta-aminolevamisole (LEWIS et al., 1987b). Mutants of the other 4 had abnormal binding activities and appeared to lack the allosteric activation of specific binding by mecamylamine (LEWIS et al., 1987b). Mutants of the genes associated with partial resistance to levamisole had grossly normal receptor binding activities (LEWIS et al., 1987b).

*Haemonchus contortus.*

Levamisole has also been reported to inhibit the fumarate reductase system in the nematode *H. contortus* with a potency less than that of cambendazole or thiabendazole (PRITCHARD, 1973; ROMANOWSKI et al., 1975). The fumarate reductase system of thiabendazole resistant strains of *H. contortus* was inhibited by levamisole to the same degree, as the fumarate reductase system of thiabendazole susceptible strains (PRITCHARD, 1973; ROMANOWSKI et al., 1975), suggesting that thiabendazole and levamisole did not react at the same site to initiate inhibition of the fumarate reductase system.

Atchison et al. (1992) reported that levamisole (1-10 µM) stimulated an initial contraction followed by a sustained paralysis of the musculature in *H. contortus*. Levamisole increased the muscle tone of the nematode and decreased the frequency of spontaneous contractions. The structural analogues of levamisole: 3-amino-levamisole and 3-bromo-levamisole, were equipotent in mimicking the actions of levamisole on *H. contortus*. The rank order of potency was determined as levamisole = pyrantel > morantel > nicotine (Atchison et al., 1992). *H. contortus* was 10-100 times more sensitive than the rat to: levamisole; its structural analogues; pyrantel; morantel; and nicotine, and the order of potency for these compounds was different to that found in rat (Atchison et al., 1992). Atchison et al. (1992) also tested the nicotinic.
antagonists d-tubocurarine (10 μM), gallamine (100 μM), pentolinium (100 μM),
decamethonium (10 μM), hexamethonium (10 μM) and mecamylamine (100 μM) and
found that they did not block the affect of levamisole on either muscle tension or
contraction in *H. contortus*. They also tested the muscarinic antagonists muscarine (10
μM), arecoline (100 μM), pilocarpine (10 μM), and atropine (100 μM), which were
also found to have no affect on the levamisole induced response in *H. contortus*.

*ATCHISON et al.* (1992) concluded that it was no possible to classify the *H. contortus*
receptor in the same way as the mammalian cholinergic receptors.

The blocking effects of levamisole on *H. contortus* were associated with a
depolarisation of the muscle fibres. The depolarising actions were not significantly
tested a wide range of ligands specific for other neurotransmitters receptors, including
those for GABA, glutamate, serotonin, several catecholamines and neuropeptides and
found that the response to levamisole in *H. contortus* was unique to nicotinic agonists.

*ATCHISON et al.* (1992) further highlighted the pharmacological difference between
mammalian and nematode cholinergic receptors by the fact that 3-bromo or 3-amino
substitution on levamisole brought about changes in the potency of levamisole in rat
(section 3.4.1.1) but not in *H. contortus*.

The findings of *ATCHISON et al.* (1992) are consistent with the findings in *Ascaris
suum* (HARROW & GRATTON, 1985) where the anthelmintics levamisole, pyrantel,
morantel were 10-100 times more potent than ACh.

**Litomosoides carinii.**

Spastic paralysis was noted seconds after the addition of levamisole to *L. carinii*,
followed by flaccid paralysis 10-20 minutes later (KOMUNIECKI & SAZ, 1982). This
particular filarial nematode lacks the fumarate reductase system (WANG & SAZ, 1974),
suggesting that the flaccid paralysis stage in nematodes treated with levamisole, was not associated with inhibition of the fumarate reductase system. KOMUNIECKI & SAZ (1982) reported no measurable affect on glycogen metabolism in L. carinii during the initial contraction, but a direct stimulation of glycogen synthesis was noted in associated with the flaccid paralysis. These results are consistent with results observed in Ascaris suum (DONAHUE et al., 1983), where the initial contraction is thought to be due to direct stimulation of the muscle and the flaccid paralysis due to interference with the c-AMP dependent regulation of glycogen metabolism (see section 3.4.1.2.2).

**Dipetalonema viteae**

ROHRER et al. (1988) have investigated the neuromuscular electrophysiology of the filarial nematode Dipetalonema viteae. They concluded that the muscle of this nematode had similar properties to that of Ascaris muscle cells: spontaneous slow waves and spikes were recorded from muscle cells; ACh or cholinergic agonists stimulated the muscle cells; the anticholinesterase, physostigmine, increased spike frequency; GABA inhibited the somatic muscle depolarisations. However, unlike Ascaris, the vertebrate antagonists, d-tubocurarine and hexamethonium were shown to be ineffective on D. viteae (ROHRER et al., 1988). Results obtained from H. contortus (ATCHISON et al., 1992), C. elegans (LEWIS et al., 1980) and D. viteae (ROHRER et al., 1988) on somatic muscle cells indicate that Ascaris may be unique among nematodes in terms of the response of the receptor to nicotinic agonists.

Levamisole was found to be one of the most potent agonists tested on D. viteae. (ROHRER et al., 1988). At low concentrations levamisole increased muscle activity, but at high concentrations it blocked muscle activity (ROHRER et al., 1988): concentrations of 0.5 to 0.05 nM did not alter the resting membrane potential of the muscle but increased the spike frequency; a concentration of 5 nM decreased the resting membrane potential and increased the spike frequency; with concentrations of 50 nM
and above there was an initially decrease in the spike rate, then a drop in resting membrane potential and a complete cessation of spike and slow wave activities.

Thus it would appear that levamsiole acts as a depolarising blocker in D. viteae, as reported earlier for Ascaris suum.

3.4.2 Pyrantel and morantel

Pyrantel is the name given to the tetrahydropyrimidine compound (E)-1,4,5,6-tetrahydro-1-methyl-2-[2-(2-thienyl)vinyl]pyrimidine. A close analogue is the compound morantel which differs from pyrantel in that it has an additional methyl group. Morantel is the name for the compound (E)-1,4,5,6-tetrahydro-1-methyl-2-[2-(3-methyl-2-thienyl)vinyl]pyrimidine.

Both pyrantel and morantel are considered in this next section as they have similar actions.

3.4.2.1 Vertebrates

Eyre (1970) showed that pyrantel (5 \( \mu \text{g ml}^{-1} \) ie. 14 \( \mu \text{M} \)) induced a marked sustained contraction of guinea-pig ileum preparations, which was followed for 5 to 10 minutes after washing the preparation, by a number of spontaneous "spikes" (contractions). The contracture produced by ACh (0.02 - 0.2 \( \mu \text{g ml}^{-1} \) ie. 0.1 - 1.1 \( \mu \text{M} \)) was potentiated following treatment of the preparation with pyrantel (5\( \mu \text{g ml}^{-1} \) ie. 14 \( \mu \text{M} \)) (Eyre, 1970), but not with pretreatment of the preparation with methyridine or tetramisole. Although methyridine (10-20 \( \mu \text{g ml}^{-1} \) ie. 73 - 146 \( \mu \text{M} \)) caused slight spontaneous movement of the ileum, and tetramisole (5 to 10 \( \mu \text{g ml}^{-1} \) ie. 21 - 42 \( \mu \text{M} \)) produced a more pronounced irregular spontaneous activity when applied to the preparation (Eyre, 1970). Hexamethonium (1 \( \mu \text{g ml}^{-1} \) ie. 3.5 \( \mu \text{M} \)) blocked the response to pyrantel (5\( \mu \text{g ml}^{-1} \) ie. 14 \( \mu \text{M} \)) but not to tetramisole (5 to 10 \( \mu \text{g ml}^{-1} \) ie. 21 - 42 \( \mu \text{M} \)), suggesting that pyrantel acted by the stimulation of nicotinic receptors at the ganglionic synapses of the
ileum. Atropine (0.1 - 0.2 μg ml⁻¹ ie. 0.1 - 0.3 μM) antagonised the pyrantel (5 μg ml⁻¹ ie. 14 μM) response completely (Eyre, 1970), suggesting that pyrantel stimulated muscarinic receptors either directly or indirectly to induce contraction.

On the cat nictitating membrane, pyrantel (2 mg Kg⁻¹ iv) contracted the membrane and induced a rise in blood pressure. Hexamethonium (5 mg Kg⁻¹) abolished both the contraction of membrane due to preganglionic electrical stimulation and the rise in blood pressure and membrane contracture due to application of pyrantel (Eyre, 1970).

Pyrantel reduces and eventually blocks the twitch response of the rat hemidiaphragm to electrical stimulation of the phrenic nerve (Aubry et al., 1970; Atichison et al., 1992). Pyrantel is less active than d-tubocurarine but more active than decamethonium on this preparation (Aubry et al., 1970). d- Tubocurarine is 50 times more active than decamethonium and 100 times more active than nicotine in blocking the twitch response of the rat hemidiaphragm preparation. These observations were confirmed by Atichison et al. (1992) who showed that pyrantel was equipotent with morantel, more potent than nicotine which was more potent than levamisole on isolated rat hemidiaphragm preparations. After blocking the response of the rat hemidiaphragm to electrical stimulation by the phrenic nerve with d-tubocurarine (8 μg ml⁻¹ ie. 12 μM); pyrantel (20 μg ml⁻¹ ie. 56 μM), nicotine (300 μg ml⁻¹ ie. 960 μM) (Aubry et al., 1970); or levamisole (50 μM) (Atichison et al., 1992) had no affect on direct electrical stimulation of the diaphragm, a result explained by a lack of effect in the electrical contraction coupling of the muscle. The twitch depression of the rat hemidiaphragm (induced by pyrantel, levamisole, nicotine etc.), was reversed by washing and not by application of physostigmine (Atichison et al., 1992), suggesting that these compounds had a direct action on the ACh receptors and did not act to inhibit cholinesterases.
In the soleus and anterior tibialis muscles of cat, injection of pyrantel (1 mg Kg\(^{-1}\)) induced an initial depolarisation followed by short lived neuromuscular block (AUBRY et al., 1970). Arterial injection of ACh (25-50 \(\mu\)g ie. 69 - 138 \(\mu\)M), during the period of impaired neuromuscular transmission induced by pyrantel, produced a deepening of the block in place of the contracture, if arterial injection of ACh was given when neuromuscular transmission was not impaired by pyrantel. Pyrantel (1 mg ie. 1.4 \(\mu\)M) enhanced the failure of neuromuscular transmission induced by both succinylcholine (50 \(\mu\)g ie. 69 \(\mu\)M) and d-tubocurarine (200 \(\mu\)g ie. 147 \(\mu\)M) (AUBRY et al., 1970), suggesting that the nature of the neuromuscular block produced by pyrantel was mixed in character, showing properties possessed by competitive and depolarising neuromuscular blocking drugs.

Pyrantel (1.15 mg Kg\(^{-1}\)) in common with nicotine (1.17 mg Kg\(^{-1}\)) and decamethonium (0.032 mg Kg\(^{-1}\)) induced spastic paralysis in chicks and contracted the chick semispinalis and toad rectus abdominis muscle (AUBRY et al., 1970). Pyrantel however had only a weak nicotinic activity.

Thus AUBRY et al. (1970) showed that pyrantel had activity which was characteristic of depolarising neuromuscular blocking agents on the rat phrenic nerve diaphragm preparation, chick semispinalis and toad rectus abdominis muscles. They also concluded that at the concentrations required to produce the anthelmintic effect of pyrantel, side effects in the host should be negligible.

3.4 2.2 Invertebrates
3.4.2.2.1 Insects

Pyrantel and morantel (10 \(\mu\)M to 1 mM), like levamisole (10 \(\mu\)M to 1 mM), were shown to induce a dose dependent change in conductance and a weak depolarisation of
the neuronal membrane of the fast coxal depressor motoneurone in the cockroach (*Periplaneta americana*) (Pinnock et al., 1988). In some experiments, no response was found when pyrantel or morantel was applied to the insect neuronal preparation, this was thought to result from the low potency of these compounds on insect nicotinic receptors (Pinnock et al., 1988). Pyrantel was shown to be 33 times less potent and morantel 66 times less potent than carbachol (5 - 100 μM) on this preparation. Mecamylamine (100 μM) abolished the depolarising response of pyrantel (100 μM) and morantel (300 μM) but not GABA, which suggested that like levamisole, pyrantel and morantel acted at the cholinergic receptors (Pinnock et al., 1988). The acetylcholinesterase inhibitor, neostigmine (0.1 μM) potentiated the actions of ACh but had no effect on responses to pyrantel, carbachol, levamisole, and morantel (Pinnock et al., 1988). This further suggested that pyrantel, carbachol, levamisole, and morantel did not induce ACh release but acted directly on the ACh receptors. Morantel, but not levamisole or pyrantel, was shown to attenuate the responses to ACh on this preparation (Pinnock et al., 1988). Test pulses of ACh, delivered ionophoretically before and during a morantel-induced depolarisation, decreased in amplitude during the morantel induced response. This suggested that morantel occupied the same receptor population as ACh and during the morantel induced depolarisation, less receptors were available for ACh to act upon.

Hassoni et al. (1988) have shown that the actions of morantel and pyrantel are virtually the same, on cells affected by ACh in the snail (*Helix aspersa*). These cells are described in section 3.4.1.2.1 which deals with levamisole action in insects. Morantel was reasonably potent on both "D" and "H" cells with potency ratios of 1.12 and 5.16 respectively, compared with ACh (Hassoni et al., 1988). Pyrantel acted in the same manner as morantel, the only difference was that of potency. Pyrantel had a relative potency of 2.56 on "D" cells and 3.53 on "H" cells compared to ACh (Hassoni et al., 1988). Both pyrantel and morantel mimicked the action of ACh on the "D" cells,
though the duration and increase in activity was greater with morantel (HASSONI et al., 1988). Pyrantel and morantel both induced an increase in conductance and appeared to act like a full agonists in these cells (HASSONI et al., 1988). HASSONI et al. (1988) also investigated the ionic mechanisms for both inhibition and excitation. In sodium free medium the responses to ACh, pyrantel and morantel on a "D" cell type were abolished. In chloride free medium the responses to ACh, pyrantel and morantel were greatly reduced or abolished on "H" cell types. D-tubocurarine blocked the affect of ACh, pyrantel and morantel. The similar ionic mechanisms and the actions of d-tubocurarine suggested that pyrantel and morantel acted at the ACh receptor complex in this preparation. The order of potency obtained for pyrantel and morantel compared to ACh in these studies on the snail, is in contrast to experiments performed in Ascaris suum where pyrantel was more potent than ACh (HARROW & GRATON, 1985). The antagonistic effects of hexamethonium and mecamylamine were investigated on "D" cells against the actions of pyrantel and ACh (HASSONI et al., 1988). Hexamethonium (4 µM) reversibly reduced the response to both compounds. Mecamylamine (1.5-3 µM), likewise reduced the ACh and pyrantel responses of both the "D" and "H" cells. The effect of these antagonists on pyrantel action suggested that the insect ACh receptor was similar to vertebrate ganglionic receptors (HASSONI et al., 1988). Thus it was concluded that the weak insecticidal activity of pyrantel (as already suggested for levamisole) compared to its potent affect in nematodes (HARROW & GRATON, 1985) may reflect a difference in ACh receptors between insects and nematodes.

3.4.2.2.2 Nematodes

Originally the anthelmintic activity of pyrantel was tested against: Nematospiroides dubius in mice; Nippostrongylus muris in rats; Haemonchus, Ostertagia and Trichostrongylus in the abomasum of sheep and cattle; Nematodirus, Cooperia and Trichostrongylum in the small intestine of sheep and cattle; Ascaris suum in pigs; and Toxocara and toxascaris in dogs (AUSTIN et al., 1966). Excellent activity after a
single oral dose of pyrantel was reported in the elimination of these parasites. AUSTIN et al., (1966) tested a series of pyrantel analogues and found that morantel was the most active followed by pyrantel. CORNWALL (1966) also reported that pyrantel was active against various adult and immature intestinal nematodes (Ostertagia, Trichostrongylus species, Nematodirus, Cooperia, Chabertia) in sheep when applied as a single oral dose. He also reported low activity of pyrantel against Trichuris. In 1967, HOWES & LYNCH evaluated the anthelmintic action of pyrantel against three sub order of nematodes (Strongylata; Ascaridata and Trichurata) and one order of Cestodes (Cyclophyllidea). Studies showed that pyrantel was active against nematodes in the sub order Strongylata and in the suborder Ascaridata but was relatively ineffective against nematodes in the suborder Trichurata (HOWES & LYNCH, 1967). Cestodes infestations were shown to be relatively unaffected by pyrantel (HOWES & LYNCH, 1967; TERADA et al., 1983a)).

Later pyrantel (1 nM) was reported to induce contraction and finally spastic paralysis in various nematodes including Angiostrongylus cantonensis (1 nM), Dinofilarial immitis (10 nM), Ancylostoma caninum (0.1 μM), toxocara canis (1 μM) and Ascaris suum (1 μM), but not against Trichuris vulpis (TERADA et al., 1983a). Preparations exposed to higher concentrations (0.1 to 1 μM) of pyrantel were incapable of restoring resting tone; preparations exposed to lower concentrations (0.01 to 0.1 μM) could easily restore resting tone when the preparation was washed (TERADA et al., 1983a). TERADA et al. (1983a) concluded that the difference in onset and potency of pyrantel was either due to the varying ability of the drug to penetrate the cuticle or to differences in susceptibility of the nematode receptor sites. Cholinergic receptors in nematodes are thought to be nicotinic, eg. Ascaris (section 3.3) and A. cantonensis (TERADA et al., 1982a). Cestodes (D. caninum) and trematodes (Schistosoma mansoni and F. hepatica) however have a cholinergic receptors which can not be classified as either
muscarinic or nicotinic. This may explain why pyrantel is potent in nematodes but shows a much lower potency in cestodes and trematodes (HOWES & LYNCH, 1967).

High concentrations of morantel tartrate (2.5 mM) were shown to inhibit the fumarate reductase system in *Haemonchus contortus* (PRITCHARD, 1973). The development of *Ascaris* larvae was shown to be sensitive to morantel (REW et al., 1986)

**Ascaris suum**

Pyrantel (25 µg ml⁻¹ ie. 70 µM) added to whole worm preparations induced a sharp contraction followed by a cessation of spontaneous movement of the worm (AUBRY et al., 1970). Pyrantel (20 µg ml⁻¹ ie. 56 µM) caused an immediate contracture and a reduction in the responses to electrical stimulation across the body wall of *Ascaris*. The resting tone of the worm was readily restored by washing the preparation. TERADA et al. (1983a) also showed that pyrantel (1 µM) induced contraction followed by spastic paralysis in whole *Ascaris suum*.

In strip preparations of *Ascaris*, pyrantel also had stimulant properties on the muscle, but concentrations required to induce contraction were lower than required in whole worm preparations: 1 to 10 ng ml⁻¹ ie. 2.8 to 28 nM (TERADA et al., 1983a) and 1.5 ng Kg⁻¹, (AUBRY et al., 1970). In contrast to the almost immediate contracture produced by ACh, pyrantel induced a slowly developing prolonged contracture of the muscle strip which was only removed by repeated washing (AUBRY et al., 1970). Pyrantel was 100 times more active at inducing contraction of *Ascaris* somatic muscle strip than ACh (AUBRY et al., 1970). Piperazine (1 mg Kg⁻¹) and d-tubocurarine (20 µg ml⁻¹, ie. 30 µM) blocked the response to pyrantel (1.5 ng Kg⁻¹, ie. 3.8 nM) on this preparation (AUBRY et al., 1970; COLES et al., 1975). The antagonistic effect with piperazine and d-tubocurarine was not observed against levamisole induced contraction (ACEVES et al., 1970) but occurred against ACh (1 µg ml⁻¹ ie. 5.5 µM) induced contraction (AUBRY et al., 1970).
Pyrantel (0.0025-1.0 μg ml⁻¹ ie. 7 nM -2.8 μM) induced depolarisation and increased spike discharge frequency in single muscle cells of *Ascaris*, accompanied by an increase in muscle tension (AUBRY et al., 1970), which could not be reversed by repeated washing of the tissue.

The use of both current-clamp and voltage-clamp techniques were used to investigate the effect of microperfusion of pyrantel and morantel onto the bag region of the muscle cell (HARROW & GRATION, 1985). Current-clamp experiments showed that pyrantel and morantel (like levamisole and ACh) increased the muscle input conductance and depolarised the muscle cell. The threshold dose for both these anthelmintics was close to 10 nM. Dose-conductance relationships for both these compounds were bell shaped, unlike those of levamisole and ACh which were sigmoid. The relative order of potency of these compounds on *Ascaris* muscle cells was morantel = pyrantel > levamisole > ACh. No dose dependent desensitisation was observed at low concentrations of pyrantel (< 100 μM) or morantel (<100 μM). At high concentrations (>100 μM) of pyrantel and morantel, HARROW & GRATION (1985) suggested that desensitisation may occur. The occurrence of desensitisation would explain the bell shaped dose-conductance curve obtained with pyrantel and morantel. To investigate the desensitisation phenomenon further, HARROW & GRATION (1985) tested the affect of morantel on the cells response to ACh. HARROW & GRATION (1985) compared the conductance changes evoked by microperfusion of ACh (10 μM) before and after exposure to morantel. They found that high doses of morantel reduced the ACh induced conductance increase, suggesting that desensitisation occurred with high morantel concentrations. Lower doses of morantel (1 μM or less) had no affect on the response of the muscle cells to ACh. Another explanation for the reduction in the conductance changes of the cell to ACh after exposure of the cell to high morantel
concentrations, is that at high concentrations morantel acted as an antagonist at the ACh receptor (HARROW & GRATION, 1985).

Voltage clamp experiments showed that pyrantel (10 μM) and morantel (10 μM) had linear and non-linear sections to the current/voltage relationship (HARROW & GRATION, 1985). Similar findings were also observed with levamisole (100 μM) and ACh (100 μM). The reversal potential for pyrantel and morantel induced currents were those similar to those of ACh and levamisole induced currents and suggested that the channel current was carried by cations, mainly sodium (HARROW & GRATION, 1985). They suggested that the non-linear sections of the current/voltage plots were due to either a voltage sensitive desensitisation, or an agonist induced channel block (HARROW & GRATION, 1985).

To further investigate the actions of pyrantel, ACh and pyrantel were applied by ionophoresis from double barrelled pipettes to the muscle bag membrane of Ascaris suum (HARROW & GRATION, 1985). Inward peak currents were recorded in response to the same ionophoretic dose of ACh and pyrantel, at a holding potential of -30 mV. The larger peak inward current induced by application of pyrantel to the muscle cell compared to that of ACh, indicated that pyrantel was a more potent agonist, which agreed with the relative potencies found from dose conductance experiments (HARROW & GRATION, 1985). The application of pyrantel as a single ionophoretic pulse to the muscle membrane, resulted in a reversible block of ACh induced currents, suggesting that competition between ACh and pyrantel for the same receptor site occurred (HARROW & GRATION, 1985).

The reports of HARROW & GRATION (1985) were consistent with the earlier observations of AUBRY et al. (1970) who suggested that pyrantel caused a prolonged contraction of A.suum muscle strips. AUBRY et al. (1970) used pyrantel concentrations up to micromolar (7 nM - 2.8 μM) which were not high enough to detect the antagonistic affect of pyrantel as reported by HARROW & GRATION (1985).
Caenorhabditis elegans.

Pyrantel (0.5 mM) or morantel (1 mM) induced sustained contraction of the wild type C. elegans but did not have any antagonistic effects, like those observed with levamisole. Levamisole treatment resulted in an initial strong contraction due to the agonistic properties, followed after several minutes by a continued but milder contraction due to the antagonistic properties. These anagonistic properties resulted in nematodes with a blocked phenotype, observed as uncoordinated motion. Even in the most resistant pseudowild types, which showed the blocked phenotype when treated with levamisole, morantel (10 mM) did not induce anagonistic properties (LEWIS et al., 1980). Pseudowild types incubated overnight with 10 mM morantel or 0.5 mM pyrantel were poorly resistant to carbachol and levamisole. If pyrantel, like levamisole, had antagonistic properties at the levamisole receptor which blocked receptor activity, you would have expected the pseudowild types incubated overnight with pyrantel to be resistant to further treatment with carbachol and levamisole (LEWIS et al., 1980).

Binding studies indicated that morantel and levamisole bound to the same site in C. elegans, since morantel powerfully inhibited [³H]Mal binding in C. elegans with an IC₅₀ value of 68 nM (LEWIS et al., 1987b).

Pyrantel (0.1 mM), morantel (1 mM), methyridine (1mM) and bephenium (1 mM) failed to induce contraction of the levamisole resistant unc-29 strain after 1 hour, although these compounds readily induce contraction of cut wild type within 1-15 minutes depending on the anthelmintic. Representative strains of each levamisole resistant loci, in experiments where whole worms were exposed to the drugs, showed the same resistance to initial contraction on plates containing 1 mM morantel and 0.5 mM pyrantel as they did on plates containing levamisole (LEWIS et al., 1980). These observations suggested that pyrantel, morantel, methyridine and bephenium acted at the same receptor as levamisole in C. elegans and were consistent with the observations of
Eyre 1970, Aubry et al., 1970; and Coles et al., 1974, who suggested that pyrantel, morantel, methyridine, and bephenium have a mode of action similar to levamisole in the nematode Ascaris suum.

**Nippostrongylus brasiliensis**

In vitro experiments with *Nippostrongylus brasiliensis* suggested that pyrantel was less active than levamisole against *Nippostrongylus brasiliensis* (Coles et al., 1975). *Nippostrongylus brasiliensis* was protected from levamisole induced spastic paralysis by pretreatment with pyrantel (Coles et al., 1974, 1975) which suggested that levamisole and pyrantel also act at the same receptor site on this parasite.

**Angiostrongylus cantonensis**

*Angiostrongylus cantonensis*, like *Ascaris suum*, is regulated by excitatory cholinergic mechanisms which appear to be nicotinic (Terada et al., 1982a, b). The motility of the nematode is affected at pyrantel concentrations of 1 nM or more (Terada et al., 1982a). D-tubocurarine (19 μM) inhibited the spontaneous motility of this parasite and also inhibited the pyrantel induced effects at lower doses (1 nM). The contractile effects induced by high doses of pyrantel (0.01 to 0.1 μM) were not inhibited by d-tubocurarine (Terada et al., 1983b), suggesting that d-tubocurarine had a low affinity for the receptor in this parasite.

Strychne inhibited the release of ACh from cholinergic nerve endings (Terada et al., 1982) and paralysed the nematode at concentrations of 1 to 3 μM (Terada et al. 1983b). Eserine (1 μM), a cholinesterase inhibitor, induced contractions of *Angiostrongylus cantonensis* muscle which were completely inhibited by strychine (0.1 - 3 μM), whereas pyrantel (0.03 μM) induced contractions were unaffected by strychine (Terada et al., 1983b). This suggested that pyrantel did not stimulate the
release of ACh from cholinergic nerve endings or inhibit cholinesterase activity, but appeared to act by stimulating the nicotinic cholinergic receptors.

TERADA et al. (1983b) concluded that pyrantel had a high affinity and or potent intrinsic activity against cholinergic receptors in Angiostrongylus cantonensis.

**Haemonchus contortus**

ATCHISON et al. (1992) reported that pyrantel (1-10 μM) had similar actions to that of levamisole in that it stimulated a spastic contraction followed by a sustained paralysis in H. contortus, mimicking the action of nicotine. ATCHISON et al. (1992) tested the nicotinic antagonists d-tubocurarine (10 μM), gallamine (100 μM), pentolinium (100 μM), decamethonium (10 μM), hexamethonium (10 μM) mecamylamine (100 μM) and found that they did not block the effect of pyrantel on either muscle tension or contraction. They also tested the muscarinic antagonists muscarone (10 μM), arecoline (100 μM), pilocarpine (10 μM), and atropine (100 μM), which were also found to have no affect on the pyrantel induced response. The blocking effects of pyrantel (like levamisole) on H. contortus were associated with a depolarisation of the muscle fibres, the depolarising actions were not significantly affected by d-tubocurarine (10 μM) (ATCHISON et al., 1992). Thus it would appear that pyrantel has similar actions to levamisole on the nicotinic ACh receptors of this parasite.

**3.4.3. Bephenium**

Bephenium (0.25 μg ml⁻¹ ie. 1 μM) induced a spastic paralysis of exposed neuromuscular preparations of Ascaris suum which was counteracted by piperazine (250 μg ml⁻¹ ie. 2.9 mM) or d-tubocurarine (20 μg ml⁻¹ ie. 31 μM) (BROOME, 1962). Bephenium at a concentration of 0.25 μg ml⁻¹ ie. 1 μM had an equivalent effect to 1 μg ml⁻¹ ie. 7 μM ACh (BROOME 1962). Bephenium at concentrations of 30 μg g⁻¹
contracted whole *Ascaris* (COLES et al., 1975). Bephenium was also reported to lower the histamine content of *Ascaris suum* (PILLIPS et al., 1976). LEWIS et al. (1980) suggested that bephenium had a similar mode of action to levamisole in *C. elegans*. Bephenium (1 mM) contracted wild type strains of *C. elegans*, but failed to contract the levamisole resistant strains.

3.4.4. Methyridine

Methyridine is [2-(2 methoxyethyl)pyridine].

3.4.4.1 Vertebrates

EYRE (1970) showed that unlike pyrantel and levamisole, methyridine did not affect the twitch response on rat isolated guinea pig ileum or chick isolated biventer nerve muscle. However, like pyrantel and tetramisole, methyridine (1 mM) induced a reversible neuromuscular block and contracture of the chick isolated biventer nerve muscle (EYRE, 1970). At concentrations of 2.8 mM, methyridine produced contractions of chick isolated semispinalis muscle, which were inhibited by d-tubocurarine (0.15 μM) (EYRE, 1970). Thus EYRE (1970) concluded that methyridine was a weak depolarisation agent which had little effect on the ileum and showed little or no tendency to augment neuromuscular transmission or antagonise curare. Methyridine like tetramisole and pyrantel also acted as an anticholinesterase but was less potent (EYRE, 1970).

3.4.4.2 Invertebrates

Methyridine has been shown to act as a more potent anthelmintic than bephenium, against *Nematodspiroides dubius, Nippostrongylus muris* and *Heterakis spumosa* (BROOME & GREENWAY 1961). It was shown that methyridine induced an irreversible paralysis in *Ascaris suum, Nematodspiroides dubius, Nippostrongylus muris* and *Heterakis spumosa* (BROOME 1961). Methyridine at concentrations of 30
μg g⁻¹ contracted whole *Ascaris* (COLES *et al.*, 1975). Methyridine (10 μg ml⁻¹ ie. 73 μM) paralysed whole nematode preparations which could not be antagonised by ACh (DEL CASTILLO 1969; VAN DEN BOSSCHE 1976; BROOME 1961). In exposed *Ascaris* tissue methyridine (20 μg ml⁻¹ ie. 146 μM) induced a rapid paralysis of rhythmic movements which was reversed by d-tubocurarine (20 μg ml⁻¹ ie. 31 μM) and piperazine (250 μg ml⁻¹ ie. 3 mM) but not by ACh (BROOME, 1962). Methyridine appeared to have a depolarising action on *Ascaris* muscle similar to that of decamethonium on the vertebrate neuromuscular junction (DEL CASTILLO, 1969).

Experiments using the nematode *Nippostrongylus brasiliensis*, showed that the nematode was protected from levamisole induced contraction by methyridine, which suggested that both levamisole and methyridine acted at the same site in this nematode (COLES *et al.*, 1974). LEWIS *et al.* (1980) also suggested that methyridine had a similar mode of action to levamisole, methyridine (1 mM) failed to induce contraction in the levamisole resistant strains of *C. elegans* after one hour, although contraction was observed in the wild type exposed to methyridine.
3.4.5 General summary of the reported differences of the actions of anthelmintics thought to act at ACh receptors

It would appear that pyrantel, morantel and levamisole act at cholinergic receptors in vertebrate and invertebrate preparations. The relative potencies of the drugs highlight pharmacological differences between receptors of the species examined in these studies. For example: in rat hemidiaphragm the order of potency was, pyrantel = morantel > nicotine > levamisole; in the cockroach the order of potency was, carbachol >> levamisole > pyrantel > morantel; in the nematode *H. contortus* the potency sequence was, levamisole = pyrantel > morantel > nicotine; and finally in *Ascaris* the order of potency was, morantel = pyrantel > levamisole > ACh. Although these compounds appear to act at the same site, certain pharmacological differences have been noted. In the guinea pig ileum, tetramisole (5-10 µg ml⁻¹ ie. 21 - 42 µM) or methyridine (10-20 µg ml⁻¹ ie. 73-146 µM) did not potentiate the ACh induced response, whereas pyrantel (5 µg ml⁻¹ ie. 14 µM) potentiated the response to ACh. Hexamethonium, at a concentration of 1 µg ml⁻¹ ie. 3.5 µM in the guinea-pig and 5 mg Kg⁻¹ in the cat nictitating membrane, blocked the pyrantel (5 µg ml⁻¹ ie. 14 µM, guinea-pig ileum; 2 mg Kg⁻¹, cat nictitating membrane) induced response but had no affect on the response to tetramisole (10 µg ml⁻¹ ie. 42 µM, guinea-pig ileum; 2 mg Kg⁻¹, cat nictitating membrane).

These anthelmintics may not act exclusively at nicotinic receptors, some evidence exists which suggests that pyrantel, methyridine and tetramisole also possessed muscarinic activity in some preparations: Atropine (0.1 to 0.2 µg ml⁻¹ ie. 0.1- 0.3 µM) antagonised the response to pyrantel completely, and tetramisole partially on the guinea-pig ileum, which suggested that stimulation of muscarinic receptors had occurred. In vivo studies in rabbit, also suggested that tetramisole and methyridine exerted both muscarinic and nicotinic effects whereas pyrantel appeared to excite mainly nicotinic receptors (EYRE 1970).
In nematodes pharmacological differences were also observed; in *Ascaris*, piperazine and d-tubocurarine blocked the response to pyrantel but not to levamisole; in *C. elegans* pyrantel and morantel had agonistic properties similar to that of levamisole but did not show any signs of antagonism reported at high levamisole concentrations.

Although these differences exist, there is strong evidence to suggest that pyrantel, levamisole, morantel, bephenium and methyridine act at ACh receptors.
3.4.6. Recovery

Levamisole was reported to induce a reversible paralysis with three nematode species: *Ascaris suum; Nippostrongylus brasiliensis; and nematodspiroides dubius* (COLES et al., 1974, 1975): some nematodes recovered motility even when continually maintained in a levamisole solution. In *Ascaris*, the worms that regained motility had a less vigorous movement (COLES et al., 1974, 1975). It should be noted that VAN NEUTEN (1972) reported that no recovery of motility was observed in nematodes after exposure to levamisole, even after 16 hours in fresh solution, except with low doses of levamisole. Levamisole (100 ppm ie. 415 μM) was used to paralyse whole *Ascaris*, worms that showed recovery of movement while continually maintained in the levamisole solutions were further treated with several compounds known to induce contraction of *Ascaris* muscle similar to the contraction produced by levamisole. The nematodes that showed recovery were resistant to further treatment with pyrantel (30 μg g⁻¹), bephenium (30μg g⁻¹), methyridine (30 μg g⁻¹) but contracted when treated with ACh (1 μg g⁻¹); and choline phenyl ether (6 mg g⁻¹) (COLES et al., 1974, 1975). Exposure of the recovered worms to nicotine induced an initial relaxation followed by contraction (COLES et al., 1974, 1975).

The recovery of motility of nematodes continually maintained in levamisole, and their resistance to further treatment with pyrantel, bephenium and methyridine may be explained in several ways: treatment of the nematodes with levamisole may induce a conformational change in the receptor resulting in a desensitised state of the receptor which is insensitive to some of the receptor agonists; alternatively ACh, choline phenyl ether and nicotine may also act on a different receptor to the nicotinic receptor, which is insensitive to the compounds levamisole, pyrantel, methyridine and bephenium.

The presence of muscarinic receptors has been suggested in the nematode *C. elegans* (WHITE et al. 1976; SEGERBERG & STRETTON, 1986); and in the nervous system of
Ascaris suum, where the muscarinic antagonist N-methyl-scopolamine has been shown to block activity of the motorneurones DE1 and DE3 (SEGERBERG & STRETTON, 1986).

LEWIS et al. (1980, 1987) have reported that mutants of the nematode C.elegans are resistant to levamisole and suggested that these mutants lack functional cholinergic muscle receptors (see section 4.3.2.2). The mutants had an uncoordinated visible phenotype and responded poorly to cholinergic agonists. The uncoordinated motor behaviour of the levamisole resistant uncs and the resistance to levamisole and other cholinergic agonists could be copied by exposing the wild type for 5-10 minutes to the antagonist mecamylamine (0.1 M) or levamisole (1 mM) and pseudowild types to levamisole and nicotine (LEWIS et al., 1980). LEWIS et al. (1980) proposed that some sort of cholinergic blockade by levamisole or mecamylamine occurred. It is possible that the results observed by COLES et al. (1974, 1975) where levamisole treated worms recovered motility, may also represent a cholinergic blockade of muscle receptors by levamisole. This would also explain why COLES et al. (1974, 1975) observed that full motility was never regained after levamisole treatment, and the resistance of the levamisole recovered nematodes to further treatment with the anthelmintics that act at the same receptor. LEWIS et al. (1980) proposed the presence of two types of ACh receptors in C. elegans: the predominant type, present on the muscle cell, was sensitive to levamisole and nicotine and affected by levamisole resistant mutations; the other type was resistant to the agonist and blocking effects of levamisole and nicotine (LEWIS et al, 1980). If similar receptors occurred in Ascaris this would explain why levamisole recovered worms would not contract when further exposed to levamisole, pyrantel, methyridine and bephenium but contracted to ACh, choline phenyl ether and nicotine.
3.5 Aims of this project

The aim of the research presented in the following chapters, was to study the mode of action of the potent anthelmintic compounds levamisole and pyrantel on the nematode *Ascaris suum*. Previous reports suggested that both pyrantel and levamisole may act at muscle receptors. I therefore decided to use the patch-clamp technique on a muscle vesicle preparation from the somatic muscle of the parasite. This experimental approach allowed kinetic analysis of levamisole-activated and pyrantel-activated currents. A comparison was also made with data obtained with ACh-activated currents on this same preparation. A detailed understanding of the mode of action of compounds such as levamisole and pyrantel may help in the design of new more potent anthelmintics.
CHAPTER 4
METHODS

4.1. Collection and maintenance of the parasite Ascaris suum.

Specimens of Ascaris suum were collected from the local slaughter house (Figure 4.1). During transport, they were kept in a thermos flask filled with Locke's solution (solution A) and maintained at 37°C. On arrival at the laboratory, they were transferred to glass tanks filled with Locke's solution. The tanks were placed in a water bath with temperature set at 37°C. The water bath was placed in a fume hood in the laboratory. This was done to protect the laboratory staff from inhalation of the volatile fatty acids which these parasites produce and to reduce exposure of the staff to antigens produced by these parasites which are known to trigger allergic reactions. The Locke's solution was changed daily. To prolong the life of parasites it was beneficial to have a small number of parasites in each tank (10-15). This reduced the contamination of the Locke's solution with their waste products. In general parasites were kept for a maximum of four days. Parasites (approximately in the size range 10-30 cm) to be used for experimentation, were selected from the tank each morning. In general small parasites were not used since dissection was difficult and sometimes resulted in damage to the muscle cells. Near mortile and very large parasites were also rejected.
Photograph of an *Ascaris suum* nematode, a coin was placed next to the parasite to indicate size.
4.2. The vesicle preparation.

A 2 cm section from the anterior region of the worm was taken, dissected along one of the lateral lines and pinned out, cuticle side down, onto Sylgard™ (Sylgard 184 silicone elastomer kit, Dow Corning Corporation, Midland, M.I. 48640, U.S.A.). The gut was gently removed with a fine pair of forceps (Figure 4.2). The colour of gut indicated the health of the parasite, the gut from a healthy specimen was yellow and could be removed in one piece. The muscle flap preparation was washed with maintenance solution (solution B) to remove small traces of the gut. The preparation was then treated with a solution containing collagenase (solution C) for about 10 minutes at 37°C or until the muscle membrane began to bud. The collagenase solution was washed off with maintenance solution. The preparation was then kept in the maintenance solution at 37°C. The vesicles formed over the next hour, as outgrowths from the bag region of the somatic muscle cells (Figure 4.3). The vesicles were harvested with a Pasteur pipette, and were used within 5 hours. For giga seal formation the cell membranes were cleaned with collagenase. Collagenase treatment of the muscle flap had two effects: (1) it induced vesicle formation; and (2) it digested away the extracellular matrix. Care was taken not to over-treat the membrane: over-treated membranes tended to produce vesicles which were weak and exploded when the patch pipette was brought in contact with the membrane.

Vesicles were transferred to the experimental chamber where recordings were made at room temperature (15-22°C). Vesicles were bathed in the experimental chamber in the bath solution (solution D). The vesicle preparation has been previously described (Martin et al., 1990).
Illustration of the muscle flap preparation of *Ascaris suum*. B. The formation of muscle vesicles from the belly region of the muscle. C. An individual vesicle at higher magnification.
A. Vesicle Formation

![Diagram of vesicle formation with labels: Bag, Spindle, Arm, Syncytium, Nerve.]

B. Recording Technique

![Diagram of recording techniques using cell-attached and isolated inside-out patch clamp configurations.]

A. Schematic diagram of vesicle formation of the muscle vesicles. C. Recording techniques using the cell-attached and isolated inside-out patch clamp configurations.
4.3. Solutions

Solutions: Concentration are in mM

Solution A. Locke's solution: NaCl (155); KCl (5); CaCl$_2$ (2);
              NaHCO$_3$ (1.5); glucose (5).

Solution B Maintenance solution: NaCl (35); Na acetate (105); KCl (2);
                  MgCl$_2$ (2); HEPES (10); glucose (3);
                  ascorbic acid (2); EGTA (1),
                  pH adjusted to 7.2 with NaOH.

Solution C Collagenase solution NaCl (35); Na acetate (105); KCl (2);
                  MgCl$_2$ (2); HEPES (10); glucose (3);
                  ascorbic acid (2); collagenase (1 mg/ml),
                  pH adjusted to 7.2 with NaOH.

Solution D. Bath solution: CsCl (35); Cs acetate (105); MgCl$_2$ (2);
                  HEPES (10); EGTA (1),
                  pH adjusted to 7.2 with CsOH.

Solution E. Pipette solution CsCl (140); MgCl$_2$ (2); HEPES (10);
                  EGTA (1),
                  pH 7.2 with CsOH.
4.4 Patch clamp technique

The patch clamp technique allows currents from single channel activations to be observed. A pipette with a small heat polished tip is pushed against a "cleaned" cell membrane, and with the aid of suction a "giga seal" is formed. This high resistance seal reduced background noise and allowed the patch of membrane to be voltage clamped without the use of microelectrodes. The patch clamp technique is described by Hamill et al. (1981).

4.4.1 Patch-electrodes

Patch electrodes were made in three stages from capillary glass, (Garner Glass 7052, internal diameter = 1.15 mm, external diameter = 1.55 mm).

(1) The pipette was pulled on a two-stage vertical puller (David Kopf Instruments). The first pull thinned the glass over a length of about 7-10 mm, the capillary was then recentered with respect to the heating element and with the second pull the capillary was pulled so the thinned part of the capillary breaks producing two pipettes. In general the pipettes had a resistance of 1-5 MΩ. The pipette tip diameter was visually checked using a light microscope (Vickers, x400), and was approximately 0.5-1.0 μm.

(2) The electrode tips were then coated to about 50 μm from the tip with Sylgard™. This reduced the pipette-bath capacitance by forming a hydrophobic surface. Sylgard coating of the electrodes was not essential for giga seal formation, but it served to reduce background noise.

(3) The pipette tip was then polished, using a heated wire installed under the microscope lens (Vickers, x400), heat polishing was stopped when a physical change in the tip geometry could be detected, usually a darkening of the tip. The tip was filled

127
with pipette solution (solution E) by suction and then the pipette was back-filled with the same solution.

### 4.4.2 Application of pyrantel or levamisole

To examine the effects of levamisole and pyrantel, different concentrations of either levamisole or pyrantel were placed in the pipette solution.

Experiments were also performed to determine the effect of intracellular application of levamisole on channel kinetics. In these experiments isolated inside out patches were obtained with 2 μM levamisole in the pipette, levamisole (30-926μM) was then added to the bath solution allowing the compound to also have access to the cytoplasmic side of the patch membrane.

### 4.4.3 Composition of pipette and bath solutions.

The channel under observation in this study was the somatic muscle nicotinic ACh receptor ion channel which is cation selective. In these experiments the pipette and bath solutions contained Cs+ as the main cation, this ion was used to avoid contamination of patch recordings with K+ channels also known to be present in this membrane. The solutions were also Ca+ free to avoid contamination of patch recordings with Ca+ activated Cl− channels known to occur in this membrane (Thorn & Martin, 1987). Solutions were filtered before use (Filter pore size < 0.5 μM).

### 4.4.4 Bath electrodes.

Ag/AgCl wires were used to make the bath electrodes which were connected to ground. To obtain these wires, a silver wire was dipped in molten silver chloride solution.
4.4.5 Mechanical set-up.

The experimental chamber was mounted on the stage of a Reichert-Jung Biostar inverted microscope and viewed at x200 magnification (Figure 4.4. Figure 4.5). The microscope was placed on an Ealing-Bec anti-vibration table. A large concrete slab was placed directly under the anti-vibration table to further reduce mechanical interference. The set up was placed inside an aluminium cage to screen out electrical interference. A List EPC-7 amplifier and head stage were used. The patch pipettes were mounted in a suction pipette holder which was connected to the head stage by a BNC connector.

The pipette holder serves two functions: (1) to connect to provide electrical connection between the BNC connector of the amplifier head stage and the pipette solution; and (2) to allow suction to be applied to the pipette interior. The head stage of the amplifier was mounted on a Narishige hydraulic micromanipulator. This in turn was mounted onto a Prior 78511 coarse manipulator. Currents were recorded unfiltered with a modified Sony digital audio processor (PCM 701ES) and a Sony betamax video recorder (SL-HF100E). Currents were viewed on a Tektronix Dual beam storage oscilloscope (model 5113) and on a two channel pen recorder. Records were analysed using a CED 1401 interface and a IBM 386 PC Computer, with PAT software kindly supplied by John Dempster, Strathclyde University. The records were filtered by an eight-pole Bessel filter, cut off frequency -3dB, 1.5 Khz (Electronic Workshop, R. (D.) S. V. S.). A digitimer was used to trigger an isolated stimulator (Digitimer, model DS 2). This was used to produce a 1 mV voltage pulse at the pipette and allowed the measurement of the pipette and seal resistance.

4.4.6 Measuring the pipette resistance.

A voltage pulse was applied between the pipette and bath ground, the resulting current was then measured. The List EPC-7, converted this current to a voltage. The voltage was displayed on the oscilloscope to enable the determination of the pipette resistance from Ohms law. Pipettes with a resistance of 1-5 MΩ were used.
were only used once, even if a seal was not obtained on the first attempt. This was due to the fact that a clean pipette tip is required for seal formation.
Photograph of the experimental set-up.
Figure 4.5

Schematic diagram showing the electrical set-up of the experimental equipment.
4.4.7 Obtaining a seal

The pipette tip was placed using the micro-manipulator onto the vesicle surface, once contact was made gentle suction was applied via a silicone tube connected to the suction holder and pipette. Suction brought the membrane and the glass pipette tip into closer contact allowing a tight seal to form. Seals with resistances of 1 GΩ or more were used. In this particular set up the best data were obtained with seal resistances of 2 or more GΩ.

When a giga seal was obtained: the area of membrane covered by the pipette known as the patch area and the rest of the vesicle are isolated electrically. If the patch area is a lot smaller than the vesicle area, as in these experiments, the small currents produced by opening of the ACh receptor channel should not affect the membrane potential.

The clamp potential was the difference between the vesicle potential and the pipette potential. It has been previously observed in whole cell recordings from vesicles that the vesicles were without any significant membrane potential (MARTIN, personal communication). So in these experiments the pipette potential represented the membrane potential, but of opposite polarity.

The giga seal is not only electrically tight but is also mechanically stable: this enables the formation of isolated inside-out patches to be used. In this study the vesicle-attached and the isolated inside-out configurations were used (Figure 4.3). Care must be taken when forming isolated inside-out patches, that a closed vesicle structure does not form. This was observed as channel opening, instead of being rectangular in shape, having abnormal channel shapes more easily described as triangular. Resealing of inside out patches can be prevented by omitting divalent cations from the bath solution or chelating the divalent cations with EGTA. EGTA was included in the bath.
solution in these experiments. On the rare occasion that the closed vesicles occurred, exposing the patch to open air and the placing the pipette back in the bath solution usually removed the outer part of the vesicle leaving an isolated inside-out patch.

4.4.8 Comparing the vesicle preparation with intact muscle cell preparation.

The formation of membrane vesicles does not significantly alter the properties of receptor gated ion channels in this membrane. Currents recorded from the GABA gated channel on the somatic muscle of *Ascaris suum* have been studied (Martin, R.J., personal communication). These experiments showed that the channel currents activated by GABA had similar properties when either a muscle flap preparation was used, where the pipette was placed directly on the bag region of the muscle cell, or when the vesicle preparation was used.

Similarly it has been shown that the formation of isolated inside out patches (Trautmann & Seigelbaum, 1983) did not effect channel kinetics in ACh activated channels.

4.4.9 Sources of Background noise.

There are theoretical limits to the resolution of the patch clamp technique due to background noise. Some of the sources of noise and steps which can reduce this noise are listed below.

1. Noise from membrane seal combination.
2. "Shot noise" from ions crossing the membrane eg. through leaky channels or pumps.
3. Intrinsic noise in pipette. The most serious source of this form of noise is from a thin film of bath solution that creeps up the outer wall of an uncoated pipette. The film apparently has a distribution resistance of the order 100 MΩ and a distributed wall capacitance of 3 pF. Sylgard™ coating the pipette reduces the noise considerably.
The hydrophobic surface of Sylgard™ prevents formation of the film and the thickness of the Sylgard™ coating reduces the capacitance. The bulk conductivity of the pipette glass can also be significant. The pipette access resistance and the capacitance of the pipette tip constitute a noise source. This can be reduced by using pipettes with a steeper taper near the tip, which reduces the access resistance, and by extending the Sylgard™ coating closer to the tip which reduces the tip capacitance.

(4) Noise in the current -voltage convertor.

The pipette current is measured as the voltage drop across the high value resistor $R_f$; the noise in this resistor is the dominant noise source in the current / voltage convertor. The other main noise source is the operational amplifier, this noise source can be reduced by minimizing the pipette capacitance, by using a low solution level in the bath and avoiding unnecessary shielding of the pipette and holder.

4.5 Determination of channel conductance and current / voltage relationships.

During each experiment the pipette potential was fixed at a steady level for periods of 100 seconds at voltages in the range of -150 mV to +150 mV. The channel amplitude was calculated as an average value of the amplitude of >20 individual channel openings measured directly from the oscilloscope screen. This value was similar to the channel amplitude calculated by fitting a gaussian curve to the plot of the amplitude histogram calculated from the Pat program. Current / voltage plots were then determined. The slope of these plots gave the channel conductance.

The ionic concentration of the bath and pipette solutions were arranged in order that the predicted reversal potentials for cation and anion selective conductances were known. The pipette and bath-solutions had symmetrical cation concentrations but non-symmetrical anion concentrations (pipette main-ions: Cs$^+$ 140 mM, Cl$^-$ 144 mM, bath main-ions: Cs$^+$ 140 mM, Cl$^-$ 39 mM). Under these conditions, with isolated inside-out patches, the predicted Nernst reversal potentials were determined by the following equations:
for an cation,
\[ E_{\text{rev}} = \frac{RT}{FZ} \log_e \frac{[X]_o}{[X]_i} \]  
 ..........(3)

and for an anion,
\[ E_{\text{rev}} = \frac{RT}{FZ} \log_e \frac{[X]_i}{[X]_o} \]  
 ..........(4)

where \( E_{\text{rev}} \) is the expected reversal potential, \( Z \) is the numerical value of the charge on the ion, \( R \) is the gas constant (8.314 J K\(^{-1}\) Mol\(^{-1}\)), \( T \) is the temperature in degrees Kelvin, and \( F \) is Faraday's number (9.6 \times 10^4 C Mol\(^{-1}\)). Inserting the concentrations of either Cl\(^-\) or Cs\(^+\) into these equations gave the predicted reversal potential for a cation selective channel as 0 mV, and for an anion selective channel as -33 mV.

As a further test of the permeability of the channel the bath-solution was diluted 50:50 with distilled water. This altered the ionic composition of the bath solution (bath main- ions becomes: Cs\(^+\) 70 mM, Cl\(^-\) 19.5 mM). The predicted effect of this was to produce a shift in the Nernst reversal potential from 0 mV to +18 mV for a cation selective channel and from -33 mV to -51 mV for an anion selective channel.

The relative permeability of Cs\(^+\) and Cl\(^-\), were calculated of using the Goldman, Hodkin, Katz constant field equation (Hille, 1984):
\[ E_{\text{rev}} = \frac{RT}{F} \ln \frac{\alpha [Cs^+]_o + [Cl^-]_i}{\alpha [Cs^+]_i + [Cl^-]_o} \]  
 ..........(5)

Where \( E_{\text{rev}} \) was the observed reversal potential; \( \alpha = \text{PCs}^+ / \text{PCI}^- \), where PC\(s^+\) was the permeability of Cs\(^+\) and PCI\(^-\) was the permeability of Cl\(^-\). Thus the relative permeabilities of Cs\(^+\) was calculated relative to Cl\(^-\).
4.6. Data processing.

The first stage of single-channel analysis was to obtain the distributions of open and closed durations. These distributions were then fitted to exponential components where the number of components can be taken as the minimum estimate of the number of open or closed states (COLQUHOUN & HAWKES, 1982). The records were clearly divided into bursts of openings where $T_{cr}$ was determined from the closed time distributions (section 4.6.3). Once this was performed a mechanism whereby the open and shut states were connected to each other was suggested. In general all of the connections cannot be worked out unambiguously, instead a hypothesis is put forward which best fits the experimental data. In these experiments the simple channel block scheme was used.

These procedures are outlined below.

4.6.1 Setting the threshold level and transition detection.

In general when selecting the threshold level, a value between 0.4 and 0.7 of the channel amplitude is used. The filter cut off frequency is then adjusted so that the rms noise is 1/3 to 1/5 of the threshold value COLQUHOUN & SIGWORTH (1983). In these experiments a 70% threshold value was used and the filter cut off frequency was set so that the rms noise was 1/3 of the threshold value.

In general the data was filtered by an eight-pole Bessel filter, -3dB at 1.5 KHz. It is best to keep the rise time of the filter as short as possible. Therefore filtering was usually at 1.5 KHz or in slightly noisier recordings 1 KHz. The data was then analysed using the Pat software. For recordings filtered at 1.5 KHz the sampling interval was set at 70$\mu$s (14.3 KHz). The sampling interval should at least be twice as fast as the filter frequency, an ideal value is ten times as fast (COLQUHOUN & SACKMANN, 1985).

The Pat software was used to construct amplitude histograms of the currents recorded. Transition detection was performed after calculation of the current
amplitudes and setting of threshold levels. A list of open and closed durations were obtained which were transferred to the university mainframe for further analysis. Histograms of the open and closed durations were obtained from the Pat program. After the determination of $T_{\text{crit}}$ value (section 4.6.3), histograms of burst durations were also obtained from the Pat program. The open-time distributions were used to ensure that the minimum resolvable interval calculated from the dead time of the filter was suitable for the particular set of data. The first few bins of the distribution are shown to fall short of the expected exponential distribution. The minimum resolvable interval must be greater than the maximum value of any of these bins.

### 4.6.2 Measurement of rise time and dead time of the filter.

At the frequencies used in these experiments (1, 1.5 and 2.5 KHz) the rise times and dead times of the filter were calculated. The rise time was measured from time taken to reach the 90% level from the 10% level of a pulse. The dead time is the time taken for a pulse when filtered to reach 50% of the original rectangular pulse.

<table>
<thead>
<tr>
<th>Filter frequency</th>
<th>rise time</th>
<th>dead time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 KHz</td>
<td>0.36 ms</td>
<td>0.20 ms</td>
</tr>
<tr>
<td>1.5 KHz</td>
<td>0.22 ms</td>
<td>0.14 ms</td>
</tr>
<tr>
<td>2.5 KHz</td>
<td>0.16 ms</td>
<td>0.08 ms</td>
</tr>
</tbody>
</table>

The minimum resolvable interval should be set at least twice the value of the dead time.

### 4.6.3 Definition of a burst

In these experiments, individual channel activations were interrupted with short closed periods (the "Nachschlag" phenomenon, COLQUHOUN & SAKMANN, 1985), so bursts of openings as well as single openings were considered. Bursts were defined as
groups of openings separated by gaps that are shorter than a specified length, \( T_{\text{crit}} \).

\( T_{\text{crit}} \) was determined by numerically solving the following equation:

\[
1 - e^{-T_{\text{crit}}/T_1} = e^{-T_{\text{crit}}/T_2}
\]  \hspace{1cm} (6)

where \( T_1 \) represented the brief closed state \( T_1 \), and \( T_2 \) represented the slightly longer closed state \( T_2 \). The equation found a value for \( T_{\text{crit}} \) which balanced the proportion of gaps within a burst misclassified (as gaps between bursts) and gaps between a burst misclassified (as gaps within a burst) (COLQUHOUN & SAKMANN, 1985). In general a value of 1 ms was used in these experiments. This particular value was not so important since gaps between bursts were several hundred fold larger than gaps within bursts.

From the distribution of shut times, it appeared that many brief events were too short to be observed, thus individual openings within a burst actually consisted of a partially resolved burst of openings where the mean length depended on the resolution of the data and the brevity of the closings. To circumvent this problem bursts of openings were considered. The length of a burst will be little effected by the failure to detect short closings (COLQUHOUN & SACKMANN, 1985).

**4.6.4 Fitting exponential components to the open, closed and burst durations.**

The idealised reconstruction of the apparent open and closed durations were transferred to the mainframe computer where further analysis was performed. Values less than the minimum resolvable duration (0.3 ms) of the set up were removed from the data files. Exponential components were fitted to the the open-, closed-, and burst-times by the method of maximum likelihood (COLQUHOUN & SACKMANN, 1981;
COLQUHOUN & SIGWORTH, 1983) to a probability density function (p.d.f.) of the form:

$$p.d.f. = \sum_{i=1}^{k} \frac{A_i e^{-t/T_i}}{T_i} \quad \ldots \ldots \ldots (7)$$

where $A_i =$ the area of the $i$th component; $T_i =$ the fitted time constant; $t =$ time and

$K =$ number of exponentials fitted. Because the lifetimes of states are random variables, their behaviour must be described by probability distributions. The p.d.f. is clearly not a probability (its dimensions are in $s^{-1}$), but it is as a function such that the area under the curve (which is dimensionless) between any specified values is the probability of observing a lifetime between these values (COLQUHOUN, 1987).

The method of maximum likelihood consists of varying the values of the hypothesis (estimates of time constants and areas) so as to maximise the following expression:

$$\text{Probability [Data given hypothesis]} = \text{likelihood [hypothesis given Data]}$$

Thus the estimates of time constants and areas were chosen that maximised the probability of observing the data. This method was used in preference to fitting the histogram data where some information is lost due to the original time intervals being pooled into groups represented as bins in the open time distribution.

The fitting of exponential components was performed on the University mainframe computer using Fortran programs which utilised NAG subroutines E04JAF & E04CCF, written and tested by Dr. R. J. Martin and Prof. B. Ginsborg.

These programs calculated an F-function value (the likelihood) which showed whether one, two or three exponential components best described the data. The F-
function value represented the sum of values obtained from the p.d.f. equation when
the estimates for the time constants (T) were compared to the actual data (t). For
example the computer would calculate a p.d.f. value from the following equation when
estimates for $A_1$, $T_1$ and $T_2$ were imputed along with the measured values (files of
open or closed durations obtained from Pat).

$$
p.d.f. = \frac{A_1 e^{-t/T_1}}{T_1} + \frac{A_2 e^{-t/T_2}}{T_2} \quad \ldots \ldots (8)$$

The computer programs found values which gave the lowest f value for the p.d.f.
when using the estimates ($A_1$, $T_1$ and $T_2$) along with the actual values. An attempt was
subsequently made to fit three exponential components to the data. The same process
was repeated where estimates for $A_1$, $A_2$, $T_1$, $T_2$, $T_3$. This also gave an F-value,
which was compared to the F-value obtained when fitting two exponential components
to the data. In general for an additional component fitted to the data to represent a
statistically significant better fit, the F-value must drop by three points (G Glasbey,
ADAS, Statistics Unit, Edinburgh).

When analysing the results obtained with the anthelmintics levamisole and pyrantel,
in general the open durations and burst durations were best fitted by two exponential
components whereas the closed durations were best fitted by three exponential
components. This indicated that there were at least two open states and two burst states
and at least three closed states. A burst distribution for example, may be fitted by 2
exponential components. In general it is not correct to identify the components with
particular physical events or to use "short bursts" or "long bursts" as though there were
2 quite separate types of phenomena. Nevertheless such terms are generally used
(Colquhoun and Sakmann, 1985) as a way of discussing the mathematical components
which were identified; in some cases the components do have an approximate physical
significance (Colquhoun and Hawkes, 1982). Therefore, in describing these results I
used the terms "short" and "long" openings when the open-time distribution have been
fitted by 2 exponentials, "short" and "long" bursts when the burst distributions have
been fitted by 2 exponentials, and "short", "intermediate" and "long" closings when the
closed-time distributions have been fitted by 3 exponentials.

4.6.5 Correction for missing events

After exponential curve fitting, the data was corrected for missing events. This was
carried out to compensate for brief events missed due to limited time resolution of the
recording apparatus. After exponential components were fitted to the apparent burst
and closed durations, it was possible to correct the data for missing events, using the
method of COLQUHOUN & SIGWORTH (1983) and COLQUHOUN and SAKMANN
(1985), which is outlined below. The assumption was, very short closings and very
short openings have been missed in a standard analysis of the open and closed
 durations because of limitations in the resolution of the recording technique. It was
possible to estimate the number of short gaps that were missed by extrapolation of the
distribution of shut times to zero time. The total open time could also be estimated by
extrapolation of the distribution of burst length. It was therefore possible to estimate the
mean length of a single opening and the mean number of openings per burst though
these quantities cannot be observed (COLQUHOUN & SIGWORTH (1983) and
COLQUHOUN and SAKMANN (1985).

The first stage in this correction process was to estimate the total number of events
from the observed number of events, this was done separately for closings and for
bursts using the general equation:

\[
N = \frac{1}{k} \sum_{i=1}^{n} a_i \left[ e^{-t_{\text{min}} / T_i} - e^{-t_{\text{max}} / T_i} \right]
\]

\[...........(9)\]
For bursts: \( n_{\text{bursts}} \) was the number of observed bursts greater than \( t_{\text{min}} \), and \( N_{\text{bursts}} \) was the estimated total number of bursts, i.e., the corrected number of bursts. For closings: \( n_{\text{closings}} \) was the number of observed closings greater than \( t_{\text{min}} \) and \( N_{\text{closings}} \) was the estimated total number of closings; \( t_{\text{min}} \) was the minimum resolvable duration, \( t_{\text{max}} \) was the length of the sample being analysed. \( K \) was the number of exponential components fitted (\( K=2 \) for bursts, \( K=3 \) for closings) and \( a \) represented the area and \( T \) represented the time constant of the \( i \)th component.

The observed closings, fitted by 3 exponentials, consisted of 3 components: brief closings (\( T_1 \)); intermediate closings (\( T_2 \)); and long closings (\( T_3 \)). Their relative proportions were represented by the areas \( A_1 \), \( A_2 \) and \( A_3 \), the sum of the areas were equal to one. Assuming that the total number of closings consisted of the same 3 components in the same ratio, you could calculate values for the number of short, intermediate and long closings. For example, the corrected number of short closings was \( N_{\text{closings}} \) multiplied by \( A_1_{\text{closings}} \). This was also applied to bursts which had 2 components (\( A_1 + A_2 = 1 \)) and gave corrected values for numbers of short and long bursts.

The next stage in the correction process was to calculate the total time spent in closings within bursts, i.e., closings shorter than \( T_{\text{crit}}(M_{\text{TS}}) \), including those that were undetected:

\[
M_{\text{TS}} = N_{\text{Closings}} \left[ \sum_{i=1}^{K} a_i T_i - \sum_{i=1}^{K} a_i (T_{\text{crit}} + T_i) e^{-T_{\text{crit}}/T_i} \right]
\]

\[\text{........(10)}\]

where \( N_{\text{closings}} \) was the total number of closings calculated from equation (9) and the other symbols have the same meanings as above. This equation used the values of \( a \) and \( T \) for closings; \( \sum a_i T_i = \text{length of overall mean closing} \).
The corrected number of short closings per long burst \( (N) \) was the corrected number of short closings divided by the corrected number of long bursts:

\[
N = \frac{N_{\text{closings}} \times A_{\text{closings}}}{N_{\text{bursts}} \times A_{\text{bursts}}} \] ...........(11)

The value for \( N \) was longer than the observed number of short gaps per burst, because allowance has been made for missed short gaps.

The corrected mean burst length was calculated by the following equation:

\[
MB = \sum_{i=1}^{k} a_i T_i \] ...........(12)

where \( a_i \) and \( T_i \) are the values estimated from the exponential components fitted to the burst duration:

\[
MB = (A_{1\text{bursts}} \times T_{1\text{bursts}}) + (A_{2\text{bursts}} \times T_{2\text{bursts}}) \] ...........(13)

The corrected mean open-time in long bursts \( (E) \) was shorter than the observed mean open-time in long bursts because an allowance was made for missed short closings:

\[
E = \frac{[(N_{\text{bursts}} \times A_{2\text{bursts}}) \times T_2] - M_{\text{TS}}}{N_{\text{closings}} \times A_{1\text{closings}} + (N_{\text{bursts}} \times A_{2\text{bursts}})} \] ...........(14)

The corrected total open-time \( (C_{\text{TOT}}) \) can be calculated by allowing for undetected gaps within bursts using the values for \( N_{\text{bursts}} \) (equation 9) \( MB \) (equation 13) and \( M_{\text{TS}} \) (equation 10):
CTOT = (N_{bursts} \times MB) - MTS \hspace{1cm} \ldots \ldots (15)

The corrected open probability (COP) was calculated as follows:

\[
COP = \frac{CTOT}{t_{\text{max}}} \hspace{1cm} \ldots \ldots (16)
\]

This made the assumption that in each sample analysed there was only one of the main state conductance channels opening. This was believed to be correct, as double openings were only observed very occasionally and such samples were not analysed. If the number of channels per patch was greater than 1, the values given for open probability were over estimates.

Corrected mean open-time (CMOT) was not calculated by the same method as used to calculate the corrected mean burst-time (equation 8), as this method would not consider unresolved closings. Instead the (CMOT) was calculated by the following equation:

\[
CMOT = \frac{CTOT}{N_{\text{closings}}} \hspace{1cm} \ldots \ldots (17)
\]

The corrected mean open-time is shorter than the mean open-time calculated in any other way, because the correction for missed short events reduced the mean value.

4.6.6 Nachschalag phenomenon

It has been observed that single openings of the ion channel were interrupted by brief closings. This phenomenon is known as "Nachschalag" (COLQUHOUN & SACKMANN, 1981, 1985). It is thought that the Nachschalag phenomenon resulted from multiple openings of the occupied receptor. It is also possible that these brief closing represent a short duration desensitised state distal to the open state or thermal fluctuations of the
channel protein which result in the pore being briefly occluded or empty of ions. If the Nachschalag phenomenon is a result of multiple openings then the qualities of these brief closings (ie. length) should be dependent on the nature of the agonist. The duration or the number of these gaps should not show dependence on agonist concentration or voltage dependence. The Nachschlag closings can thus be differentiated from gaps due to channel-block, as these blockages tend to be both voltage and concentration dependent. It is also unlikely that these gaps are caused by blockage of the channel by intracellular or extracellular constituents because the characteristics were little changed when: (1) extracellular hydrogen ion or calcium ion concentration was reduced to 1 tenth of normal; (2) extracellular calcium was replaced by magnesium; (3) intracellular chloride concentration was raised; or the normal intracellular constituents were replaced by KCl (Colquhoun & Sackmann, 1985; Colquhoun et al., 1986).

4.6.7 The simple Channel-block model.

It has been reported that certain compounds (local anesthetics, high concentration of agonist) can depress electrical activity in nerves and muscle cells by binding to sites within the ion channel and blocking the current. Evidence exists that block can occur with both cationic charged compounds (Neher & Steinbach, 1978) and uncharged local anaesthetics (Ogden et al., 1981). In the absence of the blocking substance miniature end plate currents decayed along a single exponential time course. After the addition of the channel blocker, the peak amplitude reduced and the current decline became biphasic. This decay could now be well fitted by the sum of two exponential components. This agreed well with the simple channel-block model which predicted that in the presence of drug the miniature end plate currents decayed as the sum of a rapid and slow component whose rates are speeded and slowed respectively with increasing drug concentration. Recordings of single ion channels show more directly the behaviour expected of a channel blocker. In the absence of the blocking drug,
single channel activations resembled rectangular current pulses which stayed open for a random period that was exponentially distributed. Addition of the channel blocking drug resulted in the channel openings occurring as bursts of openings composed of a series of rapid openings and closings. These bursts are interpreted as multiple cycles of the blocking-unblocking reaction which occurred when the channel reached the open state. Flickering channel-block can be analysed by fitting the data to the simple channel-block scheme. This is a sequential reaction scheme in which an open channel can either close or reversibly bind a drug molecule. The open channel with the drug molecule bound, would have a much lower conductance than the open channel. This model has been described by: ADAMS (1976, 1977); RUFF (1977); OGDEN et al. (1981); NEHER (1983); COLQUHOUN & SAKMANN (1985); and OGDEN & COLQUHOUN, 1985):

\[
\begin{array}{ccc}
  C & \xrightarrow{\alpha} & O \\
  \beta & \xrightarrow{k_{+B}X_B} & B \\
  & k_B & \\
\end{array}
\]

Where C denotes the closed state; O denotes the open state; B denotes the blocked state; \(X_B\) is the drug concentration; \(k_{+B}\) is the blocking rate constant; \(k_B\) is the unblocking rate constant; \(\alpha\) is channel closing rate and \(\beta\) is the channel opening rate. This reaction scheme can also be represented as \(nT + R \leftrightarrow TnR \leftrightarrow TnR^* \leftrightarrow TnRB\) where \(nT + R \leftrightarrow TnR\) represented the closed state, \(TnR^*\) represented the open state, and \(TnRB\) represented the blocked state. This allowed the question of how many agonist molecules were required to open the channel to remain unanswered. The open-times, closed-times and blocked-times are random variables with exponential distributions and using the simple channel block reaction scheme, predictions can be made about probability density functions and mean values of these variables. Working from the general assumption that the mean lifetime of any state can be found by adding all the values for the transitional rates that lead out of that state and taking the reciprocal of the
sum (Colquhoun & Hawkes, 1983), values for the rate constants shown in the channel block scheme were determined.

The main difference when applying the simple channel-block scheme to results from channel-block by charged and uncharged channel blockers, is that with the charged blockers the association and dissociation rate constants depend on voltage whereas with the uncharged blockers, the association and dissociation rate constants are independent of voltage.

The simple channel block model predicts that each individual opening will be shorter in the presence of the blocking agent. The mean open-time can be calculated as the reciprocal of the reaction rates away from the open state:

\[
\text{mean open-time} = \frac{1}{(\alpha + k_{+B}X_B)}, \quad \text{........(18)}.
\]

This predicts a linear dependence of the reciprocal open-time on blocker concentration which can be used as a test for the simple channel block model (Neher, 1983; Colquhoun & Hawkes, 1983). If this test was passed then a value for \(k_{+B}\) can be estimated from this plot. The slope of this plot can be used to determine values for \(k_{+B}\) and the intercept \(\alpha\).

Another approach would be to ignore these spontaneous events and to look at the whole elementary event (the duration of a burst of openings produced by a single activation of the channel). A plot of the reciprocal of the open time per burst against concentration should be linear and also give a test as to whether this simple channel block model is sufficient to describe the data (Ogden & Colquhoun, 1985). The total time on average that a channel spends in the open state after the initial opening should be invariant. This can be explained as such: once a channel enters the open state it has to spend (on average) a certain time in that state in order to find its way back into
the closed state. The time interval spent in the blocked state should not add to the chance of the channel exiting to the closed state.

The mean number of openings per burst ($M_r$):

$$M_r = 1 + \frac{k_{+B} X_B}{\alpha}$$

...............(19)

Thus a plot of $M_r$ against concentration would be linear with slope representing $k_{+B}/\alpha$.

The mean number of closings per burst, $N_b$, is represented by:

$$N_b = \frac{k_{+B} X_B}{\alpha}$$

...............(20)

The mean open-time within a burst equals the mean open-time multiplied by the mean number of openings per burst:

$$\left(1 + \frac{k_{+B} X_B}{\alpha}\right) \left(\frac{1}{\alpha + k_{+B} X_B}\right) = 1/\alpha.$$  

...............(21)

Thus the mean open-time per burst is exactly what the mean length of a burst if no blocker was present. This explains the inefficiency of channel-block in reducing the equilibrium current when agonist concentration is low. The $P_{open}$ would remain the same in the presence or absence of blocker. $P_{open}$ would only reduce if channel-block was extending the duration of bursts so that activations ran into each other i.e. with high open channel frequency.

The mean closed time per burst is calculated as the mean number of gaps per burst times the mean blocked lifetime:

$$\text{mean closed time per burst} = \left(\frac{k_{+B} X_B}{\alpha}\right) \left(\frac{1}{k_{-b}}\right) = \frac{k_{+B} X_B}{\alpha k_{-b}}.$$  

...............(22)
since $K_B = k_{-B} / k_{+B}$

\[
\text{mean closed time per burst} = \frac{X_B}{K_B} \alpha 
\]

\[
\text{mean burst length} = \frac{X_B}{K_B} \alpha + \frac{1}{\alpha} 
\]

\[
= \frac{(1 + X_B / K_B)}{\alpha} 
\]

This equation predicts that the mean burst length increases linearly with concentration of blocker. A plot of the mean burst length against concentration would have slope $1 / \alpha K_B$, and an intercept of $1 / \alpha$.

When activations of the channel are frequent (at high agonist concentrations) it is difficult to decide what represents the end of one burst and the beginning of another, so with this sort of data the prediction that the mean open-time per burst should remain unaltered by the channel blocker cannot be tested. A similar test can be performed by a blockage frequency plot (OGDEN & COLQUHOUN, 1985), as long as the component regarded as blockages can be distinguished in the closed-time distribution. Blockage frequency plots are obtained from plots of estimates of the corrected number of short gaps (equation 7) / corrected total open time (equation 11) plotted against concentration. The frequency of channel blockage per unit open time should be simply $k_{+B} X_B$. Thus a plot of blockage frequency per unit time against concentration should give a straight line with slope $k_{+B}$ (OGDEN & COLQUHOUN, 1985). If the blocker became trapped within a shut channel or if the channel were to shut directly from the blocked state without returning to the open state, a slope of less than $k_{+B}$ would be predicted.

The mean block durations were determined by selecting the distinctive blocked times seen during flickering bursts greater than 0.3 ms (the limit of resolution of the set-up). The method of maximum likelihood was then used to fit a single exponential to obtain a
value for the mean block duration. Where the mean block-time (MBT) represented the reciprocal of the unblocking rate constant.

\[ \text{MBT} = \frac{1}{k_B} \]  

(25)

This simple treatment requires that the blocked periods can be distinguished from the closed periods. It is pointed out that in these experiments there was inevitably some overlap between the blocked time and the "Nachschlag" closings, however this error is minimal as the interruptions due to channel block far exceed the number of interruptions due to the "Nachschlag" closings. The mean number of short gaps per burst increases roughly linearly with concentration. The simple channel block model predicts that for a cationic blocker, the duration of blockages should be voltage dependent, ie. the mean duration and the frequency of blocking events increased with membrane hyperpolarisation.

The above analysis can be used to estimate the channel block dissociation constant:

channel-block dissociation constant, \( K_B = \frac{k_B}{k_{+B}} \)  

(26)

For a charged blocker the model predicts that: the reaction rates \( k_B \) and \( k_{+B} \), and the dissociation constant \( K_B \) should depend exponentially on voltage; the forward and backward blocking reaction rates should have equal but opposite voltage dependencies; the apparent blocking rate increases linearly with concentration; and the unblocking rate is concentration independent.

### 4.6.8 Calculation of the blocking site within the channel pore.

At conventional membrane potentials the inside of the cell is more negative that the outside, therefore positively charged molecules (eg. levamisole, pyrantel, ACh) will tend to move from the extracellular solution towards the inside of the cell. It is not surprising that the extent of block increased as the inside of the cell was made more negative (hyperpolarised). The Boltzmann distribution can be employed to
quantitatively treat this problem. The Boltzmann distribution can be stated as follows: suppose it takes an amount of work \( w \) to move one molecule from position 1 to position 2, then the relative probability (or concentration) of a molecule being at 2 rather than 1, will be:

\[
P_2/P_1 = e^{-w/kT}
\]

(27)

where \( k \) is the Boltzmann constant, and \( T \) is the absolute temperature. \( kT \) is a measure of the thermal energy of a molecule. If position 2 has lower energy, \( w \) is negative, and molecules will tend to accumulate at position 2. This will happen to the extent that the energy difference \( w \) is substantial compared with the thermal energy \( (kT) \), because the thermal energy will tend to mix up the solution and make the concentration the same everywhere (Hille, 1984).

The above theory can be applied to channel-block if you assume that the binding site for the channel is part way through the channel pore in a position where the electrical potential, relative to the external solution is \( \delta E \), where \( E \) is the membrane potential. For example the molecule has to move through a fraction \( \delta \) of the electrical field across the membrane to reach its binding site. The work needed to move one unit charge through a potential difference \( \delta E \) is:

\[
w = Ec
\]

(28)

where \( c \) is the unit charge \( (1.602 \times 10^{-19} \text{ C}) \). The ratio of the equilibrium constant for binding at membrane potential \( E \), \( K(E) \) say, relative to that for binding in absence of a potential difference, \( K(0) \) is given as:

\[
K(E) / K(0) = e^{-\delta Ezc / kT} = e^{-\delta EzcF / RT}
\]

(29)

where \( F \) is Faraday number and \( R \) is the gas constant, \( N_a = \text{Avagadro's number} \) and \( z \) is the number of charges on the molecule. Results can be described by the equation which is written as:

\[
K(E) / K(0) = e^{-E/H}
\]

(30)
where \( H \) is the number of millivolts needed for a e-fold change in the equilibrium constant.

\[
H = \frac{RT}{zF} \tag{31}
\]

where \( \frac{RT}{F} = kT/c = 25.26 \text{ mV at } 20.\text{°C} \)

If one assumes a constant membrane field, then you can determine the relative position of the binding site (6) for either levamisole or pyrantel across the membrane.

4.7. Determination of the pKa of levamisole

A plot of the dependence of the pH on the addition of \( \text{OH}^- \) to a solution of levamisole yields a titration curve. The inflection point of this curve is the pKa. In these experiments the pKa of levamisole was determined by titrating a 10 mM solution of levamisole (pH adjusted to ~2.0 with HCl) with Molar NaOH. The pKa was then determined from the resulting graph.

4.8 Statistical methods

4.8.1 Mean and standard error

Values are presented in this thesis as the sample mean \( \pm \) the standard error.

4.8.2 Student's T-tests

Levels of significance were obtained using Student's two tailed T-test, a level of \(< 0.05 \) was used to indicate significance.

4.8.3 Linear regression

The method of least squares was used to fit a straight line to data (Current / voltage relationships). A Spearman correlation coefficient \( (r) \) was used as a test to indicate if the data could be represented a straight line.
4.8.4 Exponential distribution

The method of least squares was used to obtain the best fits for the exponential equations used to describe the voltage-sensitivity of the rate constants $k_{+B}$ and $k_{-B}$ and the dissociation constant $K_B$. A Spearman correlation coefficient ($r$) was used to test the fit of the equation.

4.9.0 Drugs

Levamisole, (-)-(5)-2,3,5,6-tetrahydro-6-phenylimadaz[2,1-b]thiazole was obtained as levamisole hydrochloride from Sigma chemical company, Fancy Road, Poole, Dorset. BH17 7NH.

Pyranthel was obtained as pyranthel tartrate, kindly supplied by Pfizer, Sandwich, Kent. CT13 9NJ.

Collagenase, Sigma type 1A, was also obtained from Sigma chemical company.
CHAPTER 5
RESULTS SECTION 1

5. Levamisole activated single channel currents from muscle of the nematode parasite
Ascaris suum.

5.1 Introduction

Studies on the mode of action of levamisole indicate it is a selective agonist at
acetylcholine receptors present on the muscle cells of parasitic nematodes. One such
nematode is Ascaris suum. Levamisole and acetylcholine treatment of Ascaris suum
results in depolarisation and spastic paralysis of the muscle (Aceves et al., 1970; Van
Neuten, 1972; Coles et al., 1975; Martin, 1982; Harrow & Gration, 1985;
Colquhoun et al., 1991).

The aim of the present study was to investigate the action of levamisole at the single-
channel level in Ascaris using the extrasynaptic ACh receptors present in isolated
muscle vesicles (Martin et al., 1990; Pennington & Martin, 1990). Our results
show that levamisole opens cation selective channels, but in addition, produces open
channel-block and desensitisation.

5.2 Results

With levamisole in concentrations between 1-90 μM, in the patch-pipette, channel
currents in 43% of 143 cell-attached and isolated inside-out patches were recorded.
These channels had apparent mean open-times in the millisecond range and amplitudes
of around 2.5 pA at -75 mV. Detailed analysis of channel data from a single patch
recorded from both the cell-attached and the isolated inside-out configuration showed
no significant differences in channel kinetics. In 20 experiments with levamisole
omitted from the patch pipette solution no such channel openings were observed. It was
therefore concluded that levamisole activated these currents.
5.2.1 Channel conductance and Current / Voltage relationships

The pipette and bath-solutions had symmetrical cation concentrations but non-symmetrical anion concentrations (pipette main-ions: Cs+ 140 mM, Cl- 144 mM, bath main-ions: Cs+ 140 mM, Cl- 39 mM). Under these conditions with isolated inside-out patches, the predicted Nernst reversal potential for a cation selective channel is 0 mV, and for an anion channel is -33 mV. In 26 out of 31 experiments the current / voltage plots of levamisole activated channels were linear with reversal potentials near 0 mV (Figure 5.1, Table 5.1) indicating that these channels were cation selective and could conduct Cs+. The slopes had an average conductance of 32.9±1.23 pS (mean± s.e., n=26) and ranged between 19-46 pS.

As a further test of the permeability of the channel the bath-solution was diluted 50:50 with distilled water. The predicted effect of this is to produce a shift in the Nernst reversal potential from 0 mV to +18 mV for a cation selective channel and from -33 mV to -51 mV for an anion selective channel. In the experiment illustrated in Figure 5.1, the reversal potential shifted from 0 mV to +13 mV.

Calculation of the relative permeability of Cs+ and Cl- using the Goldman, Hodgkin, Katz constant field equation (HILOE, 1984), revealed that Cs+ was ten times more permeable than Cl-. Similar results were obtained in three other experiments.
Figure 5.1

A

B

157
Table 5.1  Conductance and reversal potential for levamisole activated channels

<table>
<thead>
<tr>
<th>Conductance (pS)</th>
<th>Reversal potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>5.59</td>
</tr>
<tr>
<td>39</td>
<td>1.99</td>
</tr>
<tr>
<td>32</td>
<td>6.53</td>
</tr>
<tr>
<td>39</td>
<td>3.76</td>
</tr>
<tr>
<td>34</td>
<td>-7.44</td>
</tr>
<tr>
<td>32</td>
<td>13.79</td>
</tr>
<tr>
<td>34</td>
<td>13.64</td>
</tr>
<tr>
<td>32</td>
<td>-2.95</td>
</tr>
<tr>
<td>35</td>
<td>2.83</td>
</tr>
<tr>
<td>31</td>
<td>-2.97</td>
</tr>
<tr>
<td>28</td>
<td>3.06</td>
</tr>
<tr>
<td>26</td>
<td>16.70</td>
</tr>
<tr>
<td>46</td>
<td>3.92</td>
</tr>
<tr>
<td>31</td>
<td>6.31</td>
</tr>
<tr>
<td>40</td>
<td>-6.04</td>
</tr>
<tr>
<td>27</td>
<td>-1.70</td>
</tr>
<tr>
<td>36</td>
<td>4.70</td>
</tr>
<tr>
<td>36</td>
<td>2.80</td>
</tr>
<tr>
<td>35</td>
<td>-8.24</td>
</tr>
<tr>
<td>39</td>
<td>1.83</td>
</tr>
<tr>
<td>44</td>
<td>4.17</td>
</tr>
<tr>
<td>24</td>
<td>-8.98</td>
</tr>
<tr>
<td>19</td>
<td>6.37</td>
</tr>
<tr>
<td>25</td>
<td>2.79</td>
</tr>
<tr>
<td>25</td>
<td>11.96</td>
</tr>
<tr>
<td>26</td>
<td>2.72</td>
</tr>
</tbody>
</table>

32.9 ± 1.23 pS  2.97 ± 1.30 mV
5.2.2 Exponential curve-fitting

Channel data from 31 patches were analysed at various membrane potentials, ranging from +150 mV to -150 mV. The data were best-fitted by the sum of exponentials (see Methods).

With 2-10 μM levamisole in the pipette the open- and burst-time distributions were best-fitted by two exponentials, while closed-time distributions were best-fitted by three exponentials (Table 5.2, 5.3, 5.4). Since the number of open- and closed- states must at least equal the number of exponential components fitted, levamisole-activated channels in Ascaris have at least two open-states, at least two burst-states and at least three closed-states. Higher concentrations of levamisole (30 μM & 90 μM) produced substantial changes in the distribution of both the open and closed-times, these changes are considered in the sections on channel-block and desensitisation.
Table 5.2. Open-time kinetics for levamisole activated channels.

<table>
<thead>
<tr>
<th>Levamisole Conc.</th>
<th>membrane potential</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-10 μM</td>
<td>-50 mV</td>
<td>0.63±0.10</td>
<td>0.37±0.10</td>
<td>0.87±0.12</td>
<td>2.55±0.30</td>
</tr>
<tr>
<td>30 μM</td>
<td></td>
<td>0.83±0.08</td>
<td>0.17±0.08</td>
<td>0.19±0.08</td>
<td>0.91±0.06</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>-75 mV</td>
<td>0.66±0.06</td>
<td>0.34±0.06</td>
<td>0.88±0.10</td>
<td>2.30±0.24</td>
</tr>
<tr>
<td>30 μM</td>
<td></td>
<td>0.78±0.09</td>
<td>0.22±0.09</td>
<td>0.37±0.09</td>
<td>0.69±0.08</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>-100 mV</td>
<td>0.59±0.08</td>
<td>0.41±0.08</td>
<td>0.86±0.12</td>
<td>2.54±0.36</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>+50 mV</td>
<td>0.45</td>
<td>0.55</td>
<td>0.81</td>
<td>1.44</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>0.90</td>
<td>0.10</td>
<td>0.22</td>
<td>1.00</td>
</tr>
<tr>
<td>3-10 μM</td>
<td>+75 mV</td>
<td>0.62±0.10</td>
<td>0.38±0.10</td>
<td>0.71±0.10</td>
<td>1.60±0.14</td>
</tr>
<tr>
<td>30 μM</td>
<td></td>
<td>0.66±0.07</td>
<td>0.34±0.07</td>
<td>0.48±0.07</td>
<td>1.22±0.29</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>0.66±0.08</td>
<td>0.34±0.08</td>
<td>0.64±0.10</td>
<td>2.02±0.28</td>
</tr>
<tr>
<td>3-10 μM</td>
<td>+100 mV</td>
<td>0.79±0.12</td>
<td>0.21±0.12</td>
<td>0.71±0.08</td>
<td>2.35±0.50</td>
</tr>
<tr>
<td>30 μM</td>
<td></td>
<td>0.81±0.04</td>
<td>0.19±0.04</td>
<td>0.50±0.10</td>
<td>2.03±0.47</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td>0.67±0.06</td>
<td>0.33±0.06</td>
<td>0.65±0.07</td>
<td>1.88±0.24</td>
</tr>
</tbody>
</table>

Values for T1 and T2 are in ms. T1 and T2 are the time constants representing the two open states. The proportion of brief openings (T1) and the long openings (T2) are represented by the areas A1 and A2. Values are mean±s.e.
Table 5.3. Closed time kinetics for levamisole-activated channels.

<table>
<thead>
<tr>
<th>levamisole Conc.</th>
<th>membrane potential</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-50 mV</td>
<td>(n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 µM</td>
<td>0.47±0.02</td>
<td>0.33±0.06</td>
<td>0.21±0.05</td>
<td>0.30±0.03</td>
<td>251±44</td>
<td>1062±266</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 µM</td>
<td>0.48±0.04</td>
<td>0.15±0.03</td>
<td>0.37±0.04</td>
<td>0.32±0.02</td>
<td>22±11</td>
<td>152±63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.53</td>
<td>0.18</td>
<td>0.19</td>
<td>0.10</td>
<td>0.18</td>
<td>3</td>
<td>55</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>0.10</td>
<td>0.35</td>
<td>0.12</td>
<td>0.33</td>
<td>4</td>
<td>23</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.48±0.04</td>
<td>0.26±0.05</td>
<td>0.28±0.05</td>
<td>0.35±0.04</td>
<td>237±41</td>
<td>821±127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.06</td>
<td>0.20±0.04</td>
<td>0.28±0.04</td>
<td>0.76±0.16</td>
<td>24±10</td>
<td>228±80</td>
<td>1586±446</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane potential</td>
<td>-75 mV</td>
<td>(n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 µM</td>
<td>0.38±0.05</td>
<td>0.26±0.04</td>
<td>0.34±0.08</td>
<td>0.37±0.04</td>
<td>224±52</td>
<td>1081±139</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.51±0.07</td>
<td>0.25±0.03</td>
<td>0.18±0.06</td>
<td>1.57±0.32</td>
<td>20±12</td>
<td>223±131</td>
<td>3684±2579</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.04</td>
<td>0.26±0.05</td>
<td>0.28±0.04</td>
<td>0.76±0.16</td>
<td>24±10</td>
<td>228±80</td>
<td>1586±446</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.06</td>
<td>0.20±0.04</td>
<td>0.28±0.04</td>
<td>0.76±0.16</td>
<td>24±10</td>
<td>228±80</td>
<td>1586±446</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane potential</td>
<td>-100 mV</td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 µM</td>
<td>0.22±0.05</td>
<td>0.56±0.09</td>
<td>0.34±0.08</td>
<td>0.38±0.23</td>
<td>8±4</td>
<td>137±73</td>
<td>675±261</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.22±0.09</td>
<td>0.34±0.06</td>
<td>0.34±0.06</td>
<td>0.35±0.15</td>
<td>32±11</td>
<td>127±30</td>
<td>961±380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22±0.05</td>
<td>0.36±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22±0.09</td>
<td>0.36±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane potential</td>
<td>+50 mV</td>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-10 µM</td>
<td>0.26</td>
<td>0.13</td>
<td>0.61</td>
<td>0.42</td>
<td>67</td>
<td>165</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.01</td>
<td>0.90</td>
<td>0.09</td>
<td>0.20</td>
<td>211</td>
<td>523</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17±0.05</td>
<td>0.57±0.06</td>
<td>0.24±0.09</td>
<td>0.57±0.27</td>
<td>30±6.4</td>
<td>226±86</td>
<td>1013±37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22±0.05</td>
<td>0.36±0.09</td>
<td>0.34±0.06</td>
<td>0.36±0.15</td>
<td>32±11</td>
<td>127±30</td>
<td>961±380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22±0.05</td>
<td>0.36±0.09</td>
<td>0.34±0.06</td>
<td>0.35±0.15</td>
<td>32±11</td>
<td>127±30</td>
<td>961±380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane potential</td>
<td>+75 mV</td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-10 µM</td>
<td>0.22±0.05</td>
<td>0.56±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.22±0.09</td>
<td>0.34±0.06</td>
<td>0.34±0.06</td>
<td>0.35±0.15</td>
<td>32±11</td>
<td>127±30</td>
<td>961±380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22±0.05</td>
<td>0.56±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane potential</td>
<td>+100 mV</td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 µM</td>
<td>0.31±0.04</td>
<td>0.52±0.08</td>
<td>0.15±0.05</td>
<td>0.23±0.06</td>
<td>146±22</td>
<td>672±189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 µM</td>
<td>0.10±0.02</td>
<td>0.42±0.26</td>
<td>0.34±0.14</td>
<td>0.13±0.10</td>
<td>0.44±0.01</td>
<td>51±38</td>
<td>172±74</td>
<td>526±247</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10±0.03</td>
<td>0.40±0.07</td>
<td>0.46±0.65</td>
<td>0.38±0.08</td>
<td>16±4</td>
<td>87±36</td>
<td>427±128</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values for T1, T2, T3, and T4 are in ms. Closed states are represented by three exponentials at 2-10 µM levamisole. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. At 30-90 µM there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. All values are the mean ± s.e., n is the number of experiments. Values for the time constants are in ms.
Table 5.4 Burst-time kinetics for levamisole activated channels.

Levamisole

<table>
<thead>
<tr>
<th>Conc.</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mem. pot. -50 mV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>0.48±0.06</td>
<td>0.52±0.06</td>
<td>1.15±0.16</td>
<td>3.38±0.33</td>
</tr>
<tr>
<td>30 μM</td>
<td>0.77±0.01</td>
<td>0.23±0.01</td>
<td>0.30±0.15</td>
<td>1.99±0.38</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.66±0.15</td>
<td>0.34±0.15</td>
<td>0.99±0.19</td>
<td>2.85±0.51</td>
</tr>
<tr>
<td>mem. pot. -75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>0.49±0.07</td>
<td>0.51±0.07</td>
<td>1.24±0.19</td>
<td>3.07±0.35</td>
</tr>
<tr>
<td>30 μM</td>
<td>0.61±0.09</td>
<td>0.39±0.09</td>
<td>0.59±0.15</td>
<td>2.06±0.42</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.59±0.07</td>
<td>0.41±0.07</td>
<td>0.74±0.19</td>
<td>2.23±0.32</td>
</tr>
<tr>
<td>mem. pot. -100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>0.56±0.07</td>
<td>0.44±0.07</td>
<td>1.29±0.22</td>
<td>3.78±0.50</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.44±0.08</td>
<td>0.56±0.08</td>
<td>0.30±0.15</td>
<td>2.93±0.96</td>
</tr>
<tr>
<td>mem. pot. +50 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-10 μM</td>
<td>0.57</td>
<td>0.43</td>
<td>1.13</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>0.85</td>
<td>0.15</td>
<td>0.25</td>
<td>0.47</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.63±0.11</td>
<td>0.37±0.11</td>
<td>0.90±0.13</td>
<td>2.41±0.20</td>
</tr>
<tr>
<td>mem. pot. +75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-10 μM</td>
<td>0.46±0.08</td>
<td>0.54±0.08</td>
<td>0.69±0.03</td>
<td>1.89±0.29</td>
</tr>
<tr>
<td>30 μM</td>
<td>0.77±0.08</td>
<td>0.23±0.08</td>
<td>0.66±0.19</td>
<td>2.33±0.79</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.73±0.05</td>
<td>0.27±0.05</td>
<td>0.93±0.23</td>
<td>2.73±0.64</td>
</tr>
<tr>
<td>mem. pot. +100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-10 μM</td>
<td>0.71±0.06</td>
<td>0.29±0.06</td>
<td>1.06±0.21</td>
<td>2.54±0.48</td>
</tr>
<tr>
<td>30 μM</td>
<td>0.82±0.08</td>
<td>0.18±0.08</td>
<td>0.69±0.21</td>
<td>4.01±1.52</td>
</tr>
<tr>
<td>90 μM</td>
<td>0.69±0.06</td>
<td>0.31±0.06</td>
<td>0.80±0.22</td>
<td>2.33±0.52</td>
</tr>
</tbody>
</table>

Values for T1 and T2 are in ms. T1 and T2 are the time constants representing the two burst states. The proportion of brief bursts (T1) and the long bursts (T2) are represented by the areas A1 and A2. Values are mean±s.e..
5.2.3 Voltage-sensitivity of open- and burst-time durations

At lower concentrations of levamisole (2-10 µM) the corrected mean open-times (Figure 5.2, 5.3) and corrected mean burst-times exhibited a degree of voltage-sensitivity, decreasing with depolarisation. For example the value at -75 mV for the corrected mean open-time was 1.47±0.11 ms (mean ± s.e., n=10), this value was significantly greater (P < 0.03) than the value for the corrected mean open-time at +75 mV of 1.10 ± 0.26 ms (mean ± s.e., n=4). The corrected mean burst duration at -75 mV was 2.50±0.28 ms (mean ± s.e., n=16), this compared to a value of 1.50 ± 0.25 ms (mean ± s.e., n=4) at +75 mV where the corrected mean burst durations were also significantly reduced (P < 0.05) (Table 5.5, 5.6).
<table>
<thead>
<tr>
<th>levamisole Conc.</th>
<th>-50 mV</th>
<th>-75 mV</th>
<th>-100 mV</th>
<th>+50 mV</th>
<th>+75 mV</th>
<th>+100 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-10 μM</td>
<td>1.57</td>
<td>2.02</td>
<td>1.78</td>
<td>1.42</td>
<td>1.23</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>0.92</td>
<td>1.26</td>
<td>1.87</td>
<td>0.92</td>
<td>0.91</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>1.19</td>
<td>2.22</td>
<td>1.19</td>
<td>1.40</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>1.11</td>
<td>2.23</td>
<td>2.17</td>
<td>0.86</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>1.82</td>
<td>1.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.43</td>
<td>0.96</td>
<td>1.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.06</td>
<td>1.26</td>
<td>1.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.07</td>
<td>1.59</td>
<td>1.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.36</td>
<td>1.55</td>
<td>1.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.07</td>
<td>0.98</td>
<td>1.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>1.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.34</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± 0.11±</th>
<th>0.47±</th>
<th>1.52±</th>
<th>1.17±</th>
<th>1.10±</th>
<th>0.93±</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.e.</td>
<td>0.07±</td>
<td>0.11±</td>
<td>0.14±</td>
<td>0.13±</td>
<td>0.20±</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>30 μM</th>
<th>0.81</th>
<th>0.64</th>
<th>0.37</th>
<th>0.73</th>
<th>0.59</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.88</td>
<td>0.71</td>
<td>0.42</td>
<td>0.58</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>0.76</td>
<td>0.73</td>
<td>0.76</td>
<td>1.27</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>1.10</td>
<td>0.79</td>
<td>0.90</td>
<td>0.82</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>0.52</td>
<td>0.50</td>
<td>1.19</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± 0.81±</th>
<th>0.55±</th>
<th>0.73±</th>
<th>0.85±</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.e.</td>
<td>0.09±</td>
<td>0.08±</td>
<td>0.15±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.08±</td>
<td>0.17±</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>90 μM</th>
<th>0.69</th>
<th>0.77</th>
<th>0.37</th>
<th>0.73</th>
<th>2.02</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.42</td>
<td>0.72</td>
<td>0.53</td>
<td>1.70</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
<td>0.51</td>
<td>0.48</td>
<td>2.85</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>0.61</td>
<td>0.63</td>
<td>1.40</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.78</td>
<td>0.37</td>
<td>1.14</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.03</td>
<td></td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td></td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.76</td>
<td></td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± 0.43±</th>
<th>0.63±</th>
<th>0.48±</th>
<th>1.30±</th>
<th>1.36±</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.e.</td>
<td>0.09±</td>
<td>0.06±</td>
<td>0.05±</td>
<td>0.26±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.06±</td>
<td>0.26±</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.6 Corrected mean burst duration.

<table>
<thead>
<tr>
<th>Levamisole Conc.</th>
<th>Membrane Potential</th>
<th>-50 mV</th>
<th>-75 mV</th>
<th>-100 mV</th>
<th>+75 mV</th>
<th>+100 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-10 μM</td>
<td></td>
<td>4.24</td>
<td>2.16</td>
<td>2.19</td>
<td>1.80</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.71</td>
<td>2.62</td>
<td>2.22</td>
<td>1.01</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.91</td>
<td>2.24</td>
<td>3.80</td>
<td>2.05</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.25</td>
<td>3.29</td>
<td>3.97</td>
<td>1.16</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.75</td>
<td>5.99</td>
<td>2.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.39</td>
<td>2.38</td>
<td>1.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.95</td>
<td>2.45</td>
<td>3.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.34</td>
<td>3.27</td>
<td>2.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.80</td>
<td>2.47</td>
<td>1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00</td>
<td>2.39</td>
<td>1.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.38</td>
<td>2.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.85</td>
<td>2.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.93</td>
<td>1.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>2.34± 0.21 ms</td>
<td>2.50± 0.28 ms</td>
<td>2.52± 0.27 ms</td>
<td>1.50± 0.25 ms</td>
<td>1.50± 0.33 ms</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Levamisole Conc.</th>
<th>Membrane Potential</th>
<th>30 μM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.17</td>
<td>1.13</td>
<td></td>
<td>0.42</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.29</td>
<td>0.54</td>
<td>0.83</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.10</td>
<td>0.87</td>
<td>0.86</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.95</td>
<td>2.70</td>
<td>1.67</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98</td>
<td>0.92</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>1.30± 0.17 ms</td>
<td>1.14± 0.32 ms</td>
<td></td>
<td>0.94± 0.26 ms</td>
<td>0.98± 0.21 ms</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Levamisole Conc.</th>
<th>Membrane Potential</th>
<th>90 μM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3.09</td>
<td>1.17</td>
<td>1.38</td>
<td>1.30</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.10</td>
<td>1.49</td>
<td>3.74</td>
<td>2.44</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.50</td>
<td>1.70</td>
<td>1.56</td>
<td>1.37</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.15</td>
<td>0.76</td>
<td>0.73</td>
<td>1.75</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.81</td>
<td>0.98</td>
<td></td>
<td>0.83</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.62</td>
<td>0.81</td>
<td>0.81</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.81</td>
<td>1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>1.54± 0.33 ms</td>
<td>1.40± 0.21 ms</td>
<td>1.85± 0.65 ms</td>
<td>1.37± 0.24 ms</td>
<td>1.39± 0.39 ms</td>
<td></td>
</tr>
</tbody>
</table>

165
Levamisole (3 μM) activated channels from a cell-attached patch illustrating the voltage sensitivity of open-time durations. C: denotes the closed state. O: denotes the open state. A: individual channels recorded at -75 mV. B: individual channels recorded at +75 mV.
Figure legend

Histogram of open, closed and burst -times from the experiment illustrated in Figure 5.2. A. membrane potential -75 mV: (I) Histogram of the open time distribution, which was best described by the sum of two exponential with time constants T1 of 0.92 ms and T2 of 2.14 ms, The relatives areas of these components were A1, 0.54 and A2, 0.46. The corrected mean open-time was 1.59 ms. (II) Histogram of the closed-time distribution which was best described by the sum of three exponential components (T1, T2, & T3) with respective areas (A1, A2, & A3). T1 = 0.33 ms, T2 = 165 ms, T3 = 500 ms, A1= 0.37, A2= 0.27, A3= 0.36. (III) Histogram of the burst time distribution which was best described by the sum of two exponential components with time constants, T1 (1.45 ms) and T2 (3.61 ms). The relative areas of these components were A1 (0.53) and A2 (0.47). The corrected mean burst length was 2.46 ms. T_{crit} had a value of 1 ms.

B. membrane potential +75 mV, (I) Histogram showing the open time distribution. The open-times were fitted by two exponential with time constants of 0.53 ms and 1.45 ms and relative areas of 0.70 and 0.30. The corrected mean open-time was 0.91 ms. (II) Histogram of the closed-time distribution which was best described by the sum of three exponential components (T1, T2, & T3) with respective areas (A1, A2, & A3). T1 = 0.1 ms, T2 = 187 ms, T3 =990 ms, A1= 0.51, A2= 0.47, A3= 0.02. (III) Histogram of the burst-time distribution which was best described by the sum of two exponential components with time constants, T1 (0.65 ms) and T2 (1.67 ms). The relative areas of these components were A1 (0.65) and A2 (0.35). The corrected mean burst length was 1.01 ms. T_{crit} had a value of 1 ms.
5.2.4 Probability of channel opening.

The probability of the channel being open ($P_{\text{open}}$) was concentration-dependent at depolarised potentials where $P_{\text{open}}$ increased with concentration. However at hyperpolarised potentials concentration had no significant effect on $P_{\text{open}}$. Figure 5.4 illustrates the effect of concentration on $P_{\text{open}}$ at ±75 mV. Similar results were observed at ±100 mV (Table 5.7). It is suggested subsequently that the explanation for the failure of $P_{\text{open}}$ to increase at -75 mV and -100 mV is due to the blocked state of the channel, which occurs with high concentrations of levamisole at hyperpolarised potentials, directly closing without re-entering the open-state.

At low agonist concentrations the membrane potential had little affect on $P_{\text{open}}$. For example, in one experiment with 3 μM levamisole, $P_{\text{open}}$ was 0.0061 at +75 mV compared to 0.0078 at -75 mV; similar results were obtained in 8 other experiments with 2-10 μM levamisole. This lack of effect of membrane potential on $P_{\text{open}}$ contrasts with the effect of membrane potential on open-times; the lack of effect on $P_{\text{open}}$ is explained by the compensating increase in the rate of channel opening at depolarised potentials. For example in one experiment with 3 μM levamisole the channel opening rate at -75 mV was 8.6 channel openings per second compared to 13.6 channel openings per second at +75 mV.
Figure 5.4.

Graph showing $P_{\text{open}}$ versus levamisole concentration, (■) -75 mV membrane potential, (○) +75 mV membrane potential. All values are mean ± s.e. (n=4 to n=9).
<table>
<thead>
<tr>
<th>Levamisole conc.</th>
<th>membrane potential</th>
<th>$	ext{P}_{\text{open}}$ values.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-50 mV</td>
<td>-75 mV</td>
</tr>
<tr>
<td>2 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0064</td>
<td>0.0064</td>
</tr>
<tr>
<td></td>
<td>0.0039</td>
<td>0.0046</td>
</tr>
<tr>
<td></td>
<td>0.0031</td>
<td>0.0040</td>
</tr>
<tr>
<td></td>
<td>0.0057</td>
<td>0.0042</td>
</tr>
<tr>
<td></td>
<td>0.0032</td>
<td>0.0120</td>
</tr>
<tr>
<td></td>
<td>0.0083</td>
<td>0.0043</td>
</tr>
<tr>
<td>mean±</td>
<td>0.0051±</td>
<td>0.0058±</td>
</tr>
<tr>
<td>s.e.</td>
<td>0.0008</td>
<td>0.0009</td>
</tr>
<tr>
<td>3 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>0.0026</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>0.0033</td>
<td>0.0097</td>
</tr>
<tr>
<td></td>
<td>0.0049</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>0.0076</td>
<td>0.004</td>
</tr>
<tr>
<td>mean±</td>
<td>0.0038±</td>
<td>0.0043±</td>
</tr>
<tr>
<td>s.e.</td>
<td>0.0009</td>
<td>0.0015</td>
</tr>
<tr>
<td>10 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0048</td>
<td>0.0083</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
<td>0.0034</td>
</tr>
<tr>
<td></td>
<td>0.0032</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>0.0099</td>
<td>0.0033</td>
</tr>
<tr>
<td></td>
<td>0.0027</td>
<td>0.0053</td>
</tr>
<tr>
<td>mean±</td>
<td>0.0047±</td>
<td>0.0062±</td>
</tr>
<tr>
<td>s.e.</td>
<td>0.0013</td>
<td>0.0014</td>
</tr>
<tr>
<td>30 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>0.0038</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>0.0067</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>0.0053</td>
<td>0.0021</td>
</tr>
<tr>
<td></td>
<td>0.0340</td>
<td>0.0038</td>
</tr>
<tr>
<td>mean±</td>
<td>0.0164±</td>
<td>0.0046±</td>
</tr>
<tr>
<td>s.e.</td>
<td>0.0068</td>
<td>0.0014</td>
</tr>
<tr>
<td>90 $\mu$M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0220</td>
<td>0.0066</td>
</tr>
<tr>
<td></td>
<td>0.0170</td>
<td>0.0059</td>
</tr>
<tr>
<td></td>
<td>0.0160</td>
<td>0.0070</td>
</tr>
<tr>
<td></td>
<td>0.0110</td>
<td>0.0082</td>
</tr>
<tr>
<td></td>
<td>0.0053</td>
<td>0.0064</td>
</tr>
<tr>
<td>mean±</td>
<td>0.014±</td>
<td>0.0067±</td>
</tr>
<tr>
<td>s.e.</td>
<td>0.0024</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

* indicates values for 2-10 $\mu$M
5.2.5 Evidence of Channel-Block

At higher concentrations of levamisole (30 µM & 90 µM), there was a recognisable change in the kinetics of channel opening (Figure 5.5, 5.6). At hyperpolarised potentials, channel openings were briefer than at lower concentrations and appeared as sequences of openings separated by brief closings characteristic of a 'flickering' channel-block. This behaviour may be explained by levamisole acting to produce a voltage-sensitive open channel-block, in addition to its action as an agonist. The cationic charge and large size of levamisole is consistent with the direction of the voltage-sensitivity of the block. In order to describe the effect further, a simple channel-block model was used.

The presence of open channel-block reduced the duration of the open-states at hyperpolarised potentials. Table 5.2 shows that this reduction in open-times at 30 µM levamisole was associated with a reduction of both the brief and longer components of the open-time distribution. At -75 mV with 90 µM levamisole where the open-times were also reduced, only one open-state could be detected. The loss of one component at this concentration is most likely due to the brief openings reducing to a level below the limit of resolution of the recording apparatus, as a result of channel-block. At 90 µM the corrected mean open-time at -75 mV was 0.63 ± 0.06 ms (mean ± s.e., n=11), a value significantly lower than that at +75 mV which was 1.30 ± 0.26 ms (mean ± s.e., n=8), (P < 0.02) (Table 5.5). Thus mean open-times showed a reversed direction of voltage-sensitivity at the higher levamisole concentrations. At +75 mV where no channel-block was observed, there was no significant reduction in the mean duration of the two open-time components compared to values at lower concentrations (2-10 µM).
Figure legend

Levamisole-(30 μM) activated channels recorded from an isolated inside-out patch. A: membrane potential -75 mV. B. membrane potential +75 mV. The open-times in this patch were fitted with two exponentials (not shown). A: T1 = 0.14 ms T2 = 0.69 ms; B: T1 = 0.41 ms T2 = 1.91 ms. Note the presence of flickering open channel-block in A.
Figure 5.5.

A

B

2 pA

10 ms
Figure legend

A. membrane potential -75 mV: (I) Histogram of the open time distribution, which was best described by one exponential with time constant 0.31 ms. (II) Histogram of the closed-time distribution which was best described by the sum of four exponential components (T1, T2, T3, & T4) with respective areas (A1, A2, A3, & A4). T1 = 0.79 ms, T2 = 8 ms, T3 = 102 ms, T4 = 3260 ms, A1 = 0.62, A2 = 0.20, A3 = 0.02. (III) Histogram of the burst time distribution which was best described by the sum of two exponential components with time constants, T1 (0.57 ms) and T2 (2.10 ms). The relative areas of these components were A1 (0.40) and A2 (0.60). T_{crit} had a value of 1.41 ms.

B. membrane potential +75 mV, (I) Histogram showing the open time distribution. The open-times were fitted by two exponential with time constants of 0.044 ms and 1.20 ms and relative areas of 0.64 and 0.36. (II) Histogram of the closed-time distribution which was best described by the sum of three exponential components (T1, T2, & T3) with respective areas (A1, A2, & A3). T1 = 0.29 ms, T2 = 16 ms, T3 = 46 ms, T4 = 361, A1 = 0.04, A2 = 0.41, A3 = 0.56, A4 = 0.01. (III) Histogram of the burst-time distribution which was best described by the sum of two exponential components with time constants, T1 (0.47 ms) and T2 (1.43 ms). The relative areas of these components were A1 (0.62) and A2 (0.38). T_{crit} had a value of 1 ms.
5.2.6 Estimation of blocked-times

Mean block durations at membrane potentials of -35 to -100 mV for both 30 μM and 90 μM are shown in Table 5.8. Comparison of values at both 30 μM and 90 μM showed concentration did not effect mean block-times. The lack of effect of concentration on blocked-times is consistent with predictions of the simple channel-block model. Values obtained for mean block durations at both 30 μM and 90 μM were subsequently combined and their reciprocals used to estimate k_B values (Figure 5.7).

5.2.7 Estimation of k_{B+}

The forward blocking-rate constants k_{B+} were determined from plots of the reciprocals of the mean duration of long open-times (T2) against drug concentration, Figure 5.8. Long open-times were used in preference to the brief open-times because they are more accurately determined, the short open durations are close to the limit of resolution of the set-up and at 90 μM values for T1 appear to fall below the limit of resolution of the recording apparatus. In calculating k_{B+}, results obtained with 90 μM levamisole were not included. These points deviated from the straight line obtained with results at lower concentrations (2-30 μM). This may be due to the fact that at higher concentrations the mechanism of block may be more complex than the simple channel-block mechanism. However it is more likely that at the higher concentration (90 μM) some of the open-time durations are lower than the limit of resolution of the recording apparatus, making the open-time duration an over-estimation at this concentration. This interpretation is further supported by the fact that at 90 μM with hyperpolarised potentials open-times distributions could only be fitted by a single exponential.
Figure legend

A. Illustration of channel activity with 30 μM levamisole; isolated inside-out patch, -75 mV, fast flickering channel-block can be clearly observed. C: denotes the closed state. O: denotes the open state. The mean blocked-times were calculated from the measurements of blocked durations (indicated by the line between the arrows). In this experiment the mean blocked-time was 0.43 ms. B. Semi-log plot of $k_B$ against membrane potential, all values are mean ± s.e.. The voltage sensitivity of $k_B$ was described by the equation: $k_B = k_{B0} \cdot \exp\left(\frac{E}{V_k}\right)$. Where $k_B$ is the unblocking rate constant at the membrane potential E, $k_{B0}$ is the value for $k_B$ at 0 mV and $V_k$ is a constant. The value for $k_{B0}$ was 11.99 ms$^{-1}$ and $V_k$ was 35.02 mV.
Figure 5.7.

A

B

Membrane potential (mV)

k_B (ms⁻¹)
Table 5.8 The effect of membrane potential on mean block time

<table>
<thead>
<tr>
<th>levamisole Conc.</th>
<th>Membrane potential</th>
<th>mean block time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-35 mV</td>
<td>-50 mV</td>
</tr>
<tr>
<td>30 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.38</td>
<td>0.30</td>
<td>1.59</td>
</tr>
<tr>
<td>0.29</td>
<td>1.12</td>
<td>1.61</td>
</tr>
<tr>
<td>0.31</td>
<td>1.36</td>
<td>2.95</td>
</tr>
<tr>
<td>0.28</td>
<td>1.54</td>
<td>2.15</td>
</tr>
<tr>
<td>0.65</td>
<td>0.20</td>
<td>0.62</td>
</tr>
<tr>
<td>0.20</td>
<td>0.62</td>
<td>0.42</td>
</tr>
<tr>
<td>mean±s.e.</td>
<td>0.38±0.05</td>
<td>0.87±0.18</td>
</tr>
<tr>
<td>90 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.28</td>
<td>0.61</td>
<td>0.46</td>
</tr>
<tr>
<td>0.33</td>
<td>0.43</td>
<td>0.75</td>
</tr>
<tr>
<td>0.12</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>0.35</td>
<td>0.24</td>
<td>0.51</td>
</tr>
<tr>
<td>0.65</td>
<td>0.31</td>
<td>1.46</td>
</tr>
<tr>
<td>0.64</td>
<td>1.32</td>
<td>1.23</td>
</tr>
<tr>
<td>0.89</td>
<td>0.84</td>
<td>0.63</td>
</tr>
<tr>
<td>mean±s.e.</td>
<td>0.27±0.05</td>
<td>0.50±0.07</td>
</tr>
<tr>
<td>30+90 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean±s.e.</td>
<td>0.27±0.05</td>
<td>0.44±0.05</td>
</tr>
</tbody>
</table>
Reciprocal plot of open-time constant $T_2$ against concentration, at -50 mV (Δ), -75 mV (○) and -100 mV (■). The intercept represents the closing rate constant ($\alpha_2$) and the slope represents the forward blocking rate ($k_B$). Each point is the mean ± s.e. Lines were fitted to 2-30 μM by least square regression without including the results at 90 μM. Values for $k_B$ were determined as -50 mV, $2.06 \times 10^7 \text{M}^{-1}\text{s}^{-1}$; -75 mV, $3.82 \times 10^7 \text{M}^{-1}\text{s}^{-1}$; and -100 mV, $6.58 \times 10^7 \text{M}^{-1}\text{s}^{-1}$. The closing rate constant $\alpha_2$ was also determined: -50 mV, 447 s$^{-1}$; -75 mV, 396 s$^{-1}$; and -100 mV, 143 s$^{-1}$ from the intercept on the ordinate.
Values for the forward blocking-rate constant, $k_B$ at $-50$ mV, was $2.06 \times 10^7$ M$^{-1}$s$^{-1}$; at $-75$ mV it was $3.82 \times 10^7$ M$^{-1}$s$^{-1}$; and at $-100$ mV it was $6.58 \times 10^7$ M$^{-1}$s$^{-1}$. As predicted by the simple channel block model $k_B$ increased with membrane hyperpolarisation. The voltage sensitivity of $k_B$ was described by the equation:

$$k_B = k_{B0} \exp\left(\frac{E}{V_k}\right)$$

Where $k_B$ is the forward blocking rate constant at the membrane potential $E$, $k_{B0}$ is the value for $k_B$ at 0 mV and $V_k$ is a constant. The value for $k_{B0}$ was $0.82 \times 10^7$ M$^{-1}$ s$^{-1}$ and $V_k$ was $-35.5$ mV. The correlation coefficient ($r$) was 0.97. Therefore $k_B$ showed an e-fold change every 36 mV. It was also possible to estimate the closing rate constants ($\alpha_2$) for the long openings. This was the intercept of the plot of $1 / T_2$ versus concentration. At $-50$ mV the value for $\alpha_2$ was $447$ s$^{-1}$, at $-75$ mV it was $396$ s$^{-1}$; and at $-100$ mV it was $143$ s$^{-1}$. As can be observed the closing rate decreases with membrane hyperpolarisation, this supports earlier observations where open durations were longer at hyperpolarised potentials.

### 5.2.8 Determination of $K_B$

The values for $k_B$ and $k_B$ determined above were used to calculate the channel-block dissociation constant, $K_B$. At $-50$ mV, $K_B$ was $2.55 \times 10^3$ s$^{-1}$ / $2.06 \times 10^7$ M$^{-1}$s$^{-1}$ or $123$ $\mu$M; at $-75$ mV, $K_B$ was $1.75 \times 10^3$ s$^{-1}$ / $3.82 \times 10^7$ M$^{-1}$s$^{-1}$ or $46$ $\mu$M; and at $-100$ mV, $K_B$ was $0.62 \times 10^3$ s$^{-1}$ / $6.58 \times 10^7$ M$^{-1}$s$^{-1}$ or $9.4$ $\mu$M. The semi-log plot of $K_B$ versus membrane potential is shown in Figure 5.9, this shows an e-fold change in $K_B$ every 19.4 mV.
Figure 5.9

Semi-log plot of $K_B$ versus membrane potential. The voltage sensitivity of $K_B$ was described by the equation: $K_B = K_{B0} \exp\left(\frac{E}{V_k}\right)$. Where $K_B$ is the dissociation rate constant at the membrane potential $E$, $K_{B0}$ is the value for $K_B$ at 0 mV and $V_k$ is a constant. The value for $K_{B0}$ was 1780 $\mu$M and $V_k$ was 19.4 mV.
5.2.9 Burst durations

As stated earlier the burst durations exhibit a degree of voltage-sensitivity. The bursts consisted of two components; the brief bursts (mean T1) and the longer bursts (mean T2). Values for T1 and T2, with their respective proportions A1 and A2 are listed in Table 5.4. The simple channel-block model predicts that the mean burst-length should increase with concentration. In order to examine this prediction the corrected mean burst lengths were determined at different levamisole concentrations. For example at -75 mV with 2-10 μM levamisole the corrected mean burst length was 2.50 ± 0.28 ms (mean ± s.e, n=16); with 30 μM levamisole it was 1.14 ± 0.32 ms (mean±s.e, n=6); and with 90 μM levamisole it was 1.40 ± 0.21 ms (mean±s.e, n=9). Thus there was no significant increase in the corrected mean burst duration. Similar results were obtained at -100 mV. It was concluded that the mean burst durations did not increase with increasing levamisole concentration, a result not predicted by the simple channel-block model.

5.2.10 Desensitisation

At higher concentrations of levamisole (30 μM & 90 μM) long closed-times were seen in some patches between clusters of channel opening (Figure 5.10). These closed-times lasted many seconds and were observed at both depolarised and hyperpolarised potentials. The details of the closed-time components observed at higher concentrations are summarised in Table 5.3. These very long closed periods appear to correspond to a slow desensitisation process and are similar to the very long closed-times observed with high concentrations of ACh in Ascaris (Pennington & Martin, 1990). In addition to the long closed durations, shorter closed times (between 10 and 50 ms) were detected at high levamisole concentrations. These might also be part of a desensitised state as they occur at both depolarised and hyperpolarised potentials but only with high concentrations.
Figure legend

Channel currents showing long (seconds) closed-times between clusters of openings. C: denotes closed state, O: denotes open state. 90 μM levamisole, cell attached patches. A: -75 mV, B: +75 mV. These long closed periods were interpreted as desensitisation.
5.3 DISCUSSION

5.3.1 The simple block model.

The simple channel-block model was used in this paper in order to describe quantitatively some of the effects observed with levamisole. The predictions of this model, for a charged blocker, include the expectations that: the corrected mean open-times decrease with increasing levamisole concentration; burst durations increase with increasing drug concentration; the forward blocking rate constant \( k_{+B} \), the unblocking rate \( k_{-B} \), and the dissociation constant \( K_B \) all vary with membrane potential (\( k_{+B} \) and \( k_{-B} \) should have equal but opposite voltage dependences); and the mean block duration and therefore \( k_{-B} \) are independent of drug concentration. The data presented in this report are consistent with the above predictions except that burst durations did not increase with concentration. This implies that the model used is a useful approximation but not a completely sufficient model.

I did not observe the predicted increase in the average durations of bursts, although there were frequent examples of long flickering bursts (see Figure 5.7). One possible explanation for the absence of increased mean burst durations could be that the channel might enter a closed state directly from the blocked state without reopening, i.e., the channel closes trapping levamisole or the blocked channel enters into a desensitised state. To investigate the properties of the rate of entry to the blocked closed state, a plot of blockage frequency against concentration was obtained from the levamisole data (Figure 5.11). For a simple channel block the blockage frequency should increase in a linear manner with concentration. Figure 5.11 shows that for the levamisole data this does not happen. I felt unable to take this analysis further because the blockage frequency plot does not separate the "Nachschlag" closings from the blocked closings.
Blockage frequency plots versus conc. at: A, -50 mV; B, -75 mV; and C, -100 mV.
One further difficulty in using the simple channel-block model is the presence of two open-states (O1 & O2). In our analysis it seems probable at 90 μM that the brief open-state (O1) fell below the limit of resolution of the recording set up. Thus the values for $k_B$ would be appropriate for the open-state O2 at 90 μM. Since the values for $k_B$ were not significantly different from the values for $k_B$ at 30 μM where the open-state O1 could still be resolved, it implies that the presence of O1 did not affect our estimation of $k_B$ for the open-state O2. Since the values for $k_B$ were determined from the values of the open-state O2 (see methods), our determination of $K_B$ is likely to be an estimate of the dissociation constant for the block of the open-state O2.

5.3.2 Calculating the blocking site within the pore.

The equilibrium constant for levamisole blocking the channel was voltage sensitive with an e-fold change every 20 mV. Using the equation given in methods where $H = RT / zF\delta$, and $RT / F = 25.26$ mV at 20 °C. This gave the result $\delta$ of 1.26. The high voltage sensitivity requires that the single charge on levamisole senses more than the total applied field when it passes through the channel. This high voltage sensitivity may result from structural changes in the barrier at extreme hyperpolarisations, reducing the internal barrier to levamisole passage. Alternatively the AChR channel may be a multi-ion pore with more than one levamisole molecule involved, hyperpolarisation would increase the occupancy of an ion-binding site external to the blocking site, which could an increase in the rate of levamisole passage (SINE & STEINBACH, 1984, HILLE & SCHWARZ, 1978, HILLE, 1984).

5.3.3 Comparison with acetylcholine-activated channels in Ascaris.

At the single-channel level both ACh (PENNINGTON & MARTIN, 1990) and levamisole activate channels in the same concentration range: ACh 1-100 μM; levamisole 1-90 μM. The current / voltage relationship with both levamisole and ACh
are linear, both agonists showed evidence of desensitisation with long closed periods appearing at higher concentrations.

Although ACh and levamisole appear to activate the same channels there are some differences in the behaviour of the single-channel currents. ACh activates channels with two distinguishable conductance levels (25-35 ps & 40-50 pS) (Pennington & Martin, 1990); in contrast single-channel currents activated by levamisole had a range of detectable conductance levels between 19-46 pS with a mean of 32.9 ± 1.23 pS (mean ± s.e., n=26).

The open-, closed-, and burst-time durations for levamisole or ACh activated channels in this preparation show that both compounds produced two open-states and two burst-states and at least three closed-states. However the mean duration of the apparent open-times and burst-times for levamisole were shorter than for ACh. The mean duration of apparent brief and long openings produced by ACh (1-10 μM) were 1.18 ms and 4.89 ms, which compares with 0.80 ms and 2.30 ms for 2-10 μM levamisole under similar conditions. The mean brief and long burst duration for 1-10 μM ACh were 1.17 ms and 7.62 ms compared to 1.24 ms and 3.70 ms for 2-10 μM levamisole.

A further difference between the effects of ACh and levamisole is related to channel-block. Pennington & Martin (1990) did not find significant evidence for channel-block with ACh on this preparation, although they did observe a reduction in the mean open-time at higher concentrations (25 μM) which might be interpreted as signs of block. Our results clearly show that levamisole blocks the channel at hyperpolarised potentials.
5.3.4 Comparison with previous voltage-clamp experiments in *Ascaris*

Harrow & Graton (1985) examined the effects, in *Ascaris*, of both ACh and levamisole using two-microelectrode current-clamp and voltage-clamp. They found that the current / voltage relationship obtained from two-microelectrode voltage-clamp experiments obtained by applying levamisole by microperfusion or iontophoresis to *Ascaris* muscle had both linear (0 to -30 mV) and non-linear sections (> -30 mV). They suggested that the non-linearity was due to a voltage-sensitive channel-block and / or desensitisation. In our experiments we observed some desensitisation at higher concentrations of levamisole, but this appeared at both depolarised and hyperpolarised membrane potentials, not just at membrane potentials greater than -30 mV. Channel-block was clearly observed in our experiments, the degree of block increased on membrane hyperpolarisation. This is consistent with the voltage-sensitivity of the non-linearity observed by Harrow & Graton (1985) and suggests that their observation reflects channel-block but not desensitisation.

Harrow & Graton (1985) have shown that levamisole is an agonist at all concentrations (1 μM-10 mM) producing a sigmoid dose-response curve obtained under current clamp conditions with a membrane potential of -30 mV. Our results clearly show that channel-block occurs at -35 mV and at this potential $K_B$ was calculated at 285 μM. It is interesting to note that Harrow & Graton (1985) did not observe a reduction in the maximum conductance response with high concentrations (10mM). This observation of Harrow & Graton (1985) is consistent with our observations at hyperpolarised potentials which showed that $P_{open}$ failed to decrease at higher concentrations, despite the presence of channel block.

5.3.5 Possible therapeutic significance

Receptor desensitisation produced by levamisole may have significant effects therapeutically; some *Ascaris* parasites continuously treated with higher doses of
levamisole recover from the levamisole induced paralysis (ACEVES et al., 1970; COLES et al., 1974, 1975). This recovery may be due to desensitisation since like desensitisation recovery occurs only at higher doses. Consequently it is suggested that lower doses which do not produce desensitisation, potentially have a greater therapeutic effect. Also the fact that $P_{\text{open}}$ in these experiments did not increase at hyperpolarised potentials when 2-90 $\mu$M levamisole was used suggests that high concentrations would not be therapeutically advantageous.

Therefore in conclusion, these experiments confirm that levamisole acts at the ACh receptor on Ascaris suum muscle, both opening and blocking the channel.
CHAPTER 6
RESULTS SECTION 2

6. Application of levamisole to the cytoplasmic surface of the muscle membrane leads to activation of nicotinic acetylcholine receptors in the nematode parasite Ascaris suum

6.1 Introduction

Analyses of levamisole-activated single-channel currents in Ascaris suum on application of levamisole to the extracellular surface of the patch has shown: (i) the channels are cation selective with linear I/V plots; (ii) the channel conductance is approximately 35pS; (iii) generally there are two open-states and three closed-states; and (iv) at high concentrations (>30μM) desensitisation and open channel-block occur. Desensitisation occurs at both depolarised and hyperpolarised potentials whereas channel-block is only observed at hyperpolarised potentials (results section 1).

GALZI, et al. (1991) have reviewed the structure of nicotinic receptors from a variety of vertebrate preparations. They report common properties of acetylcholine gated channels among the species. In general the receptor is thought to be a 290 Kd glycoprotein composed of five subunits with a stoichiometry α2βγδ. Several charged rings have been located within the channel-pore. If the Ascaris nicotinic channel has a similar structure to that reported by GALZI et al. (1991), it is possible that open channel-block occurs when levamisole enters the channel from the extracellular solution and binds to one of these charged rings. It was therefore of interest to determine if levamisole could also give rise to open channel-block by entering from the cytoplasmic solution.

6.2 Results

Application of levamisole (2 μM) to the extracellular surface of the patch resulted in channel activation. Kinetic analyses of these channels (Table 7.1) confirmed results
found from previous investigations (chapter 5, results section 1). At both depolarised and hyperpolarised membrane potentials, the distributions of open-times were best described by two exponentials and the distributions of closed-states were best described by three exponentials. Since the number of open- and closed- states must at least equal the number of exponential components fitted. (see methods) it follows that these channels have at least two open-states and three closed-states.

6.2.1 Application of levamisole to the cytoplasmic surface of the membrane results in an increase in channel activity.

Levamisole (2 μM) was applied in the pipette to induce nicotinic channel activation as described above. In 8 out of 9 experiments, addition of levamisole (30-926 μM) to the cytoplasmic surface of the patch via the bath solution resulted in an increase in channel activation. Figure 6.1 illustrates an experiment from an isolated inside-out patch: initially 2 μM levamisole was present in the extracellular solution. The addition of 71 μM levamisole to the cytoplasmic face of the patch is marked with an arrow. In this particular experiment the probability of a channel being open (P\textsubscript{open}) increased from 0.0038 to 0.0225 on addition of levamisole to the bath solution. Control experiments showed that washing the cytoplasmic side of patch membranes with bath solution alone did not lead to an increase in channel activation (n=3).

Cytoplasmic addition of levamisole increased P\textsubscript{open} in a concentration dependent manner at both depolarised and hyperpolarised membrane potentials (Table 6.2). There was a positive correlation between P\textsubscript{open} and concentration, at both depolarised (correlation coefficient (r): 0.96; p < 0.001) and hyperpolarised potentials (correlation coefficient (r): 0.94; p < 0.005).
Table 6.1 Open time kinetics for levamisole activated channels.

<table>
<thead>
<tr>
<th>Membrane Potential</th>
<th>Levamisole Conc.</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pipette</td>
<td>Bath</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-75 mV</td>
<td>2-10 μM</td>
<td>0 μM n=17*</td>
<td>0.66±0.06</td>
<td>0.34±0.06</td>
<td>0.88±0.10</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>0 μM n=3</td>
<td>0.49±0.18</td>
<td>0.51±0.18</td>
<td>0.42±0.18</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>30 μM</td>
<td>0.85</td>
<td>0.15</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>90 μM</td>
<td>0.24</td>
<td>0.76</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>120 μM</td>
<td>1</td>
<td></td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>120 μM</td>
<td>1</td>
<td></td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>142 μM</td>
<td>1</td>
<td></td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>926 μM</td>
<td>1</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>+75 mV</td>
<td>3-10 μM</td>
<td>0 μM n=4*</td>
<td>0.65±0.10</td>
<td>0.38±0.10</td>
<td>0.71±0.10</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>0 μM</td>
<td>0.56</td>
<td>0.44</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>0 μM</td>
<td>0.59</td>
<td>0.41</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>30 μM</td>
<td>0.42</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>60 μM</td>
<td>0.91</td>
<td>0.08</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>71 μM</td>
<td>0.52</td>
<td>0.48</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>90 μM</td>
<td>0.51</td>
<td>0.49</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>807 μM</td>
<td>0.62</td>
<td>0.58</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>807 μM</td>
<td>0.80</td>
<td>0.20</td>
<td>1.23</td>
</tr>
</tbody>
</table>

T1 and T2 are the time constants (in ms) which represent mean durations of the open states, their areas are represented by A1 and A2 respectively; A1+A2=1. Data marked * comes from chapter 5, results section 1.
Channel records from an isolated inside-out patch at membrane potential of +75 mV. Levamisole (2 μM) was present in the pipette to activate the nicotinic channels, the addition of 71.4 μM levamisole to the bath is shown by the arrow. In this experiment $P_{\text{open}}$ increased from 0.0038 to 0.0225.
Table 6.2. % increase in $P_{\text{open}}$ after the addition of levamisole to the extracellular solution.

<table>
<thead>
<tr>
<th>Levamisole Conc in bath solution</th>
<th>Membrane potential</th>
<th>% increase in $P_{\text{open}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-75 mV</td>
<td>+75 mV</td>
<td></td>
</tr>
<tr>
<td>30 μM</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td>71.4 μM</td>
<td>---</td>
<td>558</td>
</tr>
<tr>
<td>90 μM</td>
<td>123</td>
<td>480</td>
</tr>
<tr>
<td>120 μM</td>
<td>690</td>
<td>1200</td>
</tr>
<tr>
<td>120 μM</td>
<td>287</td>
<td>250</td>
</tr>
<tr>
<td>142 μM</td>
<td>49</td>
<td>40</td>
</tr>
<tr>
<td>807 μM</td>
<td>1733</td>
<td>2967</td>
</tr>
<tr>
<td>926 μM</td>
<td>1614</td>
<td>4614</td>
</tr>
</tbody>
</table>

At both potentials this data can be fitted to a straight line using the method of least squares. At -75 mV the correlation coefficient ($r$) was 0.94 ($p < 0.005$) for a straight line relationship. At +75 mV the correlation coefficient ($r$) was 0.96 ($p < 0.001$) for a straight line relationship.
6.2.2 Current-voltage relationships before and after bath application of levamisole.

The increase in channel activity may be explained in one of two ways. Levamisole added to the cytoplasmic surface of the membrane may activate another type of channel or, in some way cause an increase in opening of the nicotinic channels. Current / voltage relationships were therefore determined to see if the channels before and after cytoplasmic application of levamisole had the same conductance (Figure 6.2). This experiment illustrates that there was no detectable change in the current / voltage relationship after the addition of levamisole to the bath and hence the experiment suggests that the increase in channel activity when levamisole is added to the bath is due to an increased opening of the nicotinic acetylcholine channels already activated by levamisole in the pipette.
The current voltage relationship for an experiment with 2 $\mu$M levamisole in the pipette (●) compared to the current-voltage relationships after the addition of 142 $\mu$M levamisole to the bath solution (O).
6.2.3 High cytoplasmic concentrations of levamisole lead to desensitisation and open channel-block.

6.2.3.1 Desensitisation

The addition of levamisole (30-926 μM) to the bath solution produced long closed periods between clusters of channel openings (Figure 6.3, Table 6.3). These closed-time durations lasted many seconds and were observed at both depolarised and hyperpolarised potentials. This desensitised condition was observed as an additional long closed-state (T4) resulting in the distribution of closed-times being best described by four exponentials. In one experiment, the addition of 807 μM levamisole to the bath solution (at +75mV) resulted in the distribution of closed-times being best described by four exponentials represented by the mean durations T1 (1.03 ms); T2 (9 ms); T3 (102 ms) and T4 (1077 ms). These very long closed durations (represented by T4) appear to correspond to a slow desensitization process and are similar to the very long closed-times observed with high concentrations of ACh and levamisole applied to the extracellular surface of membrane patches from *Ascaris*, (chapter 5, results section 1; Pennington & Martin 1992). Similar values were recorded in 5 other experiments at both depolarised and hyperpolarised membrane potentials.
Figure 6.3.

Channel records from an isolated inside out patch with 2 μM levamisole in the pipette solution and 807 μM levamisole in the bath solution +75 mV. The long closed periods are thought to represent the desensitised state of the receptor.
Table 6.3  Closed-time kinetics for levamisole activated channels

<table>
<thead>
<tr>
<th>Membrane potential</th>
<th>Levamisole Conc.</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>+75 mV</td>
<td>3-10 µM (n=5)*</td>
<td>0.22±0.05</td>
<td>0.56±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM (n=4)</td>
<td>0.29±0.03</td>
<td>0.51±0.08</td>
<td>0.20±0.07</td>
<td>0.30±0.02</td>
<td>243±55</td>
<td>619±120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pipette</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.35</td>
<td>0.22</td>
<td>0.39</td>
<td>0.04</td>
<td>0.28</td>
<td>7.6</td>
<td>30</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.23</td>
<td>0.07</td>
<td>0.66</td>
<td>0.04</td>
<td>0.36</td>
<td>53</td>
<td>635</td>
<td>12863</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.26</td>
<td>0.45</td>
<td>0.25</td>
<td>0.04</td>
<td>0.66</td>
<td>24</td>
<td>176</td>
<td>1678</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.16</td>
<td>0.13</td>
<td>0.51</td>
<td>0.20</td>
<td>0.35</td>
<td>13</td>
<td>127</td>
<td>1724</td>
</tr>
<tr>
<td></td>
<td>2 µM 926 µM</td>
<td>0.54</td>
<td>0.22</td>
<td>0.19</td>
<td>0.05</td>
<td>0.24</td>
<td>29</td>
<td>101</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>Bath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-10 µM (n=18)*</td>
<td>0.46±0.04</td>
<td>0.26±0.05</td>
<td>0.28±0.05</td>
<td>0.35±0.04</td>
<td>237±41</td>
<td>821±127</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM (n=3)</td>
<td>0.52±0.12</td>
<td>0.24±0.10</td>
<td>0.27±0.09</td>
<td>0.23±0.03</td>
<td>338±164</td>
<td>1335±341</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pipette</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM 30 µM</td>
<td>0.69</td>
<td>0.05</td>
<td>0.06</td>
<td>0.20</td>
<td>0.37</td>
<td>3.5</td>
<td>312</td>
<td>1363</td>
</tr>
<tr>
<td></td>
<td>2 µM 90 µM</td>
<td>0.62</td>
<td>0.13</td>
<td>0.24</td>
<td>0.01</td>
<td>0.64</td>
<td>199</td>
<td>590</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.74</td>
<td>0.12</td>
<td>0.13</td>
<td>0.01</td>
<td>0.69</td>
<td>8</td>
<td>159</td>
<td>1357</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.72</td>
<td>0.09</td>
<td>0.15</td>
<td>0.04</td>
<td>0.66</td>
<td>18</td>
<td>259</td>
<td>2586</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.52</td>
<td>0.24</td>
<td>0.19</td>
<td>0.05</td>
<td>1.03</td>
<td>9</td>
<td>102</td>
<td>1077</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Membrane potential</th>
<th>Levamisole Conc.</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>-75 mV</td>
<td>3-10 µM (n=5)*</td>
<td>0.22±0.05</td>
<td>0.56±0.09</td>
<td>0.21±0.09</td>
<td>0.24±0.04</td>
<td>132±38</td>
<td>595±115</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM (n=4)</td>
<td>0.29±0.03</td>
<td>0.51±0.08</td>
<td>0.20±0.07</td>
<td>0.30±0.02</td>
<td>243±55</td>
<td>619±120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pipette</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.35</td>
<td>0.22</td>
<td>0.39</td>
<td>0.04</td>
<td>0.28</td>
<td>7.6</td>
<td>30</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.23</td>
<td>0.07</td>
<td>0.66</td>
<td>0.04</td>
<td>0.36</td>
<td>53</td>
<td>635</td>
<td>12863</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.26</td>
<td>0.45</td>
<td>0.25</td>
<td>0.04</td>
<td>0.66</td>
<td>24</td>
<td>176</td>
<td>1678</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.16</td>
<td>0.13</td>
<td>0.51</td>
<td>0.20</td>
<td>0.35</td>
<td>13</td>
<td>127</td>
<td>1724</td>
</tr>
<tr>
<td></td>
<td>2 µM 926 µM</td>
<td>0.54</td>
<td>0.22</td>
<td>0.19</td>
<td>0.05</td>
<td>0.24</td>
<td>29</td>
<td>101</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>Bath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-10 µM (n=18)*</td>
<td>0.46±0.04</td>
<td>0.26±0.05</td>
<td>0.28±0.05</td>
<td>0.35±0.04</td>
<td>237±41</td>
<td>821±127</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM (n=3)</td>
<td>0.52±0.12</td>
<td>0.24±0.10</td>
<td>0.27±0.09</td>
<td>0.23±0.03</td>
<td>338±164</td>
<td>1335±341</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pipette</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM 30 µM</td>
<td>0.69</td>
<td>0.05</td>
<td>0.06</td>
<td>0.20</td>
<td>0.37</td>
<td>3.5</td>
<td>312</td>
<td>1363</td>
</tr>
<tr>
<td></td>
<td>2 µM 90 µM</td>
<td>0.62</td>
<td>0.13</td>
<td>0.24</td>
<td>0.01</td>
<td>0.64</td>
<td>199</td>
<td>590</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.74</td>
<td>0.12</td>
<td>0.13</td>
<td>0.01</td>
<td>0.69</td>
<td>8</td>
<td>159</td>
<td>1357</td>
</tr>
<tr>
<td></td>
<td>2 µM 120 µM</td>
<td>0.72</td>
<td>0.09</td>
<td>0.15</td>
<td>0.04</td>
<td>0.66</td>
<td>18</td>
<td>259</td>
<td>2586</td>
</tr>
<tr>
<td></td>
<td>2 µM 807 µM</td>
<td>0.52</td>
<td>0.24</td>
<td>0.19</td>
<td>0.05</td>
<td>1.03</td>
<td>9</td>
<td>102</td>
<td>1077</td>
</tr>
</tbody>
</table>

Closed states are represented by three exponentials at 2-10µM levamisole. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. With 30-926 µM in the bath solution there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. All values are the mean ± s.e., n is the number of experiments, values for the time constants are in ms.

Data marked by * represent values obtained in previous experiments (results section 1).
6.2.3.2 Channel-block

Figure 6.4 illustrates channel openings at both depolarised and hyperpolarised potentials, following bath application of 926 μM levamisole. In this experiment, the mean open-time at +75 mV was 1.35 ms, compared to the mean open-time at -75 mV which was 0.16 ms.

Channel openings at +75 mV showed no evidence of alteration in mean open-times with the addition of high concentrations of levamisole to the bath (Table 6.1). This result has also been observed with high extracellular levamisole concentrations (chapter 5, results section 1).

At hyperpolarised potentials increasing cytoplasmic levamisole concentrations resulted in a marked reduction in the mean open-times when compared to control values with just 2 μM levamisole in the pipette. Openings appeared in sequences separated by brief closings, characteristic of a 'flickering' open channel-block. This behaviour only occurred during hyperpolarisation; the same phenomena has been observed with high concentrations of extracellularly applied levamisole (30-90 μM)(chapter 5, results section 1). This suggests that in both cases the block originates from a cationic blocking agent, levamisole, acting from the same side of the channel (Figure 6.4).

As stated earlier, the distribution of open-times with 2 μM levamisole in the pipette, were best described by two exponentials; however on addition of increasing concentrations of levamisole to the bath solution, the distribution of open-times at -75 mV were best described by a single exponential (Table 6.1). The loss of one exponential component has been reported in previous experiments with high extracellular concentrations of levamisole (90 μM), (chapter 5, results section 1).
Channel records from an isolated inside-out patch with 2 μM levamisole in the pipette and 926 μM levamisole in the bath solution. A. membrane potential +75 mV, mean open-time 1.35 ms. B. membrane potential -75 mV, mean open-time 0.19 ms.
6.2.4 Does levamisole pass through the membrane?.

One explanation for the increase in channel activity and alteration of channel kinetics with high concentrations of levamisole present in the bath solution is: levamisole in the uncharged form crosses the membrane, via the lipid phase, increasing the extracellular concentration of levamisole in the pipette. To determine the amount of unionised levamisole present in bath solutions, the pKa of levamisole was estimated from a simple titration of levamisole with molar NaOH (Figure 6.5). The pKa was calculated as 8.0, similar results were obtained in two other experiments. Using the Henderson Hasselbalch equation the ratio of the concentration of unionised to the concentration of ionised levamisole was 0.158 at pH 7.2. Therefore approximately 16% of the levamisole is in the unionised form which may be able to diffuse across the membrane, down a concentration gradient and increase the extracellular concentration.
Figure 6.5

Graph showing titration of levamisole with molar NaOH. The pKa of levamisole is 8.0.
6.3. Discussion

As previously reported, levamisole application (2 μM) to the extracellular face of the membrane patch activates single channel currents with at least two open-states and three closed-states, the mean durations were comparable to those obtained in previous experiments, (chapter 5, results section 1).

Application of high concentrations of levamisole (> 30 μM) to the bath solution altered channel kinetics: an increase in $P_{\text{open}}$, open channel-block and desensitisation were observed. Channel behaviour was similar to that observed in previous experiments with high extracellular levamisole concentrations (chapter 5, results section 1).

With high concentrations of levamisole in the bath solution no evidence of channel-block occurring at depolarised membrane potentials was observed. This is reflected by the fact that increasing the concentration of levamisole in the cytoplasmic solution at hyperpolarised membrane potentials did not alter the open-time constants. If levamisole could enter the channel pore from the cytoplasmic solution one would expect to observe channel-block at depolarised potentials due to the cationic nature of this drug. This finding suggests that the *Ascaris* nicotinic channel has an asymmetric structure.

Channel open-times showed a marked reduction at -75 mV as the concentration of bath applied levamisole increased. With concentrations above 120 μM, the distributions of openings were best described by a single exponential. It appears that open channel-block reduces the mean open-times T1 and T2, high concentrations (>120 μM) reduce T1 to such a level that it falls below the limit of resolution of the recording system and can no longer be detected. Such an effect has previously been reported with respect to open channel-block observed with 90 μM levamisole applied to the extracellular surface via the pipette (chapter 5, results section 1). It is thus concluded
that the reduction in open durations appears to be the result of a voltage-sensitive open channel-block, consistent with a cationic blocking agent approaching the channel from the extracellular side of the membrane.

The detection of an additional closed-state representing desensitisation was observed with both application of levamisole to the cytoplasmic surface as well as to the extracellular surface of the membrane. As desensitisation occurs with high agonist concentrations the question therefore became: did levamisole act on a receptor at a cytoplasmic site or did it diffuse across the membrane increasing the concentration in the extracellular solution? As already shown 16% of levamisole at pH 7.2 was in the unionised form, which is able to diffuse across the membrane. The membrane crossing property of levamisole is consistent with the observations that application of levamisole to the bath solution: (i) did not alter channel conductance; (ii) increased channel activity; (iii) induced a desensitised state and; (iv) produced a voltage-sensitive open channel-block on hyperpolarisation. It is pointed out that high extracellular concentrations of levamisole also produces these same effects and that levamisole has previously been reported to cross membranes in *Ascaris suum*. (VERHEYEN et al., 1976; VAN DEN BOSSCHE & JANSSEN, 1967). The ability to cross lipid barriers like membrane or cuticle has an obvious therapeutic significance for the treatment of parasitic infestations.
CHAPTER 7
RESULTS SECTION 3

7. The action of pyrantel as an agonist and an open channel blocker at nicotinic receptors in isolated Ascaris suum muscle vesicles.

7.1 Introduction
Pyrantel is thought to act as an acetylcholine (ACh) agonist in its target species (Aceves et al., 1970; Van Neuten, 1972; Coles et. al., 1975; Harrow & Gration, 1985).

Earlier studies on Ascaris suum have reported drug induced contraction of Ascaris suum muscle, when ACh or pyrantel was applied to the whole worms or to a flap preparation (Del Castillo et. al., 1963; Aceves et al, 1970; Van Neuten, 1972; Coles et. al., 1975; Harrow & Gration, 1985). Pyrantel causes depolarization and increased spike discharge frequency during intracellular recordings from the muscle cells. Studies using intracellular current-clamp and voltage-clamp techniques have shown that pyrantel increases the input conductance and depolarises the muscle cells of Ascaris suum, an action consistent with that of ACh and levamisole (Harrow & Gration, 1985). Patch-clamp techniques have been used to study actions of ACh and levamisole at the extrasynaptic receptors of Ascaris suum (Pennington & Martin, 1990; results section 1). These studies confirm that levamisole and ACh activate channels present on the muscle. Levamisole was also shown to produce a voltage-sensitive open channel-block.

This section investigates the action of pyrantel at the nicotinic ACh channels from the somatic muscle cells of Ascaris suum. The data shows that pyrantel activates these channels and is an open channel blocker.

7.2 Results

7.2.1 Single-channel currents
The results reported for 0.03-100 μM pyrantel were obtained from a total of 297 patches, with pyrantel present in the pipette from the beginning of the recording. A total of 42% of the patches contained pyrantel-activated ion-channels. The channels had short mean open-times in the range of a few milliseconds.

In 20 experiments with pyrantel omitted from the patch pipette solution no such channel openings were observed. It was therefore concluded that pyrantel activated these currents.

7.2.2 Current-Voltage relationships and channel conductance

In solutions containing symmetrical cation concentrations but non-symmetrical anion concentrations current-voltage plots were obtained which showed a linear relationship in 28 out of 30 experiments. Reversal potentials for the pyrantel-activated channels were repeatedly found to be near zero (2.77± 0.89 mV, mean±s.e., n=28) (Table 7.1). Using isolated inside-out patches the predicted reversal potential for a cationic conductance is 0 mV and an anionic conductance is -33 mV. The results indicate that the pyrantel activates a cation selective channel (Figure 7.1). This was further tested in experiments using isolated inside-out patches, by altering the cation and anion concentrations by diluting the bath solution 50:50 with distilled water. Observed shifts in reversal potential using an isolated inside-out patch were consistent with those predicted by the Nernst equation for a cation conductance.
Current / Voltage plots for pyrantel-activated cation channels, 1 μM pyrantel in the pipette. A. Cell-attached patch, slope conductance 54 pS, reversal potential -1.5 mV, correlation = 0.969, n = 8. B. Inside-out patch, initial (○) slope conductance = 31 pS, correlation = 0.994, n = 8. Slope conductance with diluted bath solution (△) = 36 pS, correlation = 0.997, n = 6. Changes in bath solution were from 140 mM to 70 mM with Cs⁺ and from 39 mM to 19.5 mM with Cl⁻. The predicted reversal potentials for an isolated inside-out patch using the Nernst equation were Cl⁻, -33 mV and Cs⁺, 0 mV with the undiluted bath solution (○) and with the diluted bath solution (△) were Cl⁻, -51 mV and Cs⁺, 18 mV. The actual reversal potentials were 5.03 mV and 23 mV which are consistent with a cation conductance as the reversal potentials would have shifted in the opposite direction for an anionic conductance.
Table 7.1 Individual values for channel conductance for pyrantel activated channels.

<table>
<thead>
<tr>
<th>Conductance (pS)</th>
<th>Reversal potential (mV)</th>
<th>Conductance (pS)</th>
<th>Reversal potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>-1.74</td>
<td>35</td>
<td>5.75</td>
</tr>
<tr>
<td>51</td>
<td>0.98</td>
<td>37</td>
<td>1.53</td>
</tr>
<tr>
<td>53</td>
<td>5.8</td>
<td>25</td>
<td>4.96</td>
</tr>
<tr>
<td>56</td>
<td>-6.59</td>
<td>23</td>
<td>3.37</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>44</td>
<td>5.77</td>
<td>23</td>
<td>3.37</td>
</tr>
<tr>
<td>48</td>
<td>9.11</td>
<td>23</td>
<td>3.37</td>
</tr>
<tr>
<td>50</td>
<td>6.27</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>25</td>
<td>4.12</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>53</td>
<td>6.17</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>43</td>
<td>-1.46</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>48</td>
<td>6.85</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>54</td>
<td>-1.5</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>44</td>
<td>4.26</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>47</td>
<td>1.58</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>41</td>
<td>6.36</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>47</td>
<td>5.61</td>
<td>17</td>
<td>5.23</td>
</tr>
<tr>
<td>33</td>
<td>8.88</td>
<td>16</td>
<td>-8.71</td>
</tr>
<tr>
<td>37</td>
<td>8.88</td>
<td>16</td>
<td>-8.71</td>
</tr>
<tr>
<td>26</td>
<td>-1.03</td>
<td>15</td>
<td>-4.02</td>
</tr>
<tr>
<td>47</td>
<td>1.43</td>
<td>15</td>
<td>-4.02</td>
</tr>
<tr>
<td>36</td>
<td>7.85</td>
<td>15</td>
<td>-4.02</td>
</tr>
<tr>
<td>34</td>
<td>0.99</td>
<td>15</td>
<td>-4.02</td>
</tr>
<tr>
<td>21</td>
<td>-2.66</td>
<td>11</td>
<td>0.954</td>
</tr>
<tr>
<td>41±2.04 pS</td>
<td>2.69±0.62 mV</td>
<td>22.4±3.4 pS</td>
<td>4.33±0.87 mV</td>
</tr>
</tbody>
</table>
Pyrantel (0.03-100 µM) activated channels with two distinguishable conductance levels: \(41\pm2.04\ \text{pS (mean±s.e., n=28)}\) and \(22.4\pm0.34\ \text{pS (mean±s.e., n=8)}\). Occasionally even smaller conductance levels were also observed. Channel openings were sometimes observed to step from one conductance level to another (Figure 7.2, 7.3). This may represent the transition from the main conductance level to a smaller subconductance level. However the presence of two channels with different conductances cannot be excluded.

### 7.2.3 Exponential curve fitting

The data files used for the subsequent analysis were obtained from experiments with a sufficiently high channel frequency, the number of events analysed varied from 200 to 1500. Only the larger conductance channel was analysed.

These experiments were performed on cell-attached or inside-out patches, with 0.1-100 µM pyrantel present in the patch-pipette. In general the open- and burst-times were best-fitted by the sum of two exponentials and the closed-times by the sum of three exponentials (Tables 7.2-7.4). Assuming that the channel can exist in several more-or-less discrete states and that the rate constants for transitions between states are constant (as does COLQUHOUN & SAKMANN, 1985), then the number of open states of the channel must at least equal the number of exponential components needed to fit the open-time distribution (COLQUHOUN & HAWKES, 1982). This indicates the presence of at least two open- and two burst-states and at least three closed-states. Higher concentrations of pyrantel (100 µM) resulted in significant changes in channel kinetics: the loss of one open state at hyperpolarised potentials and the presence of an additional closed-state at both depolarised and hyperpolarised potentials. This will be considered subsequently under the sections "open channel-block" and "desensitisation".
Figure legend

Illustration of a single channel opening and an all-points histogram showing distribution of channel amplitude. C: denotes the closed state. O: denotes the open state. Data taken from an experiment with 0.03 μM pyrantel in the pipette with the cell-attached patch configuration and membrane potential -75 mV. The amplitude histogram has two open conductance levels fitted by two gaussian curves: mean 3.3 ± 0.30 pA (44 pS) and 1.8 ± 0.52 pA (24 pS) (values are mean ± s. d.). The single opening appears to have a main conductance of 3.19 pA or 42 pS and lower conductance which appears as a shoulder on the main conductance of 1.84 pA or 24 pS. This smaller conductance appears to be a subconductance state.
Figure 7.2.

A.

B. C

209
Figure legend

Illustration of possible sub-conductance levels from channel records with 100 μM pyrantel in the patch pipette, from a cell attached patch at a membrane potential of -75 mV. C denotes the closed state and O denotes the open state. The arrows indicate possible sub-conductance levels.
Table 7.2. Open-time kinetics for pyrantel activated channels.

<table>
<thead>
<tr>
<th>pyrantel conc.</th>
<th>membrane potential</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-50 mV</td>
<td>(ms)</td>
<td>(ms)</td>
<td>(ms)</td>
<td>(ms)</td>
</tr>
<tr>
<td>0.1 μM (n=4)</td>
<td>-50 mV</td>
<td>0.61±0.19</td>
<td>0.39±0.19</td>
<td>0.56±0.11</td>
<td>2.34±0.64</td>
</tr>
<tr>
<td>1.0 μM (n=5)</td>
<td>-50 mV</td>
<td>0.41±0.14</td>
<td>0.59±0.14</td>
<td>0.48±0.04</td>
<td>0.91±0.43</td>
</tr>
<tr>
<td>10 μM (n=1)</td>
<td>-50 mV</td>
<td>0.72</td>
<td>0.28</td>
<td>0.96</td>
<td>1.99</td>
</tr>
<tr>
<td>100 μM (n=3)</td>
<td>-50 mV</td>
<td></td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td></td>
<td></td>
<td>0.38±0.09</td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=8)</td>
<td>-75 mV</td>
<td>0.78±0.05</td>
<td>0.22±0.05</td>
<td>0.52±0.19</td>
<td>2.40±0.48</td>
</tr>
<tr>
<td>1.0 μM (n=7)</td>
<td>-75 mV</td>
<td>0.54±0.10</td>
<td>0.46±0.10</td>
<td>0.61±0.14</td>
<td>3.11±0.62</td>
</tr>
<tr>
<td>10 μM (n=3)</td>
<td>-75 mV</td>
<td>0.62±0.13</td>
<td>0.38±0.13</td>
<td>0.61±0.20</td>
<td>1.71±0.37</td>
</tr>
<tr>
<td>100 μM (n=11)</td>
<td>-75 mV</td>
<td>0.62</td>
<td>1.00</td>
<td>1.25</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>1.00</td>
<td></td>
<td>0.30±0.02</td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=5)</td>
<td>-100 mV</td>
<td>0.75±0.10</td>
<td>0.25±0.10</td>
<td>0.87±0.40</td>
<td>2.80±0.82</td>
</tr>
<tr>
<td>1.0 μM (n=4)</td>
<td>-100 mV</td>
<td>0.74±0.07</td>
<td>0.26±0.07</td>
<td>0.56±0.16</td>
<td>4.04±1.96</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=5)</td>
<td>+75 mV</td>
<td>0.77±0.07</td>
<td>0.23±0.07</td>
<td>0.43±0.11</td>
<td>1.63±0.13</td>
</tr>
<tr>
<td>1.0 μM (n=4)</td>
<td>+75 mV</td>
<td>0.66±0.14</td>
<td>0.36±0.14</td>
<td>0.38±0.12</td>
<td>1.93±0.11</td>
</tr>
<tr>
<td>10 μM (n=4)</td>
<td>+75 mV</td>
<td>0.59±0.10</td>
<td>0.41±0.10</td>
<td>0.42±0.11</td>
<td>1.61±0.13</td>
</tr>
<tr>
<td>100 μM (n=8)</td>
<td>+75 mV</td>
<td>0.71±0.09</td>
<td>0.29±0.09</td>
<td>0.62±0.14</td>
<td>1.87±0.42</td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td>0.71</td>
<td>1.00</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>1.0 μM (n=6)</td>
<td>+100 mV</td>
<td>0.71±0.09</td>
<td>0.29±0.09</td>
<td>0.62±0.14</td>
<td>1.87±0.42</td>
</tr>
</tbody>
</table>

T1 and T2 are the time constants representing the two open states. The proportion of brief openings (T1) and the long openings (T2) are represented by the areas A1 and A2, A1+A2=1. Values are mean±s.e..
Table 7.3. Closed time kinetics.

<table>
<thead>
<tr>
<th>Pyrantel Conc.</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>membrane potential</td>
<td>-50 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µM</td>
<td>(n=5)</td>
<td>0.22±0.06</td>
<td>0.25±0.11</td>
<td>0.52±0.08</td>
<td>0.28±0.06</td>
<td>86±50</td>
<td>656±99</td>
</tr>
<tr>
<td>1 µM</td>
<td>(n=7)</td>
<td>0.37±0.07</td>
<td>0.35±0.07</td>
<td>0.29±0.09</td>
<td>0.32±0.06</td>
<td>217±36</td>
<td>674±95</td>
</tr>
<tr>
<td>10 µM</td>
<td>(n=3)</td>
<td>0.36±0.05</td>
<td>0.23±0.04</td>
<td>0.35±0.10</td>
<td>0.73±0.48</td>
<td>76±20</td>
<td>550±333</td>
</tr>
<tr>
<td>100 µM</td>
<td></td>
<td>0.43</td>
<td>0.21</td>
<td>0.36</td>
<td>1.09</td>
<td>123</td>
<td>438</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.68</td>
<td>0.11</td>
<td>0.19</td>
<td>0.02</td>
<td>1.38</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>620</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3798</td>
</tr>
<tr>
<td></td>
<td>membrane potential</td>
<td>-75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µM</td>
<td>(n=7)</td>
<td>0.34±0.09</td>
<td>0.29±0.08</td>
<td>0.37±0.12</td>
<td>0.25±0.07</td>
<td>182±59</td>
<td>840±258</td>
</tr>
<tr>
<td>1 µM</td>
<td>(n=7)</td>
<td>0.37±0.10</td>
<td>0.12±0.02</td>
<td>0.51±0.10</td>
<td>0.54±0.23</td>
<td>64±34</td>
<td>1226±321</td>
</tr>
<tr>
<td>10 µM</td>
<td>(n=3)</td>
<td>0.28±0.09</td>
<td>0.31±0.09</td>
<td>0.42±0.08</td>
<td>1.18±0.11</td>
<td>56±12</td>
<td>1280±826</td>
</tr>
<tr>
<td>100 µM</td>
<td>(n=6)</td>
<td>0.24±0.05</td>
<td>0.38±0.05</td>
<td>0.38±0.07</td>
<td>1.66±0.45</td>
<td>127±69</td>
<td>1992±869</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>0.22±0.05</td>
<td>0.22±0.03</td>
<td>0.45±0.10</td>
<td>0.89±0.15</td>
<td>79±38</td>
<td>645±163</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>570±2257</td>
</tr>
<tr>
<td></td>
<td>membrane potential</td>
<td>-100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µM</td>
<td>(n=4)</td>
<td>0.37±0.09</td>
<td>0.33±0.14</td>
<td>0.29±0.11</td>
<td>3.84±3.8</td>
<td>150±44</td>
<td>710±125</td>
</tr>
<tr>
<td>1 µM</td>
<td>(n=4)</td>
<td>0.37±0.13</td>
<td>0.32±0.11</td>
<td>0.25±0.16</td>
<td>2.56±2.15</td>
<td>221±163</td>
<td>1157±663</td>
</tr>
<tr>
<td></td>
<td>membrane potential</td>
<td>+75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µM</td>
<td>(n=4)</td>
<td>0.26±0.08</td>
<td>0.33±0.12</td>
<td>0.40±0.17</td>
<td>0.50±0.25</td>
<td>81±19</td>
<td>330±36</td>
</tr>
<tr>
<td>1 µM</td>
<td>(n=5)</td>
<td>0.23±0.08</td>
<td>0.14±0.03</td>
<td>0.64±0.07</td>
<td>0.59±0.24</td>
<td>42±13</td>
<td>443±135</td>
</tr>
<tr>
<td>10 µM</td>
<td>(n=3)</td>
<td>0.31±0.09</td>
<td>0.36±0.13</td>
<td>0.33±0.14</td>
<td>0.41±0.24</td>
<td>142±93</td>
<td>1093±483</td>
</tr>
<tr>
<td>100 µM</td>
<td>(n=8)</td>
<td>0.19±0.07</td>
<td>0.61±0.08</td>
<td>0.20±0.07</td>
<td>0.46±0.14</td>
<td>296±69</td>
<td>1487±296</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>0.32±0.06</td>
<td>0.22±0.09</td>
<td>0.38±0.05</td>
<td>0.70±0.42</td>
<td>94±43</td>
<td>731±361</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5182±3733</td>
</tr>
<tr>
<td></td>
<td>membrane potential</td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>(n=3)</td>
<td>0.33±0.11</td>
<td>0.20±0.09</td>
<td>0.47±13</td>
<td>0.45±0.30</td>
<td>109±82</td>
<td>1192±537</td>
</tr>
<tr>
<td>100 µM</td>
<td></td>
<td>0.07</td>
<td>0.16</td>
<td>0.74</td>
<td>0.03</td>
<td>0.24</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>542</td>
</tr>
</tbody>
</table>

Closed states are represented by three exponentials at 0.1-10 µM. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. A1+A2+A3=1, values for A3 are not shown. At 10-100 µM there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. A1+A2+A3+A4=1, values for A4 are not shown. All values are the mean ± s.e., n is the number of experiments.
Table 7.4 Burst-time kinetics for pyrantel activated channels.

<table>
<thead>
<tr>
<th>Pyrantel Conc.</th>
<th>membrane potential</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-50 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=4)</td>
<td>0.61±0.17</td>
<td>0.39±0.17</td>
<td>0.90±0.21</td>
<td>2.68±0.70</td>
<td></td>
</tr>
<tr>
<td>1 μM (n=7)</td>
<td>0.66±0.11</td>
<td>0.34±0.11</td>
<td>0.74±0.22</td>
<td>3.25±0.69</td>
<td></td>
</tr>
<tr>
<td>10 μM (n=3)</td>
<td>0.31±0.07</td>
<td>0.69±0.07</td>
<td>0.51±0.14</td>
<td>2.21±0.27</td>
<td></td>
</tr>
<tr>
<td>100 μM (n=3)</td>
<td>0.72</td>
<td>0.28</td>
<td>0.66</td>
<td>4.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>0.64</td>
<td>0.19</td>
<td>4.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td></td>
<td></td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=7)</td>
<td>0.65±0.07</td>
<td>0.35±0.07</td>
<td>0.75±0.21</td>
<td>3.49±0.43</td>
<td></td>
</tr>
<tr>
<td>1 μM (n=7)</td>
<td>0.47±0.07</td>
<td>0.53±0.07</td>
<td>0.73±0.17</td>
<td>5.28±1.07</td>
<td></td>
</tr>
<tr>
<td>10 μM (n=3)</td>
<td>0.68±0.23</td>
<td>0.32±0.23</td>
<td>0.97±0.22</td>
<td>4.80±1.45</td>
<td></td>
</tr>
<tr>
<td>100 μM (n=3)</td>
<td>0.64±0.05</td>
<td>0.36±0.05</td>
<td>0.17±0.04</td>
<td>0.80±0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=4)</td>
<td>0.64±0.13</td>
<td>0.36±0.13</td>
<td>1.8±0.43</td>
<td>3.77±1.07</td>
<td></td>
</tr>
<tr>
<td>1 μM (n=4)</td>
<td>0.60±0.01</td>
<td>0.40±0.01</td>
<td>0.76±0.22</td>
<td>3.46±0.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM (n=5)</td>
<td>0.74±0.04</td>
<td>0.26±0.04</td>
<td>0.79±0.17</td>
<td>3.18±0.52</td>
<td></td>
</tr>
<tr>
<td>1 μM (n=4)</td>
<td>0.56±0.13</td>
<td>0.04±0.13</td>
<td>0.92±0.21</td>
<td>3.76±0.90</td>
<td></td>
</tr>
<tr>
<td>10 μM (n=3)</td>
<td>0.50±0.04</td>
<td>0.50±0.04</td>
<td>0.48±0.15</td>
<td>1.70±0.08</td>
<td></td>
</tr>
<tr>
<td>100 μM (n=12)</td>
<td>0.63±0.08</td>
<td>0.37±0.08</td>
<td>0.67±0.12</td>
<td>2.19±0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μM (n=3)</td>
<td>0.68±0.05</td>
<td>0.32±0.05</td>
<td>0.64±0.18</td>
<td>2.76±0.29</td>
<td></td>
</tr>
</tbody>
</table>

T1 and T2 are the time constants representing the two burst states. The proportion of brief bursts (T1) and the long bursts (T2) are represented by the areas A1 and A2, A1 + A2 = 1. Values are mean±s.e.
7.2.4 Open-time distributions show a degree of voltage sensitivity.

The open-time durations can be described by the sum of two exponential components. A probability density function (p.d.f.) for the open-time distribution is given below for 0.1 μM pyrantel at -75 mV, values represent the mean of eight values:

\[
p.d.f. = \frac{0.78 \ e^{-t/0.52}}{0.52} + \frac{0.22 \ e^{-t/2.40}}{2.40}
\]

This shows that for pyrantel, as for ACh and levamisole, the open durations are described by 2 mathematical components, which will be referred to as short openings and long openings. In this particular example the time constant (T1) for short openings had a mean value of 0.52 ms and the time constant (T2) for long openings had a mean value of 2.40 ms. The area (A1) for short openings had a mean value of 0.78, hence 78% of openings belong to the short component, while 22% were of the longer type. Table 7.2 lists the open durations and their respective areas.

Channel openings showed a degree of voltage-sensitivity, in general the corrected mean open-times (CMOT) were longer at hyperpolarised potentials than at depolarised potentials. For example, with 0.1 μM pyrantel in the patch pipette the CMOT at -75 mV was 1.53 ± 0.22 ms (mean ± s.e., n=7), at +75 mV the value for CMOT was 0.78 ± 0.13 ms (mean ± s.e., n=5) was significantly lower than at hyperpolarised potentials (p< 0.01) (Figure 7.4., Table 7.5).

With higher concentrations of pyrantel (100 μM) the opening durations dramatically reduced; this was thought to be due to open channel-block and will be considered subsequently.
Table 7.5  Corrected mean open-times (ms)

<table>
<thead>
<tr>
<th>Pyrantel Conc</th>
<th>membrane potential</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μM</td>
<td>-50 mV</td>
<td>1.23±0.16</td>
<td>1.53±0.22</td>
<td>1.71±0.39</td>
<td>0.78±0.13</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>1.04</td>
<td>1.39</td>
<td>1.21</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.90</td>
<td>1.40</td>
<td>2.37</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>1.69</td>
<td>1.09</td>
<td>2.37</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>1.54</td>
<td>2.82</td>
<td>0.89</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td>1.00</td>
<td>1.13</td>
<td>1.24</td>
<td>0.97</td>
</tr>
<tr>
<td>1 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>1.38</td>
<td>2.20</td>
<td>0.65</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>1.60</td>
<td>0.88</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.73</td>
<td>2.61</td>
<td>1.33</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>1.12</td>
<td>1.82</td>
<td>2.03</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>1.83</td>
<td>4.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>1.04</td>
<td>1.17</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>1.04</td>
<td>1.19</td>
<td></td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.91</td>
<td>0.49</td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>1.91</td>
<td>1.69</td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>100 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.45</td>
<td>0.32</td>
<td></td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>0.42</td>
<td>0.42</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>0.34</td>
<td>0.34</td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td>0.42</td>
<td>0.42</td>
<td></td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
<td>0.37</td>
<td></td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.51</td>
<td>0.51</td>
<td></td>
<td>0.97</td>
</tr>
<tr>
<td>200 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.45</td>
<td>0.32</td>
<td></td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>0.42</td>
<td>0.42</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>0.34</td>
<td>0.34</td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>300 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>400 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>500 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>600 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>700 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>800 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-50 mV</td>
<td>0.68</td>
<td>0.22</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.68</td>
<td>0.55</td>
<td></td>
<td>0.86</td>
</tr>
</tbody>
</table>
Figure 7.4...

A.

Pyrantel-activated channels from a cell-attached patch illustrating the voltage sensitivity of open-time durations, 0.1 μM. C: denotes the closed state. O: denotes the open state. A: individual channels recorded at -75 mV. The corrected mean open-time was 1.63 ms. B: individual channels recorded at +75 mV. The corrected mean open-time was 0.97 ms.
Figure legend

Histograms of the open, burst and closed durations at ±75 mV with 0.1 μM pyrantel in the patch pipette. Recordings were made in the cell attached confirmation. A membrane potential -75 mV: (I) This histogram shows the open-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 1.56 ms and T2, 5.20 ms. The relative areas of these components were: A1, 0.62; and A2, 0.38. (II) This histogram shows the distribution of the closed-times which were best described by the sum of 3 exponential components with time constants T1, T2, & T3 and relative areas A1, A2, & A3. A1 = 0.11, A2 = 0.11, A3 = 0.78, T1 = 0.1 ms, T2 = 8 ms, T3 = 257 ms. (III) This histogram shows the distribution of the burst-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 1.60 ms and T2, 5.38 ms. The relative areas of these components were: A1, 0.56; and A2, 0.44.

B. membrane potential +75 mV: (I) This histogram shows the open-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 0.41 ms and T2, 1.87 ms. The relative areas of these components were: A1, 0.76; and A2, 0.24. (II) This histogram shows the distribution of the closed-times which were best described by the sum of 3 exponential components with time constants T1, T2, & T3 and relative areas A1, A2, & A3. A1 = 0.35, A2 = 0.10, A3 = 0.55, T1 = 0.34 ms, T2 = 8 ms, T3 = 247 ms. (III) This histogram shows the distribution of the burst-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 0.61 ms and T2, 3.49 ms. The relative areas of these components were: A1, 0.65; and A2, 0.35. T_{crit} in this experiment was 1 ms.
7.2.5 Burst-time distributions exhibit a degree of voltage-sensitivity.

Bursts were described as a sequence of openings separated by short closings smaller than a specified length $T_{\text{crit}}$ (see methods). In general the bursts were best described by the sum of two exponentials. Values for 0.1 $\mu$M pyrantel at -75 mV are illustrated below, values represent the mean of eight values:

$$p.d.f. = \frac{0.65 \ e^{-t/0.75}}{0.75} + \frac{0.22 \ e^{-t/3.49}}{3.49}$$

The results obtained for the burst-time kinetics are given in Table 7.4. The two components will be referred to as short bursts and long bursts.

With low concentrations the burst-time durations also showed a degree of voltage-sensitivity, for example with 0.1 $\mu$M pyrantel the corrected mean burst duration (CMBD) was $2.28 \pm 0.42$ ms (mean ± s.e., n=6) compared to values of $1.12 \pm 0.21$ ms (mean ± s.e. n=5) at +75 mV (Table 7.6). The value for CMBD was significantly lower than values at depolarised potentials $p<0.02$. At higher concentrations of pyrantel values for burst durations reduced at hyperpolarised potentials, for example at -75 mV the CMBD was $0.80 \pm 0.18$ ms (mean±s.e. n=10); this is significantly lower ($p<0.001$) than values for CMBD with 0.1 $\mu$M pyrantel. An explanation for this is pursued in the discussion.
<table>
<thead>
<tr>
<th>Pyrantel Conc</th>
<th>membrane potential</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
<th>mean ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μM</td>
<td>-50 mV</td>
<td>1.14 ± 0.30</td>
<td>2.28 ± 0.42</td>
<td>2.51 ± 0.36</td>
<td>1.12 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.97 ± 0.30</td>
<td>1.37 ± 0.42</td>
<td>1.94 ± 0.36</td>
<td>0.69 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>2.04 ± 0.47</td>
<td>1.11 ± 0.42</td>
<td>2.41 ± 0.36</td>
<td>0.57 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>2.48 ± 0.47</td>
<td>3.26 ± 0.91</td>
<td>P3</td>
<td>1.62 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>0.96 ± 0.47</td>
<td>3.25 ± 0.91</td>
<td>+75 mV</td>
<td>1.3 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>-50 mV</td>
<td>1.21 ± 0.30</td>
<td>3.22 ± 0.42</td>
<td>2.59 ± 0.36</td>
<td>1.85 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>2.49 ± 0.47</td>
<td>2.08 ± 0.42</td>
<td>4.17 ± 0.36</td>
<td>1.28 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>3.53 ± 0.47</td>
<td>5.83 ± 0.91</td>
<td>2.35 ± 0.36</td>
<td>3.31 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>0.99 ± 0.47</td>
<td>1.88 ± 0.91</td>
<td>+50 mV</td>
<td>1.21 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td>2.51 ± 0.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>-50 mV</td>
<td>2.13 ± 0.30</td>
<td>1.74 ± 0.42</td>
<td>1.14 ± 0.21</td>
<td>1.19 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>1.16 ± 0.30</td>
<td>1.35 ± 0.42</td>
<td>1.20 ± 0.21</td>
<td>1.03 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>1.76 ± 0.47</td>
<td>2.45 ± 0.91</td>
<td>1.90 ± 0.36</td>
<td>1.17 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td></td>
<td></td>
<td></td>
<td>0.97 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μM</td>
<td>-50 mV</td>
<td>0.35 ± 0.47</td>
<td>0.33 ± 0.47</td>
<td>0.32 ± 0.48</td>
<td>0.48 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>-75 mV</td>
<td>0.59 ± 0.47</td>
<td>0.87 ± 0.47</td>
<td>2.13 ± 0.21</td>
<td>1.27 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>-100 mV</td>
<td>0.79 ± 0.47</td>
<td>0.92 ± 0.47</td>
<td>2.45 ± 0.21</td>
<td>0.79 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+50 mV</td>
<td>2.45 ± 0.47</td>
<td>0.92 ± 0.47</td>
<td>0.79 ± 0.47</td>
<td>2.45 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>+75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean ± s.e.</td>
<td>0.80 ± 0.18</td>
<td>1.47 ± 0.27</td>
<td>0.27 ± 0.18</td>
<td>0.27 ± 0.18</td>
</tr>
</tbody>
</table>
7.2.6 Closed-time distribution

The results obtained for the closed-time kinetics are summarised in Table 7.3. In general the closed durations were best described by the sum of three exponential components, indicating the presence of at least three closed-states. These components will be referred to as short, intermediate and long gaps. Values with 0.1 $\mu$M pyrantel in the pipette at a membrane potential of -75 mV are illustrated below. Values represent mean of seven values:

\[
p.d.f. = \frac{0.34 \times e^{-t/0.25}}{0.25} + \frac{0.29 \times e^{-t/182}}{182} + \frac{0.37 \times e^{-t/840}}{840}
\]

With lower concentration (0.1 $\mu$M & 1 $\mu$M) the long closed-times were shorter at depolarised potentials compared to hyperpolarised potentials, this is consistent with the higher frequency of opening observed at depolarised potentials. As the concentration of pyrantel was increased (100 $\mu$M) an additional closed-state was observed, this was thought to due to the phenomenon of desensitisation (see below).

7.2.7 $P_{\text{open}}$

Values for $P_{\text{open}}$ were determined with concentrations of pyrantel between 0.03-100 $\mu$M. At the lower concentrations (0.1-10 $\mu$M) there was no significant difference between values for $P_{\text{open}}$ at depolarised and hyperpolarised potentials (Table 7.7). A concentration dependent effect on $P_{\text{open}}$ was observed at hyperpolarised potentials but not depolarised potentials. The value for $P_{\text{open}}$ at -75 mV with 100 $\mu$M pyrantel in the pipette solution (0.0011 ± 0.0005; mean ± s.e., $n=10$), was significantly lower than values for $P_{\text{open}}$ with lower concentrations ($p<0.03$).
<table>
<thead>
<tr>
<th>Pyrantel Conc</th>
<th>-50 mV</th>
<th>-75 mV</th>
<th>-100 mV</th>
<th>+50 mV</th>
<th>+75 mV</th>
<th>+100 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 μM</td>
<td>0.0013</td>
<td>0.0066</td>
<td>0.0109</td>
<td>0.0071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± s.e.</td>
<td>0.0065±</td>
<td>0.0020</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>0.0023</td>
<td>0.0058</td>
<td>0.0012</td>
<td>0.0039</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>0.0075</td>
<td>0.0030</td>
<td>0.0160</td>
<td>0.0007</td>
<td></td>
</tr>
<tr>
<td>mean ± s.e.</td>
<td>0.0026±</td>
<td>0.0077±</td>
<td>0.0051±</td>
<td>0.0061±</td>
<td>0.0022±</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>0.0034</td>
<td>0.0140</td>
<td>0.0070</td>
<td>0.0040</td>
<td>0.0050</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0022</td>
<td>0.0130</td>
<td>0.0080</td>
<td>0.0020</td>
<td>0.0036</td>
<td></td>
</tr>
<tr>
<td>mean ± s.e.</td>
<td>0.0045±</td>
<td>0.0055±</td>
<td>0.0119±</td>
<td>0.0037±</td>
<td>0.0045±</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>0.0030</td>
<td>0.0024</td>
<td>0.0080</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean ± s.e.</td>
<td>0.0044±</td>
<td>0.0033±</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μM</td>
<td>0.0013</td>
<td>0.0001</td>
<td></td>
<td>0.0022</td>
<td>0.0045</td>
<td></td>
</tr>
<tr>
<td>mean ± s.e.</td>
<td>0.0021±</td>
<td>0.0011±</td>
<td></td>
<td>0.0036±</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0008</td>
<td>0.0005</td>
<td></td>
<td>0.0008</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.2.8 Channel-block

With higher concentrations of pyrantel (100 μM) there was an obvious change in the open-time kinetics of the channel at hyperpolarised potentials, channel openings were briefer and occasionally appeared as a sequence of rapid openings separated by brief closing reminiscent of a flickering channel-block, (Figure 7.6). Thus it appeared that in addition to its action as an agonist, pyrantel may enter the channel pore to effect an open channel-block. The voltage-sensitive nature of this block is consistent with the cationic charge of pyrantel. In order to describe this effect further, a simple channel-block model was used.

Estimates of the unblocking rate constant $k_B$ were determined from the reciprocal of the mean block durations observed with 100 μM pyrantel. The mean block durations were $1.43 \pm 0.24$ ms (mean ± s.e., $n=11$) at -75 mV and $1.23 \pm 0.01$ ms (mean ± s.e., $n=3$) at -50 mV (Table 7.8). Thus there appeared to be an increase in the mean block-time with increasing hyperpolarisation but the results did not reach statistical significance. However in one good experiment the voltage sensitivity was quite evident. Figure 7.7. illustrates the voltage sensitivity of $k_B$ determined from values of mean blocked durations seen in this experiment. The voltage sensitivity of $k_B$ was described by the equation: $k_B = k_{B0} \exp(E/V_k)$, where $k_B$ is the unblocking rate constant at membrane potential $E$, $k_{B0}$ is the value for the unblocking rate constant at 0 mV and $V_k$ is a constant. The value for $k_{B0}$ was 1.80 ms⁻¹ and $V_k$ was 67.5 mV suggesting an e-fold change in $k_B$ every 67 mV.

The simple channel-block model predicts a decrease in open-times with increasing concentration of blocker; Table 7.5 shows that in these experiments there was a significant reduction in the corrected mean open-times at hyperpolarised potentials with increasing concentrations of pyrantel (the level of significance between 0.1 μM and 100 μM was $p<0.001$). The reduction in corrected mean open-durations was due to a
reduction in duration of both the brief (T1) and longer (T2) openings (Table 6.2). It appeared that the reduction in brief openings lead to the values of T1 falling below the limit of resolution of the recording set-up. Thus with 100 μM pyrantel, only one exponential component was resolved in the open-time distributions (Table 7.2). The long open-times (T2) were used in preference to the brief open-times, since they were better resolved. An estimate of the forward channel blocking rate, k+g, was determined from the reciprocal plot of the open-time constant, T2, against concentration (see methods). Figure 7.8 illustrates the reciprocal plot of open-time constants T2 against concentration at -50 mV and -75 mV. The slopes of these plots represent k+g and the intercepts represent α. At -50 mV, α was 840 s⁻¹ and k+g was 2.18 x 10⁷ M⁻¹ s⁻¹; at -75 mV α decreased to 380 s⁻¹ and k+g increased to 3.09 x 10⁷ M⁻¹ s⁻¹. This represents an e-fold change in k+g every 72 mV. The simple channel-block model for a charged blocking agent predicts an increase in the forward blocking rate constant with membrane hyperpolarisation. The decrease in α with membrane hyperpolarisation is consistent with earlier observations where open durations were longer at hyperpolarised potentials.

It was possible from the values determined for k−g and k+g to calculate the dissociation rate constant for block for the open channel block (Kg= k−g / k+g). At -50 mV, Kg was 37 μM (0.81 x 10³ s⁻¹ / 2.18 x 10⁷ M⁻¹ s⁻¹) and at -75 mV, Kg was 20 μM (0.61 x 10³ s⁻¹ / 3.09 x 10⁷ M⁻¹ s⁻¹), this represents an e-fold change every 40 mV. In accordance with the channel-block model for a cationic channel blocker, Kg decreased with membrane hyperpolarisation.
Table 7.8 Mean block durations (ms)

<table>
<thead>
<tr>
<th>Membrane Potential</th>
<th>Mean Block Duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-50 mV</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
</tr>
<tr>
<td>Mean</td>
<td><strong>1.23±0.1 ms</strong></td>
</tr>
<tr>
<td>-75 mV</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>1.22</td>
</tr>
<tr>
<td>Mean</td>
<td><strong>1.43±0.24 ms</strong></td>
</tr>
</tbody>
</table>

All values are with 100 μM pyrantel in the patch pipette
Pyrantel-(100 μM) activated channels  C: denotes the closed state. O: denotes the open state. A: channel openings at membrane potentials -25 mV, -50 mV, -75 mV.
Semi-log plot of $k_B$ against membrane potential. The voltage sensitivity of $k_B$ was described by the equation: $k_B = k_{B0} \exp\left(\frac{E}{V_k}\right)$. Where $k_B$ is the unblocking rate constant at the membrane potential $E$, $k_{B0}$ is the value for $k_B$ at 0 mV and $V_k$ is a constant. The value for $k_{B0}$ was 1.80 ms$^{-1}$ and $V_k$ was 67 mV, correlation coefficient $(r)$ 0.982.
Reciprocal plot of open-time constants $T_2$ against concentration, at -50 mV (○) and -75 mV (■). The intercept represents the closing rate constant ($\alpha_2$) and the slope represents the forward blocking rate ($k_{+B}$). Each point is the mean ± s.e. Lines were fitted by least square regression. Values for $k_{+B}$ were determined as -50 mV, $2.18 \times 10^7$ M$^{-1}$s$^{-1}$; -75 mV, $3.09 \times 10^7$ M$^{-1}$s$^{-1}$. The closing rate constant $\alpha_2$ was also determined: -50 mV, 840 s$^{-1}$; -75 mV, 380 s$^{-1}$. Lines were fitted by the method of least squares and had correlation coefficients of 0.913 (-50 mV) and 1.00 (-75 mV).
7.2.9 Burst durations

The simple channel-block model predicts that the corrected mean burst-duration should increase linearly with concentration of blocker at hyperpolarised but not depolarised potentials. The corrected mean burst-durations did not vary significantly at +75 mV, however in contrast to the expectations of the simple channel-block model the corrected mean burst-durations did not increase but decreased at -75 mV (Table 6.6). For example, the value obtained for the corrected mean burst-duration at 100 µM, 0.80 ± 0.18 ms (mean ± s.e., n=10), was significantly lower (p < 0.001) than the value of 2.28 ± 0.42 ms (mean± s.e., n=6) obtained with 0.1 µM pyrantel. Possible explanations for the reduction in corrected mean burst durations are considered in the discussion.

Histories of the open-, burst-, and closed-time distributions at 100 µM pyrantel are shown in Figure 7.9

7.2.10 Desensitisation

At both depolarised and hyperpolarised potentials with the highest concentrations of pyrantel (100 µM) the closed-time distributions were best fitted by four exponential components (Table 6.3). The additional component, T4, had a value of around 5000 ms and was thought to represent a desensitised state of the receptor (CACHELIN & COLQUHOUN, 1989). Desensitised states of this receptor have been observed with high concentrations of ACh (PENNINGTON & MARTIN, 1990) and levamisole (Chapter 5 results section 1) where they produce an additional long non voltage-sensitive component in the closed-time distributions.
Figure legend

Histograms of the open, burst and closed durations at ±75 mV with 100 μM pyrantel in the patch pipette. Recordings were made in the cell attached confirmation. A membrane potential -75 mV: (I) This histogram shows the open-time distributions which were best described by one exponential components (T1), with time constants T1, 0.27 ms. (II) This histogram shows the distribution of the closed-times which were best described by the sum of 3 exponential components with time constants T1, T2, T3, & T4 with relative areas A1, A2, & A3. A1 = 0.23, A2 = 0.31, A3 = 0.46, T1 = 0.91 ms, T2 =44, & T3 = 1147 ms. (III) This histogram shows the distribution of the burst-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 0.23 ms and T2, 1.39 ms. The relative areas of these components were: A1, 0.69; and A2, 0.31. T_{crit} in this experiment was 2.59 ms.

B. membrane potential +75 mV: (I) This histogram shows the open-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 0.32 ms and T2, 0.81 ms. The relative areas of these components were: A1, 0.87; and A2, 0.13. (II) This histogram shows the distribution of the closed-times which were best described by the sum of 3 exponential components with time constants T1, T2, & T3 and relative areas A1, A2, & A3. A1 = 0.09, A2 = 0.60, A3 = 0.31, T1 = 1.12 ms, T2 = 58 ms, T3 = 304 ms. (III) This histogram shows the distribution of the burst-time distributions which were best described by two exponential components (T1 & T2), with time constants T1, 0.36 ms and T2, 1.32 ms. The relative areas of these components were: A1, 0.70; and A2, 0.30. T_{crit} in this experiment was 2.24 ms.
7.3 DISCUSSION

In this study we have shown that the tetrahydropyrimidine, pyrantel, activates an ion channel in *Ascaris* muscle with similar properties to those activated by the natural excitatory transmitter ACh (Pennington & Martin, 1990) and the imidazothiazole, levamisole (results section 1). Both pyrantel and levamisole are thought to act directly as agonists at the nicotinic ACh receptors present on the muscle cells. Pyrantel and levamisole also act as open channel-blockers of the nicotinic channel in this preparation.

7.3.1 Channel-block

In order to qualitatively describe the channel-block by pyrantel, the simple channel-block model was used. The predictions of this model for a charged blocking agent include the expectations that: the corrected mean open-time decreases with increasing concentration; burst durations increase with increasing drug concentration; the forward blocking rate constant ($k_{+B}$), the unblocking rate ($k_{-B}$), and the dissociation constant $K_B$ all vary with membrane potential. The data presented in this report are consistent with the above predictions except one, the burst durations did not increase with concentration. This implies that the model used is a useful approximation but not a completely sufficient model.

At hyperpolarised potentials the burst durations decreased rather than increased with high pyrantel concentrations. The presence of another exit route from the blocked state to another closed-state without the blocked channel re-opening could explain this observation ($C \leftrightarrow O \leftrightarrow B \leftrightarrow BC$). This putative closed-state (BC) might arise if the blocked-state directly entered a desensitised state or if the blocked channel pore closed and trapped the pyrantel molecule. To investigate the properties of the rate of entry to the blocked closed state, a plot of blockage frequency against concentration was obtained from the pyrantel data (Figure 7.10). In our experiments the blockage frequency was hyperbolically related to the pyrantel concentration and therefore
increased in a less than linear manner with concentration. This observation may be explained if there is a concentration dependent reaction rate for the exit from the blocked state to the blocked closed state. However I felt unable to take this analysis further because the blockage frequency plot does not separate the "Nachschlag" closings from the blocked closings. Further limitations of the simple channel-block model are discussed by Adams (1977), Neher & Steinbach (1978) and Neher (1983).

The open durations at depolarised potentials with 100 µM pyrantel could only be fitted by a single exponential component in some experiments (Table 7.2). The loss of one open time component and the visual observation of channels occasionally "flickering" between the open and closed state suggests open channel-block at depolarised potentials. Experiments with higher concentrations of pyrantel would be needed to study this phenomenon in more detail. Channel-block by an ion against the voltage gradient has previously been reported for the blockade of GABA channels with penicillin (Twymen et al., 1992) and is not further investigated in this paper.

7.3.2 Calculating the blocking site within the pore.

The equilibrium constant for pyrantel blocking the channel was voltage sensitive with an e-fold change every 40 mV. Using the equation given in methods where \( H = \frac{RT}{zF\delta} \), and \( RT / F = 25.26 \) mV at 20 ºC. This gave the result \( z\delta \) of 0.63.

Assuming a constant membrane field and that pyrantel has a charge of 1, this voltage sensitivity of pyrantel induced block suggests that the binding site for pyrantel is slightly greater than half way through the channel pore.
The plot of blockage frequency versus concentration at a membrane potential of -75 mV. For a simple channel block model this plot should be linear. However as can be clearly observed this is not a linear plot which suggests that the blocked state of the channel can close without re-entering the open state.
7.3.3 General comparison of currents activated by ACh, pyrantel and levamisole from *Ascaris suum*

Activation of the nicotinic receptor in *Ascaris* by pyrantel (this study), levamisole (chapter 5) and ACh (PENNINGTON & MARTIN, 1990) induced cation selective conductances with linear current / voltage relationships. Channel conductances were similar for pyrantel and ACh, where two conductance levels were observed: pyrantel 41 ± 2.04 pS (mean ± s.e., n=28) and 22 ± 0.34 pS (mean ± s.e., n=8); ACh, 40-50 pS and 25 -35 pS. Levamisole activated a single conductance of 32.9 ± 1.23 pS (mean ± s.e., n=26). Evidence exists to suggest that the smaller level with pyrantel could be a subconductance state. It is probable that each agonist binds in a slightly different way to the receptor subunits inducing different stable open confirmations of the channel. Hence levamisole induces one stable open confirmation and pyrantel two, the smaller pyrantel conductance representing a subconductance level. The presence of two populations of nicotinic channels generating two discrete conductance levels as suggested by PENNINGTON & MARTIN (1990) with ACh cannot, however, be ruled out.

In general ACh, pyrantel and levamisole activate channels with at least two open-states, two burst-states and three closed-states. Channel mean open-times are in the millisecond range with values of approximately 1.5 ms. However the duration of the "brief" and "long" openings as well as their respective percentages of the total openings showed some differences. At a membrane potential of -75 mV, levamisole (1-10 μM) and pyrantel (0.1-10 μM) activated channels with similar brief and long durations: "brief" openings, pyrantel 0.57 ms, levamisole, 0.88 ms; "long" openings, pyrantel 2.40 ms, levamisole, 2.30 ms. ACh had a larger value for both the "brief" (1.18 ms) and "long" (4.89 ms) openings. However the brief openings represented 80% of the total openings compared to around 65% with levamisole and pyrantel.
Some differences in channel properties were noted between the three agonists, for example: ACh activated currents showed no voltage-sensitivity in open- and burst-durations whereas pyrantel- and levamisole- activated currents which had mean open- and burst-durations that were significantly greater at hyperpolarised potentials. Also a flickering channel-block was observed with high concentrations of both levamisole (100 µM) and pyrantel (100 µM). Although no obvious signs of channel-block was observed with ACh, a decrease in mean open durations was observed with increased ACh concentrations. This might indicate open channel-block.

Using the simple channel-block model comparison of the blocking action of pyrantel (this study) and levamisole (chapter 5) on the nicotinic ACh channels in Ascaris suum can be made. Both compounds blocked the channel at hyperpolarised potentials in a voltage-sensitive manner, the degree of voltage sensitivity was greater for levamisole than pyrantel. The dissociation rate constant for block (K_B) showed an e-fold change every 20 mV for levamisole compared to every 40 mV for pyrantel. The difference in voltage-sensitivity suggests that levamisole and pyrantel block the channel by binding to different sites within the channel pore. The forward blocking rate constant (k_B) for pyrantel and levamisole were similar, for example at -50 mV (the potential in the study nearest to the normal resting potential of the muscle cell) k_B had a value of $2.06 \times 10^7$ M$^{-1}$ s$^{-1}$ with levamisole compared to $2.18 \times 10^7$ M$^{-1}$ s$^{-1}$ with pyrantel. The unblocking rate, k_g, was slower for pyrantel compared to levamisole: for example at -50 mV k_g was $0.81 \times 10^3$ s$^{-1}$ with pyrantel compared to a value of $2.55 \times 10^3$ s$^{-1}$ with levamisole. Comparison of the dissociation constants for block reveals that pyrantel has a lower value for K_B than levamisole: pyrantel 37 µM at -50 mV and 20 µM at -75 mV compared to levamisole which has values of 123 µM at -50 mV and 46 µM at -75 mV.
The calculation of the \( P_{\text{open}} \) values using the patch clamp technique is useful as an approximation. For example in this study the \( P_{\text{open}} \) value for levamisole activated channels does not alter with concentration at hyperpolarised potentials and increases with concentration at depolarised potentials (results section 1). This was thought to be due to the fact that at hyperpolarising potentials some sort of blocked closed state exists. With pyrantel activated channels, where it is also believed that there is a blocked-closed state at hyperpolarised potentials, the \( P_{\text{open}} \) values does not increase with concentration at depolarised potentials and decreases with concentration at hyperpolarised potentials. This illustrates that the patch-clamp technique, using the single channel mode, is not always the best way to study all channel properties. The whole-cell patch-clamp mode would probably resolve these inconsistencies in \( P_{\text{open}} \) and give a greater understanding of desensitisation.
The actions of pyrantel, levamisole and ACh on *Ascaris* muscle cells have been studied using both current-clamp and voltage-clamp techniques (Harrow & Gratton, 1985). The relative potencies obtained from conductance dose relationships suggest the order of potency is pyrantel > levamisole > ACh. This is consistent with results obtained using patch-clamp techniques where pyrantel induces channel openings at lower concentrations than either ACh or levamisole. All three compounds under voltage-clamp produced current / voltage relationships which had linear (-30 to +10mV) and non-linear components (-80 to -30 mV). Harrow & Gratton (1985) suggested this could be due to either an agonist induced channel-block or voltage-sensitive desensitisation. Results with pyrantel and levamisole now show that the non-linear component is due to agonist induced channel-block.

The occurrence of desensitization and channel-block at the *Ascaris suum* ACh receptor is of particular interest because it may be relevant therapeutically. It may be that too high a dose of nicotinic anthelmintics such as pyrantel would be less effective than using a lower dose. Experiments using intracellular current-clamp on muscle cells of *Ascaris suum* (Harrow and Gratton, 1985), showed that high doses of pyrantel (>100 µM) produced smaller responses than lower concentrations implying that it produced desensitization or acted as an antagonist (possibly by a channel-block mechanism) at high concentrations. Our results suggest that desensitization does occur with pyrantel but these effects need to be studied at a wider range of pyrantel concentrations and over longer periods of time to fully establish the nature of the desensitization.
CHAPTER 8
DISCUSSION

8.1 Channel-block model

8.1.1 The simple channel-block model

The limits of the single channel-block model used in this thesis to describe the behaviour of the currents activated by levamisole and pyrantel are discussed in both chapters 5 & 7. In this section I will discuss other schemes which might have been used.

8.1.2 Can the channel enter a closed state directly from the blocked state?

One of the predictions of the simple channel-block model is that the burst durations should increase with increasing concentration of the blocker. A close agreement between this prediction and the experimental data indicates that the blocked channel cannot undergo a confirmational change into a closed state. This was not observed in the experiments reported in this thesis with levamisole or pyrantel. Similar observations were made where the charge carried by bursts, of an ACh activated channel in rat muscle preparations, declined as the channel blocker QX222 was increased above 100 μM (Neher, 1983). Alternative mechanisms which may explain these observations are: (1) a pathway to a long duration blocked state; (2) a cyclic pathway where the channel could by-pass the open state and return to the closed state; or (3) a slow reaction rate to a desensitised state. An appropriate reaction scheme which seemed to explain the data more accurately, than the simple channel-block scheme, was suggested (Ogden & Colquhoun, 1985; Neher, 1983; Murrell et al., 1991), where the presence of another exit route from the blocked state to another closed-state without the blocked channel re-opening was included:
Where $R$ represented the rate constant away from the blocked state to a "blocked closed" state without re-entering the open state. These authors suggested that this putative closed state (BC) might arise if the blocked state underwent a confirmational change to a closed state with the blocking molecule still bound. Alternatively they suggested that there may be more than one binding site involved: one near or in the channel pore which produces channel block; and another elsewhere on the channel or in the channel pore, which when occupied causes the open state to destabilise to a closed state. In either of these cases the blocked closed state may represent a desensitised state. They also suggested that this additional closed state may be obscured in the closed time distributions due to the complexity of the closed durations (ie. at high concentrations there should be three closed states, one or two blocked states and two desensitised states included in the closed time distributions).

In this scheme (A) the frequency of blocked durations were reduced because only closings followed by reopenings were defined as blockages. The frequency of the channel reopening is defined by the ratio of $k_B$ to $(k_B + R)$. Therefore the frequency of blocked durations ($f$) was defined as:

$$f = k_B \left( \frac{k_B}{k_B + R} \right) x_B$$

Where $x_B$ is the corrected number of short gaps / corrected total open time plotted against concentration may be obtained (COLQUHOUN & SAKMANN, 1985; MURRELL et. al., 1991). If this model is sufficient to describe the experimental data, the slope of the frequency plot is represented by:

$$........(32)$$
\[ k_{+B} \left( \frac{k_{-B}}{k_{-B} + R} \right) \] 

This enables the calculation of \( R \) and \( k_{B} \) since \( k_{+B} \) has already been calculated. The mean blocked time is now represented by \((1 / k_{B} + R)\) instead of \((1 / k_{B})\). The mean number of blockages per unit open time will be reduced below its value for a simple channel block model by a factor that depends on the rate that the channel goes from the blocked state to the blocked closed state.

In this thesis, blockage frequency plots were obtained from the levamisole and pyrantel data. These plots were not linear which suggested that an exit route, as suggested above, from the blocked state to a closed state was present. However as you observed in the results sections these plots are variable and very difficult to interpret. It was therefore impossible to obtain an estimate for the rate constant \( R \) in these experiments.

8.1.3 An alternative scheme for block.

Another possible reaction scheme to explain channel flickering observations is that the blocking agent binds to the channel receptor which induced a confirmational change in the membrane spanning proteins, which resulted in a "closed" confirmation. This is discussed by NEHER & STEINBACH (1978). They suggested that the scheme below could also explain the events associated with channel block.

\[
\begin{align*}
 nT + R &\leftrightarrow TnR \leftrightarrow TnR^* [\text{blocker}] \leftrightarrow TnR^*B \leftrightarrow TnR^*B \\
 \text{closed} &\leftrightarrow \text{open} \leftrightarrow \text{blocked} \\
 \end{align*}
\]

They suggested that this scheme (B) becomes plausible if instead of assigning the voltage dependence to the binding and unbinding steps, they assigned the voltage dependence to the confirmational changes. The best test of the scheme is to plot the blocking rate against concentration which should show a simple hyperbolic
dependence. NEHER & STEINBACH (1978) had insufficient data to perform this test but suggested that the scheme was unlikely since ADAMS (1976) studied uncharged drugs which showed similar blocking actions without any voltage dependence. Thus unless the confirmational change required that the drug blocking the channel was carried across a substantial fraction of the membrane, it seems unlikely that the reaction scheme which involved a confirmational change represented the molecular mechanisms known as channel block. NEHER & STEINBACH (1978) suggested that that plugging the channel by the blocker stabilised the open state. Therefore they suggested that in equilibrium thermodynamic studies, the open state should have a higher affinity for cholinergic ligands. Thus the results of COHEN et al. (1974) where local anaesthetics increased the affinity of cholinergic agonists to the ACh receptor on membrane fragments from Torpedo supported the drug plugging the channel theory.

8.1.4 Model used for examining data collected when ACh activated the channel.

The analysis was extended by PENNINGTON & MARTIN (1990) by adopting the same model of acetylcholine interacting with its receptor as COLQUHOUN & SAKMANN (1985).

\[
\begin{align*}
R & \xrightleftharpoons[k_1]{2k_{+1}} A R, \\
A R & \xrightleftharpoons[2k_{-2}]{k_{+2}} A^R, \\
A^R & \xrightleftharpoons[2k_{+2}]{k_{-2}} A^R^*, \\
A^R^* & \xrightleftharpoons[\beta]{\alpha} A^R^*, \\
A^R^* & \xrightleftharpoons[\beta]{\alpha} A^R^*.
\end{align*}
\]

R denotes the shut receptor-channel molecule, R* the open state and A the agonist (2 molecules of agonist are believed to bind to each receptor). Oscillations between the 2
open states is believed to be very slow and so this pathway could be omitted. Oscillations between AR* and AR would cause short gaps within bursts and AR* possibly represents brief openings. Estimates of values for the rate constant for channel opening (β); and the dissociation rate constant (k₂) and β the closing rate constant were then determined. Estimates are valid for any mechanism which involved AR ↔ A₂R ↔ A₂R*.

mean length of brief closing within a burst (A₂R) = \frac{1}{\beta + 2k₂} \hspace{40em} \text{(34)}

mean number of gaps per long burst = \frac{\beta}{2k₂} \hspace{40em} \text{(35)}

\[\alpha = \frac{1}{E} \hspace{30em} \text{(36)}\]

\[k₂ = \frac{1}{T₁ \text{ closings}} \times 0.5 \hspace{30em} \text{(37)}\]

\[\beta = N₀ \times 2k₂ \hspace{40em} \text{(38)}\]

where E = the corrected estimated mean open-time in long bursts, T₁ closings = time constant for short closings and N₀ = corrected number of short closings per long burst.

A value of k_D and for affinity could also be determined:

\[k_D = \frac{k_{-1}}{k₂} \hspace{40em} \text{(39)}\]

\[\text{affinity} = \frac{1}{k_D} \hspace{40em} \text{(40)}\]
Although the method described was used to analyse currents generated from nicotinic channels located on the somatic membrane of this parasite when the receptor was activated by ACh as used by Pennington & Martin (1990), I decided that this model was not entirely suitable for the analysis of single-channel recordings when either levamisole or pyrantel were activating the channel. The main reason for this was that single-channel currents, activated by both these agonists, showed clear evidence of channel-block and the above model did not take this into account. It was decided to adopt the simple channel-block model to quantitatively describe some of the effects observed with levamisole and pyrantel.

8.1.5 Comparison of rate constants estimated using the simple channel-block model for levamisole and pyrantel with those estimated for ACh agonists.

Table 8.1 lists some values for $k_{+B}$, $k_B$ and $K_B$ obtained in several studies where the authors were looking at the block of a nicotinic channels. It can be seen that the forward blocking rate constants ranged from $0.18 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ to $7.3 \times 10^7 \text{M}^{-1} \text{s}^{-1}$, the unblocking rate constant ranged from $221 \text{ s}^{-1}$ to $110000 \text{ s}^{-1}$, and the equilibrium constant for block $K_B$ ranged from $52 \mu\text{M}$ to $1.6 \text{mM}$. The values obtained in this thesis were included at the bottom of the table for a general comparison.
Table 8.1 Comparison of values obtained for the rate constants $k_{+B}$, $k_{-B}$ and $K_B$ obtained in this study, with those obtained in other studies of block of nicotinic channels.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{+B}$ (10$^7$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_{-B}$ (s$^{-1}$)</th>
<th>$K_B$ (μM)</th>
<th>membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>suberyldicholine</td>
<td>3.8</td>
<td>221</td>
<td>5.7 μM</td>
<td>-105</td>
</tr>
<tr>
<td>acetylcholine</td>
<td>3.7</td>
<td>56000</td>
<td>1.2 mM</td>
<td>-100 to -125</td>
</tr>
<tr>
<td>carbacol</td>
<td>7.3</td>
<td>110000</td>
<td>1.6 mM</td>
<td>-120 to -140</td>
</tr>
<tr>
<td>benzocaine</td>
<td>0.18</td>
<td>270</td>
<td>114 μM</td>
<td>-70</td>
</tr>
<tr>
<td>decamethonium</td>
<td>1.7</td>
<td>1000</td>
<td>59 μM</td>
<td>-130</td>
</tr>
<tr>
<td>DMPP</td>
<td>2.27</td>
<td>1186</td>
<td>52 μM</td>
<td>-100</td>
</tr>
<tr>
<td>levamisole</td>
<td>2.06</td>
<td>2550</td>
<td>123 μM</td>
<td>-50</td>
</tr>
<tr>
<td></td>
<td>3.82</td>
<td>1750</td>
<td>46 μM</td>
<td>-75</td>
</tr>
<tr>
<td></td>
<td>6.58</td>
<td>620</td>
<td>9.4 μM</td>
<td>-100</td>
</tr>
<tr>
<td>pyrantel</td>
<td>2.18</td>
<td>810</td>
<td>37 μM</td>
<td>-50</td>
</tr>
<tr>
<td></td>
<td>3.09</td>
<td>610</td>
<td>20 μM</td>
<td>-75</td>
</tr>
</tbody>
</table>

The symbol before the compound indicate the source of the data.

~ OGDEN et al, 1981. (frog muscle, end-plate).
+ NEHER & STEINBACH, 1978. (rat sympathetic neurones)
8.2. Desensitisation

Fluctuation and relaxation measurements of end-plate currents have led to the conclusion that the rate of channel opening increased with agonist concentration, and that once open, the channel closed spontaneously. KATZ & THESLEFF (1957) however showed that in the continued presence of ACh, the initial end-plate current declined to an equilibrium value with a time constant of several seconds. Later CACHÉLIN & COLQUHOUN (1989) showed that desensitisation of nicotinic receptors in frog end-plate was a reversible biphasic process. SACKMANN et al. (1980) showed that in the presence of ACh at concentrations sufficient to cause desensitisation, single channel current pulses appeared in groups or clusters. There appeared to be three states: closed, open and a desensitised non-conducting state. A single channel can convert from the open and closed states to the desensitised state. It can fluctuate rapidly between the open and the closed state before it converted to the desensitised state. This results in channel openings appearing as clusters of bursts containing many channel activations separated from each other by long closed periods. These long closed periods are thought to represent desensitised states of the receptor. This clustering behaviour of the channel due to desensitisation may be confused visually with channel block as both result in channel activations occurring in clusters or bursts. In the case of desensitisation, the behaviour of the channel in the observed clusters represents the channel fluctuating from the closed state to the open state. Clustering due to desensitisation should not affect the mean open time of the channel, channel openings should increase with agonist concentration. With channel-block, however, the cluster represents the channel going from the open-state to the blocked-state. Under these circumstances, the mean open-time would shorten as the agonist concentration increased (as opposed to an increase in the open-time within the cluster due to desensitisation) (SACKMANN et al., 1980). It is therefore possible to differentiate between clustering due to desensitisation and clustering due to channel-block based on the affect of concentration on the open duration of the channel.
Despite a great deal of research nothing is known about the physical nature of the desensitised receptor. It has been suggested that desensitisation can be influenced by calcium ions (Chemiris et al., 1982; Oswald 1983) or phosphorylation of the receptor (Huganir et al., 1986), although there is still some controversy on this subject (see Cachelin & Colquhoun (1989) for further discussion). Desensitisation probably results from receptor channel molecule adopting long lived shut confirmations in which agonists binding sites are occupied. It has been shown that desensitisation involved at least two distinct closed states in the ACh receptor, each with affinity for agonists higher than the resting or active configuration (Katz & Thelesff, 1957; Sackmann et al., 1980; Heidmann & Changeux, 1979; Neubig et al., 1982; Heidmann et al., 1983). It has been suggested that desensitisation induces a confirmational change within the ion channel which renders the channel temporarily impermeable to ions. Reva et al. (1991) showed that mutations in the highly conserved MII region of the ion channel (portion of the receptor protein thought to represent the membrane spanning ion channel, Galzi et al., 1992) suppressed inhibition by the open channel blocker QX-222 (Neher & Steinbach, 1978) and also decreased the rate of desensitisation. They suggested that a mutation of the leucine 247 group in the ion channel, when replaced by a smaller hydrophobic amino acid rendered the high affinity desensitised state permeable. Desensitisation was observed with high concentrations of levamisole and pyrantel but no further explanation was sought in this study.

Comparison of $P_{open}$ shows that ACh in this preparation has a greater $P_{open}$ than levamisole or pyrantel (Pennington, & Martin, 1992). The corrected open probability for 0.1 $\mu$M pyrantel had a value of 0.0077 ± 0.0020 (mean ± s.e., n=6) compared to a value of 0.0068 ± 0.0017 (mean ± s.e., n=4) for 2 $\mu$M levamisole. These values were significantly lower than the value of 0.029 ± 0.013 (mean ± s.e., n=12)
for 1-10 μM ACh. The value for pyrantel and levamisole are nearer to the value for 25μM ACh of 0.0052 ± 0.0037 (mean ± s.e.mean), suggesting that these concentrations may be showing some degree of desensitization. However it should be noted that desensitisation was observed with 100μM levamisole and some signs of desensitisation were observed with 100μM pyrantel.

8.3 Analysis of closed-times

If there are double openings observed in the record then obviously there was more than one active channel in this particular patch and these experiments were discarded from this study. However if the observed mean open time is small and the mean closed time is large it is still possible that there are more than one active channel in the patch. This can be treated in several ways: (1) An estimate of the number of channels in the patch can be determined by the method of COLQUHOUN & HAWKES (1983). They suppose that the channel can either be shut or open, and that n_o openings were observed, but no double openings, and that most of the time the channel was closed. Therefore the mean closed-time (M_c) is greater than the mean open-time (M_o). The question to be answered is how probable is the observation, if there were actually N channels present.

\[ \pi = \frac{1}{1 + \left( \frac{N-1}{N} \right) \frac{m_o}{m_c}} \]  

\[ \ldots \ldots (41) \]

Equation (41) calculates the probability (\( \pi \)) that when the channel is open, that the next transition is the open channel shutting rather than the opening of another channel. The observations consist of n_o consecutive occurrences of the event (opening and shutting of the same channel) with probability p. The probability of observing the channel opening and closings in an experiment given that there are n channels present is:

\[ p_{obs} \approx \pi^{n_o} \]  

\[ \ldots \ldots (42) \]
For example consider some of the data presented in this thesis with levamisole. In one experiment with 2 \( \mu \)M of levamisole, the \( m_0 \) was 2.23 ms, the \( m_c \) was 178 ms and \( n_0 \) was 231. So for \( N=2, \pi =0.993774 \) and \( p_{\text{obs}} \) equalled 0.2363. Therefore these observations would not be surprising if there were actually two channels present. With data like this it would be dangerous to make assumptions regarding the closed time distributions. Therefore any detailed interpretation of long closed times is highly unreliable, as there is probably greater than one channel. However in these experiments several openings are observed to occur in close succession followed by a much longer gap before the next burst of openings. This implied there was more than one closed state. It is likely if the gaps within the burst are short that all the openings in a burst originate from one channel, even if the next burst may originate from a different channel. In these case, the distribution of the lengths of shut periods within (not between) bursts can be interpreted. (COLQUHOUN & SACKMANN, 1983).

To study the distribution of closed times (ie. desensitisation) the channel records would have to be significantly longer than the 70 -100 s durations at each potential used in this study.

8.4 Future work

The results in this thesis have given a further insight of the mode of action of both the anthelmintics levamisole and pyrantel at the somatic muscle of the parasite Ascaris suum. It has been shown that both compounds activate the nicotinic receptor and also block the open channel. Several points still remain unanswered: (1) does levamisole act at muscarinic receptors in this parasite?; (2) what contributes to the recovery of levamisole treated parasites while they are maintained in levamisole ?; and (3) what is the physical state of the receptor during desensitisation. It would be of interest to examine the levamisole treated worms that showed signs of recovered movement, to investigate the receptors that are present and can still be activated. This is of particular
interest when you consider the results of LEWIS et al. (1980) who showed that mutant C. elegans strains can still survive although they are deficient in the levamisole receptor. These nematodes show signs of deficient motility. A study of this nature may shed some light on desensitisation. Another field which may be of interest and provide another target for anthelmintic development would be the study of muscarinic receptors in this parasite. SERBERG & STRETTON (1986) suggested that muscarinic receptors may be present in Ascaris on the DE1 and DE3 neurones, and pilocarpine and muscarine have a weak hyperpolarisation effect on Ascaris muscle which COLQUHOUN et al. (1991) suggested might indicate the presence of muscarinic receptors of lower sensitivity than the nicotinic ACh receptors on the somatic muscle. Finally the process of desensitisation and the factors affecting the rate and onset could also be studied.
CHAPTER 9
REFERENCES


Caldwell, P.C. (1972) Sodium ion and potassium ion transport and permeability: a comparison between squid giant axons and Ascaris muscle fibres. *Biochemical Journal*, **127**, 54P.


COLES unpublished, quoted by COLES, G. C (1977)


DONAHUE, M. J.; YACOUB, N. J.; MICHNOFF, C. A.; MASARACCHIA, R. A.;
HARRIS, B. G. (1981c) Serotonin (5-hydroxytryptamine): a possible regulator of
glycogenisis in perfused segments of Ascaris. Biochemical & Biophysical research
communications, 105, 112-117

activity with glycogen metabolism in muscle of Ascaris suum. American Journal of
Physiology, 242, R514-R521.

AMP-mediated regulation of glycogen metabolism in levamisole perfused Ascaris suum

DONAHUE, M. J.; MICHNOFF, C. A.; MASARACCHIA, R. A. (1985) Calcium-
dependent muscle contraction in obliquely striated Ascaris suum muscle. Comparative
Biochemistry and Physiology, 82(2), 395-403.

DUITTOZ, A. H. & MARTIN, R. J. (1991a) Effects of the arylaminopyridazine-GABA
derivatives, SR95103 and SR95531 on the Ascaris muscle GABA receptor: the relative
potency of the antagonists in Ascaris is different to that at vertebrate GABA<sub>a</sub> receptors.
Comparative Biochemistry and Physiology, 98C, 417-422.

single-channel currents from Ascaris suum muscle. Comparative Biochemistry and
Physiology, 98C, 423-432.


HILLE & SCHWARZ, (1978) Pottasium channels as multi ion single file pores. J. General Physiology, 72, 409-442.


NEUBIG, R.; BOYD, N.; & COHEN, J. B. (1982) Confirmations of Torpedo acetylcholine-receptor associated with ion transport and desensitisation. Biochemistry, 21, 3460-


ROSKIN, G. (1925) Beitrage zur Kenntniss der glatten Muskelzellen. Erste

*General Pharmacology, 11*, 141-146.


PUBLICATIONS


Robertson, S. J. & Martin, R. J. (1993) Application of levamisole to the cytoplasmic surface of muscle membrane leads to the activation of nicotinic acetylcholine receptors in the nematode parasite *Ascaris suum*. *(Pesticide Science, in press).*

Robertson, S. J.; Pennington, A. J.; Evans, A. M.; Martin, R. J. The action of pyrantel as an agonist and an open channel blocker at nicotinic receptors in isolated *Ascaris suum* muscle vesicles. Manuscript submitted to J. Experimental Biology
The physiology and pharmacology of neuromuscular transmission in the nematode parasite, *Ascaris suum*

R. J. MARTIN, A. J. PENNINGTON, A. H. DUITTOZ, S. ROBERTSON and J. R. KUSEL

Department of Pre-clinical Veterinary Sciences, R.(D).S.V.S. University of Edinburgh, Edinburgh EH9 1QH
and *Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ*

**SUMMARY**

The organization of *Ascaris* motoneurones and nervous system is summarized. There is an anterior nerve ring and associated ganglia, main dorsal and ventral nerve cords which run longitudinally, and a small set of posterior ganglia. Cell bodies of motoneurones are found in the ventral nerve cord and occur in 5 repeating 'segments'; each contains 11 motoneurones. Seven morphological types of excitatory or inhibitory motoneuron are recognized.

Each *Ascaris* somatic muscle cell is composed of the contractile spindle; the bag region, containing the nucleus; the arm; and the syncytial region, the location of neuromuscular junctions. The resting membrane potential of muscle is approximately $-30$ mV and shows regular depolarising, Ca-dependent 'spike potentials' superimposed on smaller Na+- and Ca*-dependent 'slow waves' and even slower 'modulation waves'. The membrane shows high Cl- permeability. Adjacent cells are electrically coupled so that electrical activity in the cells is synchronized. Acetylcholine (ACh) and γ-aminobutyric acid (GABA) affect the electrical activity. Bath-applied ACh increases membrane cation conductance, depolarizes the cells, alters the frequency and amplitude of spike potentials and produces contraction. Bath-applied GABA increases Cl- conductance, decreases spike activity and causes hyperpolarization and muscle relaxation.

The extra-synaptic ACh receptors on the bag region of *Ascaris* muscle can be regarded as a separate subtype of nicotinic receptor. ACh and anthelmintic agonists (pyrantel, morantel, levamisole) produce a dose-dependent increase in cation conductance and membrane depolarisation which is blocked by tubocurarine, mecamylamine but not by hexamethonium. The potency of GABA agonists, with the exception of sulphonic acid derivatives, correlates with the vertebrate GABA$_A$ receptor. The potency of antagonists does not. Thus, bicuculline, securinine, piritazepine, SR95531 and RU5135 are potent vertebrate GABA$_A$ antagonists but have little effect on GABA receptors. The potency order of the arylaminoarylazine GABA antagonists: SR95103, SR95132, SR42666, SR95133, SR95531, SR42627 and SR42640 at the *Ascaris* GABA receptors contrasts with that at vertebrate GABA$_A$ receptors. It has been suggested that the receptor is referred to as a GABA$_A$ receptor.

Patch-clamp studies show that ACh activates a non-selective cation channel which has a main conductance of 40-50 pS and apparent mean open time of 1-3 ms; a smaller channel of 20-30 pS with a similar open-time is also activated. Pyrantel and levamisole also produce openings with similar conductances and open-times. GABA activates a CI- channel with a main state conductance of 22 pS and an apparent mean open duration of 32 ms; conductance states of 10 and 15 pS are also seen. Piperazine similarly activates this channel but the mean open-time is shorter (14 ms). Ivermectin in high doses, is an antagonist which reduces the GABA channel conductance and $P_{open}$; it does, however, open 'small' Cl- channels when applied to the outside surface of membrane. These channels have a conductance of 9-15 pS and very long open times ($> 100$ ms). 5-HT does not have a direct effect on membrane potential or conductance but acts on cAMP levels and glycogen metabolism. Dopamine, octopamine and AFI may act as neurotransmitters or neuromodulators.

**Key words:** *Ascaris suum*, neuromuscular transmission, electrophysiology, pharmacology, anthelmintics.

**INTRODUCTION**

Estimates of global parasitism in the human population show that *Ascaris* may affect more than 1000 million people and give rise to considerable suffering in Third World countries as well as affecting the growth and performance of livestock (see for example, Horton, 1990). The treatment of nematode infections in man and animals with anthelmintic drugs plays a vital role in the control of these parasites.

The adult *Ascaris suum* is a common, large (up to 30 cm) nematode parasite found in the intestine of the pig. The frequent occurrence of this parasite means that it can be collected from local abattoirs on a weekly basis for study in the laboratory. The large size of the parasite, its muscle cells and some of its neurones have facilitated anatomical, biochemical, physiological and pharmacological studies, not possible on smaller nematodes. *A. suum* has been used frequently and successfully for examining the mode of action of a variety of anti-nematodal drugs.

A number of anthelmintics interfere selectively with neuromuscular transmission of the nematode parasite. The purpose of this paper is to review the physiology and pharmacology of neuromuscular transmission in the nematode parasite *A. suum*, and to provide a background useful for designing, testing and understanding the mode of action of anti-nematodal drugs.
**Lcomotory Behaviour**

The body movements made by adult Ascaris allow the parasite to maintain its position in the pig intestine and alter the position of the head for feeding. Coordinated waves of contraction and relaxation, in the dorso-ventral plane only, can propagate anteriorly or posteriorly. The most common wave is of large amplitude, which propagates anteriorly and starts about half-way along the length of the animal near the vulva. This waveform gives rise to forward propulsion of the Ascaris when contained within a tube structure like the intestine (Johnson & Stretton, 1980). The more complex behaviour of the head is believed to be under the control of the cranial ganglia and sublateral nerve cords.

**Nervous System**

**Basic structure of Ascaris nervous system**

Early studies of the nervous system of Ascaris (Hesse, 1892; Goldschmidt, 1908, 1909) showed that there are only a small number of neurones present (ca. 250) and that these are completely reproducible from animal to animal.

Goldschmidt (1908) described an anterior nerve ring, surrounding the pharynx with a series of associated ganglia. The ganglia associated with the circumferential nerve ring are the dorsal ganglion, the ventral ganglion, the two lateral ganglia, and the retroversiccular ganglion. The anatomy and electrophysiology of the retroversiccular ganglion have been examined by Angstadt, Domnoyer & Stretton (1989); this ganglion containing 13 neurones, which are interneurones (including the neurones named AVF-, GAB-, RIF- and RIG-like) or motoneurones (DE2 and V2). The two main nerve cords arising from the nerve ring pass caudally to form the dorsal and ventral nerve cords; motoneurones are found scattered in the ventral nerve cord; and a second set of ganglia is found in the tail region (Goldschmidt, 1908, 1909). The nerve cords are connected by single or double transverse nerves forming commissures (Hesse, 1892).

Stretton et al. (1978) described and classified the structure and physiology of motoneurones; they based their observations on tracing nerve fibres in serial sections, and on intracellular recordings from muscle bags during electrical stimulation. The motor nervous system was divided into segments. Each segment usually has three right-hand commissures and one left-hand commissure (Fig. 1), and contains 11 motoneurones with 6 non-segmental interneurones crossing the segments. Each motoneurone cell body has two processes; one of the processes may have a single branch. The 11 motoneurones were separated into 7 types according to the distribution of their axon and dendrites: three types (DI, DE2 and DE3) are represented only once in each segment, while the remaining four types (DE1, VI, V1 and V2) occur twice in each segment (Fig. 1).

Excitatory and inhibitory potentials recorded in muscle after selective stimulation showed that DI and VI were inhibitory and that the remainder were, or were likely to be, excitatory (Stretton et al. 1978). Histochemical studies showed that choline acetyltransferase was present in DE1, DE2 and DE3 motoneurones, a finding consistent with the suggestion that acetylcholine is the excitatory neurotransmitter (Johnson & Stretton, 1985). GABA-immunoreactivity has been identified in the inhibitory motoneurones, DI and VI (Johnson & Stretton, 1987; Sithigorngul et al. 1989), a finding consistent with the suggestion that GABA is the inhibitory neurotransmitter.

The motoneurones of Ascaris are structurally...
similar to motoneurones identified in *C. elegans* (White *et al.* 1976, 1986), suggesting that all nematodes may be organized in a similar way. Additional minor nerve cords in *Ascaris* have also been described (Johnston & Stretton, 1987). They include dorsal lateral, dorsal sublateral, ventral sublateral, and ventral lateral nerve cords which are all found adjacent to the lateral line.

**Non-spiking motoneurones of Ascaris**

Electrical stimulation of single motoneurons in dissected preparations did not produce all-or-none responses in muscle cells but responses which were graded with the intensity of the stimulus (Walrond *et al.* 1985); this implies that *Ascaris* motoneurons are non-spiking. Direct intracellular records from commissures confirmed the non-spiking nature of motoneurones; it showed that the neuronal membrane has a high resistance (61–251 KΩ cm⁻²) and a long space constant (+10 mm), suitable for passive conduction over long distances (Davis & Stretton, 1989a). Another property of the neuronal membrane is the presence of voltage-activated Ca²⁺ currents, which occur at the resting membrane potential; these currents may give rise to the tonic release of the neurotransmitter acetylcholine (Del Castillo, de Mello & Morales, 1963; Davis & Stretton, 1989b). The Ca²⁺ currents in *Ascaris* remain to be typed physiologically or pharmacologically and it is not known if they correspond to the vertebrate T, L or N type of Ca²⁺ channels.

**Physiological interconnections of motoneurones**

Walrond & Stretton (1985) showed, with the dissected preparation, that stimulation of the dorsal excitatory motoneurone, DE1, produced reciprocal inhibition in ventral muscle cells as a result of the projection onto the ventral inhibitory motoneurone, VI. They further showed that the motoneurones, DE2 and DE3 could also produce reciprocal inhibition as a result of projecting onto VIs in adjacent segments. Reciprocal inhibition in the ventral cord was suggested on anatomical grounds involving the putative excitatory motoneurones V1 and V2 and their projection onto the dorsal inhibitory motoneurone DI. In their paper, Walrond & Stretton (1985) demonstrated that stimulation of DE1, DE2 and DE3 produced, via a projection onto DI, inhibition in muscle fields adjacent to the dorsal excitatory field. The consequence of this synaptic organization, with reciprocal and adjacent inhibition, is that stimulation of a single excitatory motoneurone would produce an omega-shaped body waveform. This synaptic organization provides a basis for a model which can produce propagated waves of muscle contraction.

**MUSCLE**

**Anatomy**

*Morphology of somatic muscle cells.* The anatomy of *A. suum* body muscle was described as early as 1866 by Schneider (1866). It was then the subject of debate by several scientists (Goldschmidt, 1908, 1909; Cappe de Baillon, 1911) because of its unusual structure, with branches of the muscle passing to the nervous system, rather than branches of the nervous system reaching the muscles.

Fig. 2 shows in diagrammatic form the structure and location of *Ascaris* somatic muscle. There is the longitudinal contractile region, known as the spindle ('fuseau', Cappe de Baillon, 1911); it lies under the hypodermis and is composed of 'obliquely striated muscle' (Rosenbluth, 1965a, b, 1967, 1969). Its striaions form an acute angle, rather than the usual 90° of skeletal muscle, because of the different organization of thick and thin filaments. This organization allows the greater extensibility of smooth muscle and maintains the velocity of contraction of skeletal muscle. In *Ascaris*, fast twitch contraction is associated with Ca²⁺ fixation by actin, and is produced by acetylcholine; muscle relaxation is associated with a change in the phosphorylation state of light chain myosin (Martin & Donahue, 1987). The bag or belly ('panse', Cappe de Baillon, 1911) is a large 200 μm bag-shaped structure which
contains submembrane mitochondria, the nucleus and particulate glycogen (Rosenbluth, 1965b); it lies in the perienteric space (Fig. 2).

Each somatic muscle cell of *Ascaris* has on average 2-7 arms (Stretton, 1976). The arm is a thin process which arises from the base of the bag and passes to the syncytium which lies over the nerve cord (Fig. 2). When the arms reach the syncytium, they break
Neuromuscular transmission in Ascaris

up into a number of fine processes or 'fingers'. Tight junctions are found between adjacent arms of the muscle cells at the syncytium (Rosenbluth, 1965b) and they are responsible for the electrical coupling seen between adjacent cells (De Bell, Del Castillo & Sanchez, 1963). Neuromuscular junctions (Fig. 3) are found between the syncytial region and a longitudinally-running axon of a motoneurone (Rosenbluth, 1965b). The neuromuscular junctions are formed by 2 μm extensions of the longitudinal axons which penetrate the hypodermal chalice and closely approach the syncytium. The synaptic region is associated with clear spherical 40 nm vesicles clustered under the pre-synaptic membrane; dense core vesicles, 80–100 nm in diameter; giant mitochondria; and membrane thickenings on the pre-synaptic and post-synaptic membrane (Rosenbluth, 1965b). Except in the head region, the dorsal muscle cells are only connected to the dorsal nerve cord, the ventral muscle cells are only connected to the ventral nerve cord; this arrangement is consistent with contraction which is only possible in the dorso-ventral plane.

Electrophysiology

Electrical activity of muscle cells. Jarman (1959) was the first to make intracellular recordings from Ascaris muscle cells. He described the presence of regular spontaneous depolarizing potentials superimposed on a resting potential of around −30 mV. The ionic basis of the low resting potential has been investigated by Del Castillo, De Mello & Morales (1964a) and Brading & Caldwell (1971). In both studies, extracellular K⁺ had little effect on potential, in contrast to the effects of extracellular Cl⁻ and, to a lesser extent, Na⁺. Ion flux experiments of Caldwell & Ellory (1968) showed that the permeability ratio for K⁺, Na⁺ and Cl⁻ was 1:4:7, respectively. These studies failed to provide a satisfactory explanation for the behaviour of the membrane potential. Brading & Caldwell (1971) suggested that an active electrogenic pump, possibly moving carboxylic acids, may explain why the original Goldman-Hodgkin-Katz equation was not sufficient for describing the behaviour of the membrane potential. Caldwell (1974) subsequently considered this problem in a review. The composition of peri-enteric fluid, which normally surrounds the muscle cells, is consistent with this hypothesis. Hobson et al. (1952a, b) found the peri-enteric fluid to contain low concentrations of Cl⁻ but high concentrations of volatile fatty acids. The volatile fatty acids (including 2-methyl butyrte and 2-methyl valerate) are produced from the anaerobic metabolism of glucose (Saz & Weil, 1962; Tsang & Saz, 1973).

The spontaneous depolarizing potentials described by Jarman (1959) were shown to be myogenic in origin and to arise at the syncytium (De Bell, 1965; De Bell et al. 1963). Three types of depolarizing potential have now been described (Fig. 4): (a) spikes of varying amplitude up to 30 mV and lasting 5–50 ms (De Bell et al. 1963; Weisblat & Russel, 1976); (b) slow waves up to 20 mV in amplitude and lasting 100–1000 ms (De Bell et al. 1963; Weisblat & Russel, 1976); and (c) a long lasting modulation wave around 5 mV, lasting 3–20 s and coinciding with contraction of the body wall (Weisblat & Russel, 1976).

Ion substitution experiments have shown that Ca²⁺ currents are responsible for the spikes and that both Na⁺ and Ca²⁺ currents can produce the slow waves (Weisblat, Byerly & Russel, 1976). The Ca²⁺ spikes were not blocked by the Na⁺ channel blocker, tetrodotoxin (Jarman & Ellory, 1969), and the Ca²⁺ channel blockers, verapamil and cinnarizine unexpectedly had no effect either; however, 'high concentrations' of ivermectin did block spiking (Wann, 1987). K⁺ currents, activated by depolarization (Martin, 1982), may facilitate repolarization; these K⁺ currents are blocked by 4-aminopyridine and the anthelmintic, diethylcarbamazine (Martin, 1982). The ionic basis of the modulation waves, inferred from the behaviour of 'square waves', suggests that Ca²⁺ currents are involved (Weisblat et al. 1976).

Jarman (1959) showed that the electrical activity seen in adjacent muscle cells was correlated. He found that anteriorly placed cells depolarized before cells behind them; he also found that cells nearest to a nerve cord depolarized before those placed laterally. De Bell et al. (1963) and Del Castillo et al. (1967, 1989) have used two intracellular micropipettes with current injection to record the electrical coupling which occurs between adjacent muscle cells. The coupling is consistent with the presence of tight junctions seen with the electron microscope (Rosenbluth, 1965b). Initial studies on the coupling...
revealed that the receptors changes spike on synaptically located are (Del Castillo et al. 1982; Martin, 1980). Ionicophoresis experiments have shown that the GABA receptors responsible for this are at the synctium and extrasynaptically over the surface of the rest of the cell including the bag region (Fig. 5) (Martin, 1980). The physiological function of the extrasynaptic acetylcholine and GABA receptors is not known, but they may be exploited in pharmacological experiments designed to look at receptor properties; they may also be activated by anthelmintics during therapy of the host animal.

**Ionic basis of the electrical responses to acetylcholine and GABA.** The ionic basis of electrical responses to acetylcholine and GABA have been investigated by applying transmitter ionophoretically to the bag region of the muscle cell and making current- or voltage-clamp records of the responses. Acetylcholine increases the non-selective cation conductance of the membrane with a reversal potential near 0 mV (Martin, 1982; Harrow & Gratton, 1985). This is compatible with acetylcholine opening ion-channels permeable to both Na+ and K+. Ionophoresis of GABA onto the bag, on the other hand, produces a current which has a reversal potential near −65 mV and which is explained by the opening of ion-channels permeable to Cl− ions (Martin, 1980).

**Biochemistry**

**Biochemistry of acetylcholine.** The presence of acetylcholine in Ascaris was first described by Mellanby in 1955. Choline acetyltransferase (CAT), the enzyme responsible for the synthesis of acetylcholine has been demonstrated in the commissural branches of the excitatory motoneurones as well as in the hypodermis (Johnson & Stretton 1980, 1985). The presence of CAT in the excitatory motoneurones is consistent with acetylcholine being the transmitter. The function of CAT in the hypodermis is not clear, but it has been suggested that the synthesis and release of acetylcholine from the hypodermis may cause tonic contraction by acting on extrasynaptic ACh muscle receptors (Johnson & Stretton, 1980). The hypodermal CAT has also been reported to be concentrated in two bands within 1 mm of the tip of the head, but the physiological function of this remains obscure (Johnson & Stretton, 1980).

Cholinesterase, the enzyme inactivating acetylcholine, was first reported in Ascaris by Bueling (1952). Lee (1962) described the distribution of this enzyme using histochemical techniques; in the head region, most of the activity was associated with the contractile region of the muscle in the extracellular matrix; enzyme activity was also observed in muscle arms near their endings on the nerve cords but not on the bag region; little or no staining was seen in the
Table 1. Potency of cholinergic agonists and antagonists in *Ascaris*
(Baldwin & Moyle 1957); lowest concentration producing contraction. Natoff (1969): range of agonist concentrations producing contraction; antagonist $K_p$, Rozhkova et al. (1980): EC$_{50}$ muscle contraction; antagonist $K_G$, Harrow & Gratton (1985): IC$_{50}$ input conductance. Colquhoun et al. (1990): relative potency for agonists; IC$_{50}$ for antagonists.)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µM)</td>
<td>(µM)</td>
<td>(µM)</td>
<td>(µM)</td>
</tr>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morantel</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>Pyrantel</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>Dimethylphenylpiperazinium</td>
<td>—</td>
<td>0.2-0.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Pentyltrihyrammonium</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>—</td>
<td>—</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>1</td>
<td>6-55</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Proprionylcholine</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Succinylcholine</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>1</td>
<td>2-38</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>5</td>
<td>5-55</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Trimehtylammonium</td>
<td>—</td>
<td>2-9</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Muscarine</td>
<td>—</td>
<td>—</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Muscimol</td>
<td>—</td>
<td>—</td>
<td>0.0006</td>
<td></td>
</tr>
<tr>
<td>Cariscoline</td>
<td>—</td>
<td>—</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Arecoline</td>
<td>—</td>
<td>—</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Suberyldicholine</td>
<td>1</td>
<td>73-366</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>Decamethonium</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-methyllycaconitine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.23</td>
</tr>
<tr>
<td>Bungarotoxin</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.4</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>—</td>
<td>0.25</td>
<td>—</td>
<td>0.33</td>
</tr>
<tr>
<td>Strychnine</td>
<td>100</td>
<td>2-34</td>
<td>0.5</td>
<td>1.29</td>
</tr>
<tr>
<td>Tubocurarine</td>
<td>100</td>
<td>6-0</td>
<td>6.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Tetraethyrammonium</td>
<td>—</td>
<td>—</td>
<td>3-2</td>
<td></td>
</tr>
<tr>
<td>Pancuronium</td>
<td>80</td>
<td>80</td>
<td>6.7</td>
<td>14</td>
</tr>
<tr>
<td>Atropine</td>
<td>80</td>
<td>—</td>
<td>—</td>
<td>43</td>
</tr>
<tr>
<td>Decamethonium</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

nervous tissue. No staining was seen caudal to the vulva, an observation consistent with propagation of contraction waves starting at the vulva and passing anteriorly. Johnson & Stretton (1980) have reported the presence of two types of cholinesterase, a 13 S and a 5 S form, which are not distributed in the same way; the 5 S fragment may be regulated by muscle activity, but the 13 S fragment may not. Although cholinesterase is responsible for the breakdown of acetylcholine released from excitatory moto-neurones, it is also secreted in the external environment by *Ascaris* and has been suggested to act as a ‘biochemical holdfast’ by blocking local peristalsis (Lee, 1970).

Neostigmine, a carbamate anti-cholinesterase in other preparations has an acetylcholine-potentiating effect in *Ascaris* (Del Castillo et al. 1963). Selective organophosphorous anti-cholinesterases, including metrifonate and haloxon (Martin, 1985a), are used as effective anthelmintics. The use of these compounds against nematodes shows cholinesterase to be an effective target site for anthelmintics.

**Pharmacology**

**Pharmacology of the acetylcholine receptor.** Initial experiments on the pharmacology of *Ascaris* acetylcholine receptors used contraction of muscle strips. Low concentrations of nicotine produced contraction (Toscano-Rico, 1926; Baldwin & Moyle, 1949; Natoff, 1969) like acetylcholine (Baldwin & Moyle, 1949; Natoff, 1969; Rozhkova, Malyutina & Shishov, 1980; Grzywacz, Szkudlinska & Zandarowska, 1985; Onuaaguluchi, 1989). The acetylcholine-induced contractions were blocked by tubocurarine but not by atropine (Baldwin & Moyle, 1949; Natoff, 1969; Rozhkova et al. 1980) so that the *Ascaris* acetylcholine receptor has some of the pharmacological properties of the vertebrate nicotinic receptor. Natoff (1969) and Rozhkova et al. (1980) attempted to classify the receptor as either ganglionic or neuromuscular. These papers showed that the potent ganglionic nicotinic agonist, dimethylphenylpiperazinium (DMPP) is more potent than acetylcholine in *Ascaris*; the potent ganglionic nicotinic antagonist...
mecamylamine was the most potent acetylcholine antagonist in Ascaris, more potent than tubocurarine; in contrast, hexamethonium, a potent ganglionic antagonist in vertebrates, has a low potency in Ascaris. The Ascaris acetylcholine receptor cannot, therefore, be classified as either ganglionic or neuromuscular and can be regarded as a separate subtype of nicotinic receptor (i.e. the Ascaris ACh receptor).

Electrophysiological techniques have also been used to examine effects of cholinergic agonists and antagonists (Aubry et al. 1970; Aceves, Erlij & Martinez-Maron, 1970; Martin, 1982; Harrow & Gratton, 1985; Colquhoun, Holden-Dye & Walker, 1990); the potency of these compounds in Ascaris is summarized in Table 1. Levamisole and pyramos are more potent agonists at the Ascaris ACh receptor than at vertebrate nicotinic receptors where they have only weak nicotinic actions (Aubry et al. 1970; Eyre, 1970). The selective action of these drugs allows them to be used as effective anthelminthics, killing the nematode parasite but not the host. The degree of selectivity will obviously affect the safety and efficacy of any nicotinic drug selected for therapeutic purposes.

Table 2. GABA agonists potency in Ascaris
(Duittoz & Martin (1990): EC50 input conductance dose-response curves described by the modified Hill equation with the estimated Hill coefficient between 2 and 4; the relative potency is given in parentheses; means ± S.E., n = 3-13; Holden-Dye et al. (1988, 1989): relative potency.)

<table>
<thead>
<tr>
<th>Agonist*</th>
<th>EC50, µM (rel. potency)†</th>
<th>Rel. potency†</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-(+)-dihydromuscimol</td>
<td>8.3 ± 0.5 (3.704)</td>
<td>7.53</td>
</tr>
<tr>
<td>± dihydromuscimol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZAPA</td>
<td>24.1 ± 4.6 (1.266)</td>
<td>1.46</td>
</tr>
<tr>
<td>GABA</td>
<td>30.7 ± 2.1 (1.000)</td>
<td>0.85</td>
</tr>
<tr>
<td>R-(-)-dihydromuscimol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TACA</td>
<td>65.7 ± 14.5 (0.467)</td>
<td>0.55</td>
</tr>
<tr>
<td>IAA</td>
<td>76.1 ± 11.3 (0.403)</td>
<td>0.20</td>
</tr>
<tr>
<td>Muscimol</td>
<td>82.5 ± 20.4 (0.372)</td>
<td>0.55</td>
</tr>
<tr>
<td>Isoguvacine</td>
<td>124.2 ± 10.1 (0.247)</td>
<td>0.19</td>
</tr>
<tr>
<td>S-(+)-3-OH-GABA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GuAA</td>
<td>135.4 ± 18.0 (0.227)</td>
<td>0.16</td>
</tr>
<tr>
<td>Homo-beta-proline</td>
<td>233.6 ± 37.2 (0.131)</td>
<td>0.079</td>
</tr>
<tr>
<td>DAVA</td>
<td>366.5 ± 17.6 (0.084)</td>
<td>0.079</td>
</tr>
<tr>
<td>CACA</td>
<td>391.8 ± 83.2 (0.078)</td>
<td>0.031</td>
</tr>
<tr>
<td>Isonipecotic acid</td>
<td>409.2 ± 83.2 (0.075)</td>
<td>—</td>
</tr>
<tr>
<td>BGUpA</td>
<td>536.5 ± 49.7 (0.057)</td>
<td>0.085</td>
</tr>
<tr>
<td>THIP</td>
<td>N.A.</td>
<td>0.006</td>
</tr>
<tr>
<td>S-(+)4-methyl-TACA</td>
<td></td>
<td>0.007</td>
</tr>
</tbody>
</table>

* GABA, gamma-amino butyric acid; DHM, dihydromuscimol; ZAPA, (Z)-3-[9(aminomimethyl)thiol]-2-propanoic acid; TACA, trans aminocrotic acid; IAA, imidazole-4-acetic acid; IAA, imidazole-4-acetic acid; GuAA, guanido acetic acid; DAVA, delta-amino valeric acid; CAVA, ca aminocrotic acid; BGUpA, beta-guanidino propionic acid. 3-Aminopropene sulphonic acid, pyridine-4-sulphonic acid, baclophen, glycine and alanine were without effect at concentrations below 1 mM (Holden-Dye et al. 1988, 1989; Martin & Duittoz unpublished). N.A.: not active at 1 mM.
† Duittoz & Martin (1990).

It may be concluded that the Ascaris acetylcholine receptor is a subtype of nicotinic receptor and there are compounds which have a selective effect on the nematode acetylcholine receptor but not on the host receptors.

Pharmacology of the GABA receptor. The pharmacology of extrasynaptic GABA receptors has been studied using a two microelectrode recording technique to examine conductance dose–response relationships during the bath-application of GABA agonists and antagonists (Martin, 1980, 1982; Holden-Dye & Walker, 1988; Holden-Dye et al. 1988, 1989; Duittoz & Martin, 1989, 1990a) and at the single-channel level (Martin, 1985a, b; Martin & Pennington, 1990; Duittoz & Martin, 1990b).

Conductance dose–response relationships for a variety of bath-applied GABA agonists have been described by the modified Hill equation and the E50s determined; these values are shown in Table 2 along with estimates of the relative potency of the agonists (Holden-Dye et al. 1989).

The agonist profile of the Ascaris receptor is similar, but not identical to that of the vertebrate GABAa receptor: (1) The stereoselectivity of the
Ascaris receptor and the vertebrate GABA receptor for the enantiomers of dihydromuscimol, 3-0H-GABA, and 4-me-TACA is the same (Holden-Dye et al. 1989). (2) Most potent GABA agonists are potent in Ascaris, so that the relative potency of GABA agonists at vertebrate receptors is correlated with the relative potency of agonists at the Ascaris receptor (r = 0.74; Holden-Dye et al. 1989). (3) The potency of the sulphonic acid GABA derivatives (JAPS and P4S) as well as muscimol and isoguvacine (Table 2) is less than at the vertebrate GABA receptor. (4) The anthelmintic piperazine derivatives act as a selective GABA agonist in Ascaris (Martin, 1982).

There are, however, major pharmacological differences between the Ascaris GABA receptor and the vertebrate GABA receptor. (1) Pentobarbital and flurazepam do not potentiate GABA responses in Ascaris, as they do in the vertebrate (Holden-Dye et al. 1989). (2) The GABA antagonists bicuculline, picrotoxin, securine, pitrazepine, SR95353, dieldrin and TBPS are weak or inactive in Ascaris (Martin, 1980; Wann, 1987; Holden-Dye et al. 1988, 1989; Duittoz & Martin, 1990a). In addition, we have also tested one of the most potent GABA antagonists, the steroid RU5135, and found that it only acts as a weak non-competitive GABA antagonist in Ascaris (Fig. 6). The concentrations required to block the GABA response in Ascaris muscle (IC50 = 117 μM) were much higher than found at the GABA receptor (IC50 = 5 nM; Simmonds & Turner, 1985). The insensitivity to all these drugs, therefore, distinguishes the Ascaris GABA receptor pharmacologically from the vertebrate GABA receptor and confirms the earlier suggestion (Martin, 1987) that there is a separate type of receptor in nematodes, i.e. the GABA receptor (n = not antagonized by picrotoxin; n = nematodes).

Arylaminoypyridazine derivatives. We have investigated further the actions of arylaminoypyridazine-GABA derivatives (Fig. 7; Chambon et al. 1985; Wermuth et al. 1987) as antagonists at the Ascaris GABA receptor, using current- and voltage- and patch-clamp techniques with bath-application of the antagonists. Initially, the actions of SR95531 and SR95301 in Ascaris were examined (Duittoz & Martin, 1989, 1990). SR95301 was a more potent antagonist than SR95531 in Ascaris, this order of potency is the reverse at the vertebrate GABA receptor (Duittoz & Martin, 1989). The antagonism of SR95103 was associated with a parallel shift to the right in the GABA dose–response relationships and the dose–ratio described by a modified Schild plot (Williams et al. 1988). The data were consistent with 2 molecules of GABA but one molecule of antagonist interacting with the receptor. The Ks for SR95103 was 64 μM and the antagonism showed little voltage-sensitivity. At the single-channel level, the actions of SR95103 were also consistent with competitive antagonism, with an additional small non-competitive component, possibly a channel-blocking action (Duittoz & Martin, 1990). Again, the antagonist seen in single-channel data was consistent with the GABA receptor combining with a single molecule of SR95103 but two molecules of GABA; this latter observation may be explained if the two GABA binding sites on the receptor are not identical, only one permitting the binding of SR95103.

Subsequently, in another series of experiments the actions of a series of arylaminoypyridazine-GABA derivatives were tested and compared with the potency order seen in binding studies at the GABA receptor (Table 3). Fig. 7 shows chemical structures of the analogues tested and the mean percentage reduction in the conductance response to 30 μM GABA produced by 1 mM antagonist. Compounds producing greater than 50% reduction all possessed a methyl or phenyl moiety on the 4-position of the pyridazine ring. SR95531 is the most potent vertebrate GABA antagonist and SR95312 is the least potent. At the Ascaris GABA receptor, SR95103 and SR95132 are the most potent and SR95531 has a low potency. This difference is further evidence that the Ascaris receptor can be separated pharmacologically and suggests a rational basis for the design of new therapeutic agents.

Ariens et al. (1979) have argued that ‘accessory’ sites on receptors bind to hydrophobic regions of Neuromuscular transmission in Ascaris
Fig. 7. Structure of arylaminopyridazine-GABA antagonists and the mean percentage inhibition produced by 1 mM antagonist of the conductance response produced by 30 µM GABA. Compounds producing greater than 50% inhibition were considered active.

Table 3. Comparison of the potency of the arylaminopyridazine derivatives at the GABA<sub>a</sub> receptor by inhibiting [H<sup>3</sup>]GABA binding and the percentage inhibition of the response to 30 µM GABA by 1 mM antagonist at the Ascaris receptor. (The two potency series do not equate, showing that the two receptor types are different.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>GABA&lt;sub&gt;a&lt;/sub&gt; binding: Ki (µM)</th>
<th>Inhibition in Ascaris (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR95531</td>
<td>0-15</td>
<td>43</td>
</tr>
<tr>
<td>SR42666</td>
<td>0-31</td>
<td>76</td>
</tr>
<tr>
<td>SR42627</td>
<td>0-55</td>
<td>21</td>
</tr>
<tr>
<td>SR95103</td>
<td>2-20</td>
<td>93</td>
</tr>
<tr>
<td>SR95133</td>
<td>2-60</td>
<td>58</td>
</tr>
<tr>
<td>SR95640</td>
<td>10-40</td>
<td>7</td>
</tr>
<tr>
<td>SR95132</td>
<td>100-00</td>
<td>89</td>
</tr>
</tbody>
</table>

competitive antagonists and give rise to their antagonist nature; the structure of the hydrophobic regions is more important for the potency of competitive antagonists than the structure of the moiety binding to the agonist recognition site. The mode of action and structure activity relationships of arylaminopyridazine-GABA antagonists at the vertebrate GABA<sub>a</sub> receptor have been explained in terms of Ariens theory (Wermuth, 1987). The correlation between the agonist potencies at the GABA<sub>a</sub> and Ascaris receptors but not competitive antagonists suggests that 'accessory' sites on the vertebrate GABA<sub>a</sub> receptor and Ascaris receptor are different. Recognition and characterization of these accessory binding sites may allow the rational design of antagonists which have a selective action in Ascaris.
So tissue separation of the diffusion coefficient experiments with this ivermectin has been developed (Martin, Kusel & Pennington, 1990). Vesicles of 10–50 μm diameter then form as outgrowths of the original bag membrane over the next 1–2 h. These vesicles can be harvested using a Pasteur pipette and are suitable for patch-clamp studies. Their membranes contain high-conductance Ca²⁺-activated Cl⁻ channels (Thorn & Martin, 1987), GABA-activated channels, and acetylcholine-activated channels (Martin et al. 1990; Pennington & Martin, 1990). The vesicles are also suitable for fluorescence-recovery after photobleaching experiments (FRAP) designed to look at fluorescent-labelled probe movement in the membrane. Recent experiments with this approach have shown that ivermectin has no significant effect on the lateral diffusion coefficient (8.1 × 10⁻⁹ cm² sec⁻¹) and that the membrane is not homogeneous, being composed of coalescent lipid ‘domains’ (Martin et al. 1990). The main advantage offered by the vesicle preparation is that it is not covered by any connective tissue so that it more readily permits anthelmintic mode of action studies involving fluorescent dye techniques and single-channel recording. For example, it was shown that ivermectin has no effect on muscle membrane fluidity or the proportion of mobile lipid (Martin et al. 1990).

**ACh single-channel currents.** Acetylcholine-activated single-channel currents (Fig. 8) have been recorded from cell-attached and isolated inside-out patches from muscle vesicles (Pennington & Martin, 1990). The channels activated by acetylcholine have at least two conductances: the larger was 40–50 pS and the smaller 25–35 pS; the I/V plots of both channels were linear and the average open-times were independent of patch potential. Kinetic analysis of the larger conductance channel showed that the distributions of open and burst durations were composed of two components (short and long) and analysis of the closed-time distributions showed that they were composed of at least three components (short, intermediate and long). These observations are interpreted to indicate that there are at least two open states and three closed states for the larger conductance channel. The average open-time was similar to that of the nicotinic channel at the frog neuromuscular junction and had a corrected mean open-time of 1.26 ms. High concentrations of acetylcholine (25–100 μM) produced a reduction in open probability and caused single-channel currents to occur in clusters with long closed-times (200 s).
R. J. Martin and others

Fig. 9. Pyrantel (A) and levamisole (B)-activated channels and their open-time histograms, from cell-attached patches from muscle vesicles. Openings are shown downwards. Patch-potential: −75 mV. 0.1 μM pyrantel or 10 μM levamisole in the pipette. Pipette solution (mM): CsCl 140, MgCl₂ 2, EGTA 1, HEPES 10, pH 7.2 with CsOH. Bathing solution (mM): CsCl 35, Cs acetate 105, MgCl₂ 2, EGTA 1, HEPES 10, pH 7.2 with CsOH. Filter: −3 dB 2.5 kHz, 8-pole Bessel. The mean open-times produced by pyrantel (1.09 ms) and levamisole (1.34 ms) were similar to those produced by acetylcholine. The open-time histograms are typical of channel open-times and show that the open-times vary and that their distribution is skewed with brief openings being more common.

between clusters; this behaviour has also been reported at the frog neuromuscular junction and described as desensitization. Earlier intracellular recordings and voltage-clamp experiments have shown that levamisole (Coles, East & Jenkins, 1975; Harrow & Gratton, 1985), pyrantel (Aubry et al., 1970; Harrow & Gratton, 1985) and morantel (Harrow & Gratton, 1985) act as agonists at the extrasynaptic acetylcholine receptor on the bag. Recent experiments have now shown activation of acetylcholine receptors at the single-channel level by pyrantel and levamisole (Fig. 9). These experiments have also demonstrated that levamisole also acts at higher concentrations to produce a voltage-sensitive flickering channel block.

GABA single-channel currents. GABA- and piperazine-activated channels have been recorded using cell-attached and outside-out patches (Martin, 1985a, b). The channels opened by both agonists had a mainstate conductance of 22 pS but two subconductance states were also observed. The average duration of the effective openings (bursts) produced by GABA was in the region of 32 ms while the average duration of effective openings (bursts) produced by the anthelmintic piperazine was 14 ms (Fig. 10). High concentrations of GABA or piperazine produced desensitization, which was seen as a decrease in open probability due to the appearance of long closed-times. Piperazine is some 100 times less potent than GABA in Ascaris (Martin, 1982) and this difference in potency may be explained by the fact that piperazine requires a higher concentration to achieve a similar opening rate to GABA and, in addition, the average duration of openings produced by piperazine is shorter. The density of the extrasynaptic receptors was also estimated in these experiments (Martin, 1985b): only one channel was found in each area of membrane of about 2.4 μm².
Fig. 10. GABA- and piperazine-activated channels. Cell-attached patches. Transpatch potential —75 mV.
GABA: 3 μM GABA as agonist. Piperazine: 500 μM piperazine as agonist. Effective channel open times:
GABA 33 ms; piperazine 18 ms. Channel conductance 22 pS for both agonists. Bathing solution (mm): NaCl 135, KCl 3.0, MgCl₂ 15.7, glucose 3.0, and Tris adjusted to pH 7.6 with maleic acid. Pipette solution (mm): CsCl 140, MgCl₂ 15.7 and Tris adjusted to pH 7.6 with maleic acid.

Ivermectin

Effects of dihydroavermectin. Electrophysiological effects of the anti-parasitic agent dihydroavermectin on Ascaris muscle have been studied with intracellular micropipette recordings (Kass et al. 1980, 1982, 1984; Holden-Dye et al. 1988) and at the single-channel level with the patch-clamp technique (Martin & Pennington, 1989). In contrast to effects on other invertebrate preparations, dihydroavermectin does not act like GABA on Ascaris muscle (i.e. it does not produce a marked hyperpolarization with an increase in membrane conductance), even when high concentrations (20 μM-0.2 μM) are used. However, dihydroavermectin depresses GABA-activated channel currents by reducing mean conductances and probability of opening (Martin, 1987; Martin & Pennington, 1989). This GABA-blocking effect was also seen in current-clamp experiments (Holden-Dye et al. 1988).

Low concentrations of dihydroavermectin applied to the outside membrane by bath application to an outside-out muscle patch did produce opening of up to 13 channels (Martin & Pennington, 1989). The progressive opening of the channels over a period of 1–3 min produced a staircase effect. (Fig. 11). These channels conducted Cl⁻ ions and had conductances of 9–15 pS and long average open-times (greater than 100 ms). A long delay (greater than 15 s) between the application of dihydroavermectin and channel opening and its lipophilic nature suggest a site of action in the lipid phase of the membrane. The action of low concentrations of dihydroavermectin on inside-out patches was to produce, after a long delay (mean 2.5 min), noisy cation-selective channels which had conductances of 5–30 pS. It did not produce Cl⁻ channels. The study concluded that dihydroavermectin may act to open non-GABA-activated Cl⁻ channels by acting from the external membrane, but the speed of onset of the effect is slow. Thus, the mode of action of ivermectin appears to be to open a particular type of Cl⁻ channel, possibly only present in susceptible animals, rather than to act as a GABA agonist.

Electrophysiological effects of dihydroavermectin on Ascaris neurones have been determined using differential application of drugs to dorsal and ventral muscle flaps in split-chamber experiments (Kass et al. 1984). It was reported that 6 μM dihydroavermectin blocked the response of DE1 to indirect but not direct stimulation. These results suggest action of dihydroavermectin on interneurones in Ascaris nerve cords.

OTHER TRANSMITTERS

The action of 5-HT

In addition to the neurotransmitters, GABA and acetylcholine there is evidence that 5-HT (serotonin) may play a role in regulating muscle metabolism. 5-HT can be absorbed from the host or synthesized by Ascaris (Martin, Chaudhuri & Donahue, 1988); 5-HT receptors in muscle and intestine of Ascaris have been demonstrated using radiolabelled binding techniques (Chaudhuri & Donahue, 1989).

5-HT has no observable effect on muscle membrane potential (unpublished observations) but appears to regulate glycogen metabolism. In Ascaris, glycogen metabolism depends on glycogen synthetase for glycogen synthesis from glucose-6-phosphate, and glycogen phosphorylase for glycogen breakdown. 5-HT inactivates glycogen synthetase and activates glycogen phosphorylase by increasing the level of cAMP, resulting in breakdown of glycogen (Donahue et al. 1981).

The receptor binding studies of Chaudhuri & Donahue (1989) suggest that in Ascaris the muscle receptor is similar to the mammalian 5-HT₂ receptor. Ketanserin, a selective 5-HT₂ antagonist has a high affinity (Kᵦ = 167 μM) for the muscle receptor but not the Ascaris intestinal receptor. The recognition of a 5-HT receptor in Ascaris muscle suggests another target site for the design of new anthelmintics.
Other possible neurotransmitters

Other putative transmitters have been suggested to occur in nematodes but their presence in *Ascaris* remains to be confirmed. These transmitters include: dopamine (Sulston, Dew & Brenner, 1975); adrenaline and noradrenaline (Willett, 1980); octopamine (Hovitz et al. 1982). Immunoreactivity to FMRFamide-like peptides has been demonstrated in the pharyngeal nerves and the four major nerve cords of *Ascaris* (Davenport et al. 1988). One purified form has been sequenced and called AF1 (A for *Ascaris*, F for FMRFamide and 1 for the first purified) and has been shown to be biologically active and to reduce input resistance of inhibitory motoneurones (Cowden et al. 1989). Peptidergic transmission remains to be studied in greater detail; it seems likely that more peptides as transmitters may be recognized in future studies.

Second messengers

The involvement of second messengers in neurotransmission is at present not known. Donahue et al. (1982, 1983) have correlated changes in muscle activity produced by piperazine and levamisole with changes in glycogen metabolism. They have also proposed a role for cyclic AMP-mediated regulation. The involvement of other second messengers including Ca\(^{2+}\), cyclic GMP and IP\(_3\) has not yet been established in *Ascaris*.

CONCLUSION

The physiology and pharmacology of the neuromuscular transmission in *Ascaris* is an interesting subject area which may be studied for a variety of reasons. The nematode is a model parasite, readily collected from many abattoirs, and large enough for electrophysiological study. Anthelmintic mode of action studies have identified sites of action for piperazine, dihydroavermectin, pyrantel and levami-
Neuromuscular transmission in Ascaris

muscle cells of the parasitic nematode Ascaris suum. British Journal of Pharmacology 99, 253P.


(5-hydroxytryptamine): a possible regulator of
glycogenolysis in perfused muscle segments of *Ascaris
suum*. *Biophysics and Biochemical Research

GABA antagonist in *Ascaris suum* muscle. *British
Journal of Pharmacology* **97**, 490P.

DUITTOZ, A. H. & MARTIN, R. J. (1990a). Effects of the
arylaminopyridazine-GABA derivatives, SR95103 and
SR95531 on the *Ascaris* muscle GABA receptor: the
relative potency of the antagonists in *Ascaris* is
different to that at vertebrate GABA<sub>a</sub> receptors.
*Journal of Comparative Biochemistry and Physiology
(In Press).*

DUITTOZ, A. H. & MARTIN, R. J. (1990b). Effects of
SR95103 on GABA-activated single-channel currents
from *Ascaris suum* muscle. *Journal of Comparative
Biochemistry and Physiology (In Press).*

EYRE, P. (1970). Some pharmacodynamic effects of the
nematocides: methyridine, tetramisole and pyrantel.

GOLDSCHMIDT, R. (1908). Das Nervensystem von
*Ascaris lumbricoides* und Megalocephala. Ein Versuch, in den
Aufbau eines einfachen Nervensystems einzudringen,
Zweiter Teil. *Zeitschrift für wissenschaftliche Zoologie
90*, 73–136.

GOLDSCHMIDT, R. (1909). Das Nervensystem von
*Ascaris lumbricoides* und Megalocephala. Ein Versuch, in den
Aufbau eines einfachen Nervensystems einzudringen,
Zweiter Teil. *Zeitschrift für wissenschaftliche Zoologie
92*, 306–57.

GRZYWACZ, M., SZKUDLINSK, J. & ZANDROWSKA, E.
(1985). Pharmacological receptors of *Ascaris

of the anthelminetics morantel, pyrantel and
levamisole on the muscle cell membrane of the

HEISE, R. (1892). Über das Nervensystem von *Ascaris
lumbricoides* und *Ascaris megalocephala*. *Zeitschrift für

Studies on the physiology of *Ascaris lumbricoides*. I.
The relation of total osmotic pressure, conductivity
and chloride content of the body fluid to that of the
external environment. *Journal of Experimental Biology
29*, 1–21.

Studies on the physiology of *Ascaris lumbricoides*. II.
The inorganic composition of the body fluid in
relation to that of the environment. *Journal of

HOLDER-DYE, L., HEWITT, G. M., WANN, K. T.,
involving avermectin and the 4-aminobutyric acid
(GABA) receptor of *Ascaris suum* muscle. *Pesticide

HOLDER-DYE, L., KROGGJAARD-LARSEN, P., NEILSEN, L. &
WALKER, R. J. (1989). GABA receptors on the somatic
muscle cells of the parasitic nematode, *Ascaris suum*:
stereoselectivity indicates similarity to a GABA<sub>a</sub>-type
agonist recognition site. *British Journal of Pharmacology
98*, 841–50.

HOLDER-DYE, L. & WALKER, R. J. (1988). ZAPA, (Z)-3-
[(amino iminomethyl)thio]-2-propenoic acid
hydrochloride, a potent agonist at GABA receptors on
the *Ascaris* muscle cell. *British Journal of Pharmacology
95*, 3–5.


HORVITZ, R. H., CHALFIE, M., TREAT, C., SULSTON, J. E. &
EVANS, P. D. (1968). Serotonin and octopamine in the


control of locomotion in *Ascaris*: anatomy,
electrophysiology and biochemistry. In *Nematodes as

of choline acetyltransferase within identified
motoneurons of the nematode *Ascaris*. *Journal of

immunoreactivity in inhibitory motor neurons of the

KASS, I. S., LARSEN, D. A., WANG, C. C. & STRETON, A. O.
B1a on the intact animal and neuromuscular strip

effects of avermectin and drugs related to
acetylcholine and 4-aminobutyric acid on
neuromuscular transmission in *Ascaris suum*.

KASS, I. S., WANG, C. C., WALDROND, J. P. & STRETON,
A. O. W. (1980). Avermectin B<sub>1a</sub>, a paralysing
anthelminthic that affects interneurones and inhibitory
motorneurones in *Ascaris*. *Proceedings of the National

LEE, D. L. (1962). The distribution of esterase enzymes in

system in adult *Nippostrongylus brasiliensis* (Nematoda) and a suggested function for the excretory
system of *Anisakis* larva (Nematoda: Anisiakidae).

Serotonin (5-hydroxytryptamine) turnover in adult
female *Ascaris suum* tissue. *Comparative Biochemistry

light chain phosphorylation and gamma aminobutyric
acid receptors in *Ascaris suum* muscle. *Comparative

MARTIN, R. J. (1980). The effect of y-aminobutyric acid on
the input conductance and membrane potential of

MARTIN, R. J. (1982). Electrophysiological effects of
piperazine and diethylcarbamazine on *Ascaris suum*
Neuromuscular transmission in Ascaris


WHITE, J. G., SOUTHGATE, E., THOMSON, J. N. & BRENNER, S. (1976). The structure of the ventral cord of...


Pyrantel and levamisole activated single channel currents in isolated muscle vesicles from *Ascaris suum*.

Susan J. Robertson, Anne J. Pennington, A. Mark. Evans & Richard J. Martin.
Dept. of Preclinical Veterinary Sciences, R.(D.)S.V.S., University of Edinburgh, Summerhall, Edinburgh, EH9 1QH.

A variety of anthelmintic drugs, including levamisole and pyrantel, are thought to be nicotinic agonists acting on the parasitic muscle (1). Acetylcholine opens non-selective cation channels (25-50pS), mean open time 4.9ms, in isolated muscle vesicles in the nematode parasite *Ascaris suum* (2). Our study shows that both levamisole and pyrantel also activate cation channels in this preparation (Fig.1). Pyrantel (0.03-1.0μM) activated channels (12-40pS) had a mean open time around 1.5ms. Levamisole (2-30μM) activated channels (20-45pS) had a mean open time around 2.3ms, the mean open-time was voltage sensitive, decreasing with depolarisation. At higher concentrations of levamisole (30μM) a "rapid flickering" channel block was observed, the block was voltage sensitive increasing with increasing hyperpolarisation (range 0.2-5.0ms).


WORK SUPPORTED BY MERK, SHARP & DOHME, THE SERC & THE WELLCOMME TRUST.
Levamisole activated single channel currents in isolated muscle vesicles from *Ascaris suum*.

S. J. Robertson* & R. J. Martin, Dept. of Preclinical Veterinary Sciences, R. (D). S. V. S., Summerhall, University of Edinburgh, Edinburgh, EH9 1 QH

A variety of anthelmintic drugs, including levamisole, are thought to be nicotinic agonists acting on parasite muscle (Harrow and Gration, 1985). Acetylcholine has been shown to open non-selective cation channels (mean open time 4.9ms) in isolated muscle vesicles in the nematode parasite *Ascaris suum* (Pennington & Martin, 1990). The aim of this study was to examine the properties of levamisole activated channels in this preparation. Levamisole (2-30μM) activated non-selective cation channels (conductance 20-45pS, mean open time 2.3ms). The open times exhibited a degree of voltage sensitivity, decreasing with depolarization. At the higher concentrations there was evidence of a voltage sensitive channel block (Figure 1) and desensitization.

![Figure 1: Levamisole (30μM) activated channels. Isolated inside-out patch from muscle vesicle (for methods see Pennington & Martin, 1990).](image_url)

A: -75mV membrane potential, mean open time 0.69ms. 
B: +75mV membrane potential, mean open time 1.9ms.

Work supported by Merck, Sharp and Dohme.

Levamisole-activated single-channel currents from muscle of the nematode parasite Ascaris suum

S.J. Robertson & R.J. Martin

Dept of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall, University of Edinburgh, Edinburgh, EH9 1QH

1 The patch-clamp technique was used to examine levamisole-activated channels in muscle vesicles from Ascaris suum. Cell-attached and isolated inside-out patches were used.

2 Levamisole (1–90 μM), applied to the extracellular surface, activated channels which had apparent mean open-times in the range 0.80–2.85 ms and linear I/V relationships with conductances in the range 19–46 pS. Ion-replacement experiments showed the channels to be cation selective.

3 The kinetics of the channels were analysed. Generally open- and closed-time distributions were best fitted by two, and three exponentials respectively, indicating the presence of at least two open states and at least three closed states. The distributions of burst-times were best-fitted by two exponentials.

4 Channel open- and burst-times were voltage-sensitive: at low levamisole concentrations (1–10 μM), they increased with hyperpolarization. At higher concentrations of levamisole (30 μM and 90 μM) flickering channel-block was observed at hyperpolarized potentials. Using a simple channel-block model, values for the blocking dissociation constant, $K_b$ were determined as 123 μM at $-50$ mV, 46 μM at $-75$ mV and 9.4 μM at $-100$ mV.

5 At the higher concentration of levamisole (30 μM and 90 μM) long closed-times separating 'clusters' of bursts were observed, at both hyperpolarized and depolarized membrane potentials and this was interpreted as desensitization.

Keywords: Levamisole; Ascaris; nicotinic-channels

Introduction

Nematode parasites inflict much suffering on human and animal hosts. Approximately one in five of the world's human population is infected with intestinal nematodes (Standen, 1975), while infestation in domestic animals is a major source of economic loss. Anthelmintic drugs, such as levamisole, are used to control these infestations.

Studies on the mode of action of levamisole indicate it is a selective agonist at acetylcholine receptors present on the muscle cells of parasitic nematodes. One such nematode is Ascaris suum. Levamisole and acetylcholine treatment of Ascaris suum results in depolarization and spastic paralysis of the muscle (Aceves et al., 1970; Van Nen, 1972; Coles et al., 1975; Martin, 1982; Harlow & Gratton, 1985; Colquhoun et al., 1991).

The acetylcholine receptors of Ascaris muscle have been classified as nicotinic, as nicotinic agonists produce contractions antagonised by tubocurarine, whereas muscarinic agonists and antagonists have little effect on this preparation (Natoff, 1969; Rozkova et al., 1980; Martin, 1982; Colquhoun et al., 1991). The pharmacology of the acetylcholine receptors on Ascaris muscle is most like that of the nicotinic ganglionic receptors of vertebrates, most notably mecamylamine is a potent antagonist at both receptor sites (Natoff, 1969; Rozkova et al., 1980).

The aim of the present study was to investigate the action of levamisole at the single-channel level in Ascaris by use of the extrasynaptic acetylcholine receptors present in isolated muscle vesicles (Martin et al., 1990; Pennington & Martin, 1990). Our results show that levamisole opens cation selective channels, but in addition, produces open channel-block and desensitization.

Methods

Single-channel recordings of levamisole-activated currents were made from Ascaris muscle vesicles. In general the data were analysed by the same methods as used in previous studies with acetylcholine (Pennington & Martin, 1990).

The vesicle preparation

Ascaris suum were collected from the local abattoir, maintained in Locke solution (replaced daily) at 32°C and used within 4 days. To prepare the vesicles, a 2 cm section from the anterior region of the worm was taken, cut along one of the lateral lines and pinned out to form a muscle-flap preparation. This muscle flap was treated with collagenase solution for about 10 min at 37°C. (composition as follows): mM NaCl 35, Na acetate 105, KC1 2, MgCl2 2, HEPES 10, glucose 3, ascorbic acid 2 and collagenase (1 mg ml−1) pH adjusted to 7.2 with NaOH. The preparation was then kept in a maintenance solution at 37°C (composition as follows): mM NaCl 35, Na acetate 105, KC1 2, MgCl2 2, HEPES 10, glucose 3, ascorbic acid 2 and EGTA 1; pH adjusted to 7.2 with NaOH. The vesicles formed over the next hour, as outgrowths from the bag region of the muscle cells. The vesicles were used within 5 h.

Electrical recordings

The vesicles were transferred to the experimental chamber where recordings were made at room temperature (15–22°C). The experimental chamber was mounted on the stage of a Reichert-Jung Biostar inverted microscope and viewed at ×200 magnification. Vesicles were bathed in the experimental chamber in the following solution (composition mM): CsCl 35, Cs acetate 105, MgCl2 2, HEPES 10 and EGTA 1; pH adjusted to 7.2 with CsOH.

Recordings were made from either isolated inside-out patch.
lices or cell-attached patches. Patch-electrodes were made from micro-haemocytocar capillary glass (Garner Glass 7052) with a resistance of 1–3 MΩ. The pipettes were coated with Sylgard to improve frequency responses. Electrodes were filled with pipette solution (composition, mm) CsCl 140, MgCl₂ 2, HEPES 10 and EGTA 1; pH 7.2 with CsOH. To examine the effects of levamisole, different concentrations of levamisole were placed in the pipette solution. All of the recordings were made with Cs⁺ as the main cation to avoid contamination of patch recordings with K⁺ channels known to be present in the membrane. The solutions were also Ca²⁺-free to avoid contamination of patch recordings with Ca²⁺-activated Cl⁻ channels known to occur in this membrane (Thorn & Martin, 1987).

In experiments to determine ion-selectivity of the channel, the ionic composition of the bath solution was altered by diluting 50:50 with distilled H₂O. Inside-out patches were used for these experiments to ensure changes in ionic concentration at the cytoplasmic membrane surface.

Currents were recorded with a List EPC-7 and a modified Sony digital audio processor with a Betamax video recorder.

Data processing

Records were analysed with a CED 1401 interface and a DCS286 PC Computer, with PAT software kindly supplied by John Dempster, Strathclyde University. The records were filtered by an eight-pole Bessel filter, 3 dB, 1.5 kHz; the sampling time was 70 μs and the minimum detectable interval was 0.3 ms. The threshold for channel opening was set at 70% of the single channel amplitude determined from channel amplitude histograms so as to include only full openings. In some experiments a 50% threshold was also used to examine the effect on open times. Little practical difference was observed. It is pointed out that corrected open values were determined (Colquhoun & Sakmann, 1985) for levamisole concentrations between 2 and 10 μM and found to be not significantly different from apparent open values obtained from the Dempster software. Subsequently in this manuscript only apparent open values are quoted.

In these experiments, individual channel activations were interrupted with short close periods (the 'Nachschlag' phenomenon, Colquhoun & Sakmann, 1985), so bursts of openings as well as single openings were considered. Bursts were defined as groups of openings separated by gaps that are shorter than a specified length, Tᵢ, Tᵢ was determined by numerically solving the following equation:

\[ 1 - e^{-Tᵢ/Tᵢ} = e^{-Tᵢ/Tᵢ} \]

where T₁ represents the brief closed state T₁, and T₂ represents the slightly longer closed state T₂. The equation finds a value for Tᵢ which balances the proportion of gaps within a burst misclassified (as gaps between bursts) and gaps between a burst misclassified (as gaps within a burst) (Colquhoun & Sakmann, 1985). In general a value of 1 ms was used in these experiments. This particular value was not so important since gaps between bursts were several hundred fold larger than gaps within bursts.

Exponential curve fitting

Exponential curves were fitted to the open-, closed-, and burst-time by the method of maximum likelihood (Colquhoun & Sigworth, 1983; Martin, 1985) to a probability density function (p.d.f) of the form:

\[ p.d.f. = \sum_{i=1}^{k} \frac{Ae^{(-t/T_i)}}{T_i} \]

where Aᵢ = the area of the ith component; Tᵢ = the fitted time constant; t = time and K = number of exponentials fitted.

After exponential curve fitting, the data were corrected for missing events (Colquhoun & Sakmann, 1985; Pennington & Martin, 1990). This was carried out to compensate for brief events missed due to limited resolution of the recording apparatus.

The flickering channel-block seen at high levamisole concentrations was analysed by the simple channel-block scheme described by Adams (1976) and Colquhoun & Sakmann (1985):

\[ C \times \beta \times O \times \frac{k_{-B}X_B}{k_{-B}} \times B \]

where C denotes the closed state; O denotes the open state; B denotes the blocked state; X_B is the drug concentration; k₋B is the blocking rate constant; k₋B is the unblocking rate constant; x is channel closing-rate and B is the channel opening-rate. The model predicts that:

mean open-time = \(1/(x + k_{-B}X_B)\),

mean block-time = \(1/k_{-B}\).

channel-block dissociation constant, \(K_B = k_{-B}/k_{-B}\).

The slope of the reciprocal plot of mean durations of long openings (T₂), against levamisole concentration was used to determine values for k₋B.

The mean block durations were determined by selecting the distinctive blocked times seen during flickering bursts greater than 0.3 ms (our limit of resolution). The method of maximum likelihood was then used to fit a single exponential to obtain a value for the mean block duration. It is pointed out that there was inevitably some overlap between the blocked time and the 'Nachschlag' closings, however this error is minimal as the interruptions due to channel block far exceed the number of interruptions due to the 'Nachschlag' closings.

Drugs

Levamisole ((-)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole) was obtained as levamisole hydrochloride from Sigma chemical company, Fancy Road, Poole, Dorset, BH17 7NH. Collagenase, Sigma type 1A, was also obtained from Sigma chemical company.

Statistical analysis

The method of least squares was used to obtain the best fits for the exponential equations used to describe the voltage-sensitivity of the rate constants k₋B and k₋B and the dissociation constant Kᵣ, a Spearman correlation coefficient (r) was used to test the quality of the fits. Values are given as mean ± s.e.mean. Levels of significance were obtained by use of Student’s t tests, a significance level of 0.05 was used.

Results

With levamisole in concentrations between 1–90 μM, in the patch-pipette, channel currents in 43% of 143 cell-attached and isolated inside-out patches were recorded. These channels had apparent mean open-times in the millisecond range and amplitudes of around 2.5 pA at -75 mV. Detailed analysis of channel data from a single patch recorded from both the cell-attached and the isolated inside-out configuration showed no significant differences in channel kinetics. In 20 experiments with levamisole omitted from the
patch pipette solution no such channel openings were observed. It was therefore concluded that levamisole activated these currents.

Channel conductance and current/voltage relationships

The pipette and bath-solutions had symmetrical cation concentrations but non-symmetrical anion concentrations (pipette main-ions: Cs+, 140 mM; Cl−, 144 mM; bath main-ions: Cs+, 140 mM; Cl−, 39 mM). Under these conditions with isolated inside-out patches, the predicted Nernst reversal potential for a cation selective channel is 0 mV, and for an anion channel is −33 mV. In 26 out of 31 experiments the I/V plots of levamisole-activated channels were linear with reversal near 0 mV (Figure 1) indicating that these channels were cation-selective and could conduct Cs+. The slopes had an average conductance of 32.9 ± 1.23 pS (mean ± s.e., n = 26) and ranged between 19-46 pS.

As a further test of the permeability of the bath-solution the bath-solution was diluted 50:50 with distilled water. The predicted effect of this is to produce a shift in the Nernst reversal potential from 0 mV to +18 mV for a cation selective channel and from −33 mV to −51 mV for an anion selective channel. In the experiment illustrated in Figure 1, the reversal potential shifted from 0 mV to +13 mV.

Calculation of the relative permeability of Cs+ and Cl− with the Goldman constant field equation (Hille, 1984), revealed that Cs+ was ten times more permeable than Cl−. Similar results were obtained in three other experiments.

![Figure 1](image)

Figure 1 (a) Individual channel openings recorded from an isolated inside-out patch, at a membrane potential of −75 mV and with levamisole 3μM. The conductance of these channel currents was 26 pS. C: denotes the closed state. O: denotes the open state. (b) I/V plot of channel currents activated by 3μM levamisole in an isolated inside-out patch. The initial plot (O) has a conductance of 31 pS and a 0 mV reversal potential. Dilution of the bath solution (●) produced a shift in the reversal potential to +13 mV. The predicted Nernst potentials during the initial I/V plot were: for Cs+, 0 mV; for Cl−, −33 mV. The predicted Nernst potentials for the I/V plot obtained with the diluted solution were: for Cs+, 18 mV; for Cl−, −51 mV.

Exponential curve-fitting

Channel data from 31 patches were analysed at various membrane potentials, ranging from +150 mV to −150 mV. The data were best-fitted by the sum of exponentials (see Methods). Files contained between 200 and 10.000 apparent open-, burst- and closed-times.

With 2–10μM levamisole in the pipette the open- and burst-time distributions were best-fitted by two exponentials, while closed-time distributions were best-fitted by three exponentials (Table 1,2,3). Since the number of open-, burst- and closed- states must at least equal the number of exponential components fitted (Colquhoun & Hawkes, 1982), levamisole-activated channels in Ascerts have at least two open-states, at least two burst-states and at least three closed-states. Higher concentrations of levamisole (30μM and 90μM) produced substantial changes in the distribution of both the open- and closed-times, these changes are considered in the sections on channel-block and desensitisation.

Voltage-sensitivity of open- and burst-time durations

At lower concentrations of levamisole (2–10μM) the corrected mean open-times (Figure 2) and corrected mean burst-times exhibited a degree of voltage-sensitivity, decreasing with depolarization. For example the value at −75 mV for the corrected mean open-time was 1.47 ± 0.11 ms (mean ± s.e., n = 10), this value was significantly greater (P<0.03) than the value for the corrected mean open-time at +75 mV of 1.10 ± 0.25 ms (n = 4). The corrected mean burst duration at −75 mV was 2.50 ± 0.28 ms (n = 16), this compared to a value of 1.50 ± 0.25 ms (n = 4) at +75 mV where the corrected mean burst durations were also significantly reduced (P<0.05).

Probability of channel opening

The probability of the channel being open (Popen) was concentration-dependent at depolarized potentials where Popen increased with concentration. However at hyperpolarized potentials concentration had no significant effect on Popen. Figure 3 illustrates the effect of concentration on Popen at ±75 mV. Similar results were observed at ±100 mV. It is suggested subsequently that the explanation for the failure of Popen to increase at −75 mV and −100 mV is due to the blocked state of the channel, which occurs with high concentrations of levamisole at hyperpolarized potentials, directly closing without re-entering the open-state.

At low agonist concentrations the membrane potential had little affect on Popen. For example, in one experiment with 3μM levamisole, Popen was 0.0061 at +75 mV compared to 0.0078 at −75 mV; similar results were obtained in 8 other experiments with 2–10μM levamisole. This lack of effect of membrane potential on Popen contrasts with the effect of membrane potential on open-times; the lack of effect of Popen is explained by the compensating increase in the rate of channel opening at depolarized potentials. For example in one experiment with 3μM levamisole, the channel opening rate at −75 mV was 8.6 channel openings per second compared to 13.6 channel openings per second at +75 mV.

Evidence of channel block

At higher concentrations of levamisole (30μM and 90μM), there was a recognisable change in the kinetics of channel opening (Figure 4). At hyperpolarized potentials, channel openings were briefer than at lower concentrations and appeared as sequences of openings separated by brief closings characteristic of a 'flickering' channel-block (see, Neher & Steinbach, 1978). This behaviour may be explained by levamisole acting to produce a voltage-sensitive open-channel-block, in addition to its action as an agonist. The cationic charge and large size of levamisole is consistent with...
### Table 1 Open-time kinetics for levamisole-activated channels

<table>
<thead>
<tr>
<th>Levamisole conc. (µM)</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential - 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.66 ± 0.06</td>
<td>0.34 ± 0.06</td>
<td>0.88 ± 0.10</td>
<td>2.30 ± 0.24</td>
</tr>
<tr>
<td>30</td>
<td>0.78 ± 0.09</td>
<td>0.22 ± 0.09</td>
<td>0.37 ± 0.09</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>90</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane potential - 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.59 ± 0.08</td>
<td>0.41 ± 0.08</td>
<td>0.86 ± 0.12</td>
<td>2.54 ± 0.36</td>
</tr>
<tr>
<td>90</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane potential + 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.62 ± 0.10</td>
<td>0.38 ± 0.10</td>
<td>0.71 ± 0.10</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>90</td>
<td>0.66 ± 0.07</td>
<td>0.34 ± 0.07</td>
<td>0.48 ± 0.07</td>
<td>1.22 ± 0.29</td>
</tr>
<tr>
<td>Membrane potential + 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.79 ± 0.12</td>
<td>0.21 ± 0.12</td>
<td>0.71 ± 0.08</td>
<td>2.35 ± 0.50</td>
</tr>
<tr>
<td>90</td>
<td>0.81 ± 0.04</td>
<td>0.19 ± 0.04</td>
<td>0.50 ± 0.10</td>
<td>2.03 ± 0.47</td>
</tr>
</tbody>
</table>

T1 and T2 are the time constants representing the two open states. The proportion of brief openings (T1) and long openings (T2) are represented by the areas A1 and A2. Values are mean ± s.e.

### Table 2 Closed time kinetics for levamisole-activated channels

<table>
<thead>
<tr>
<th>Levamisole conc. (µM)</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential - 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.26 ± 0.05</td>
<td>0.28 ± 0.05</td>
<td>0.35 ± 0.04</td>
<td>237 ± 41</td>
<td>821 ± 127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.20 ± 0.04</td>
<td>0.28 ± 0.04</td>
<td>0.06 ± 0.02</td>
<td>0.76 ± 0.16</td>
<td>24 ± 10</td>
<td>228 ± 80</td>
<td>1586 ± 446</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.17 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.63 ± 0.08</td>
<td>12 ± 3</td>
<td>251 ± 107</td>
<td>2528 ± 714</td>
<td></td>
</tr>
<tr>
<td>Membrane potential - 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.26 ± 0.04</td>
<td>0.34 ± 0.08</td>
<td>0.37 ± 0.04</td>
<td>224 ± 52</td>
<td>1081 ± 139</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.25 ± 0.03</td>
<td>0.18 ± 0.06</td>
<td>0.07 ± 0.02</td>
<td>1.57 ± 0.32</td>
<td>20 ± 12</td>
<td>223 ± 131</td>
<td>3684 ± 2579</td>
<td></td>
</tr>
<tr>
<td>Membrane potential + 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.56 ± 0.09</td>
<td>0.21 ± 0.09</td>
<td>0.24 ± 0.04</td>
<td>132 ± 38</td>
<td>595 ± 115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.57 ± 0.08</td>
<td>0.12 ± 0.03</td>
<td>0.38 ± 0.23</td>
<td>8 ± 4</td>
<td>137 ± 73</td>
<td>675 ± 261</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane potential + 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.38 ± 0.05</td>
<td>0.34 ± 0.06</td>
<td>0.56 ± 0.15</td>
<td>32 ± 11</td>
<td>127 ± 30</td>
<td>961 ± 380</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.30 ± 0.02</td>
<td>0.13 ± 0.10</td>
<td>0.44 ± 0.01</td>
<td>51 ± 38</td>
<td>172 ± 74</td>
<td>526 ± 247</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Closed states are represented by three exponentials at 2 – 10 µM levamisole. Brief closings (T1), intermediate closings (T2) and long closings (T3), their relative proportions are represented by the areas A1, A2 and A3. At 30 – 90 µM there are four closed states represented by the time constants T1, T2, T3 and T4. Their relative proportions are represented by the areas A1, A2 and A3 and A4. All values are the mean ± s.e., n is the number of experiments.

### Table 3 Burst-time kinetics for levamisole-activated channels

<table>
<thead>
<tr>
<th>Levamisole conc. (µM)</th>
<th>A1</th>
<th>A2</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential - 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.49 ± 0.07</td>
<td>0.51 ± 0.07</td>
<td>1.24 ± 0.19</td>
<td>3.07 ± 0.35</td>
</tr>
<tr>
<td>30</td>
<td>0.61 ± 0.09</td>
<td>0.39 ± 0.09</td>
<td>0.59 ± 0.15</td>
<td>2.06 ± 0.42</td>
</tr>
<tr>
<td>90</td>
<td>0.59 ± 0.07</td>
<td>0.41 ± 0.07</td>
<td>0.74 ± 0.19</td>
<td>2.23 ± 0.32</td>
</tr>
<tr>
<td>Membrane potential - 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 – 10</td>
<td>0.55 ± 0.07</td>
<td>0.44 ± 0.07</td>
<td>1.29 ± 0.22</td>
<td>3.78 ± 0.50</td>
</tr>
<tr>
<td>90</td>
<td>0.44 ± 0.08</td>
<td>0.56 ± 0.08</td>
<td>0.30 ± 0.15</td>
<td>2.93 ± 0.96</td>
</tr>
<tr>
<td>Membrane potential + 75 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.46 ± 0.08</td>
<td>0.54 ± 0.08</td>
<td>0.69 ± 0.03</td>
<td>1.89 ± 0.29</td>
</tr>
<tr>
<td>90</td>
<td>0.72 ± 0.08</td>
<td>0.23 ± 0.08</td>
<td>0.66 ± 0.19</td>
<td>2.33 ± 0.79</td>
</tr>
<tr>
<td>Membrane potential + 100 mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 10</td>
<td>0.73 ± 0.05</td>
<td>0.27 ± 0.05</td>
<td>0.93 ± 0.23</td>
<td>2.73 ± 0.64</td>
</tr>
<tr>
<td>90</td>
<td>0.71 ± 0.06</td>
<td>0.29 ± 0.06</td>
<td>1.06 ± 0.21</td>
<td>2.54 ± 0.48</td>
</tr>
</tbody>
</table>

T1 and T2 are the time constants representing the two burst states. The proportion of brief bursts (T1) and the long bursts (T2) are represented by the areas A1 and A2. Values are mean ± s.e.
the direction of the voltage-sensitivity of the block. In order to
describe the effect further, a simple channel-block model
(Adams 1976; Colquhoun & Sakmann, 1985) was used.
The presence of open channel-block reduced the duration
of the open-states at hyperpolarized potentials. Table 1
shows that this reduction in open-times at 30 µM levamisole
was associated with a reduction of both the brief and longer
components of the open-time distribution. At −75 mV with
90 µM levamisole where the open-durations were also
reduced, only one open-state could be detected. The loss of
one component at this concentration is most likely due to
the brief openings reducing to a level below the limit of resolu-
tion of the recording apparatus, as a result of channel-block.
At 90 µM the corrected mean open-time at −75 mV was
0.63 ± 0.06 ms (mean ± s.e., n = 11), a value significantly
lower than that at +75 mV which was 1.30 ± 0.26 ms
(mean ± s.e., n = 8), (P<0.02). Thus mean open-times
showed a reversed direction of voltage-sensitivity at the
higher levamisole concentrations. At +75 mV where
channel-block was observed, there was no significant reduc-
tion in the mean duration of the two open-time components
compared to values at lower concentrations (2–10 µM).

Estimation of blocked-times

Mean block durations at membrane potentials of −35 to
−100 mV for both 30 µM and 90 µM are shown in Table 4.
Comparison of values at both 30 µM and 90 µM showed
concentration did not affect mean block-times. The lack of
effect of concentration on blocked-times is consistent with
predictions of the simple channel-block model. Values
obtained for mean block durations at both 30 µM and 90 µM
were subsequently combined and their reciprocals used to
estimate k[B,] values (Figure 5).

Estimation of k[B,]
The forward blocking-rate constants k[B,] were determined
from plots of the reciprocals of the mean duration of long
open-times (T2) against drug concentration (Figure 6). Long
open-times were used in preference to the brief open-times
because they are more accurately determined, the short open
durations are close to the limit of resolution of the setup
and at 90 µM value for T1 appears to fall below the limit of
resolution of the recording apparatus. In calculating k[B,]
results obtained with 90 µM levamisole were not included.
These points deviated from the straight line obtained with
results at lower concentrations (2–30 µM). This may be
due to the fact that at higher concentrations the mechanism
of block may be more complex than the simple channel-block
mechanism. However it is more likely that at the higher
concentration (90 µM) some of the open-time durations are
lower than the limit of resolution of the recording apparatus,
making the open-time durations an overestimation at this
concentration. This interpretation is further supported by the
fact that at 90 µM with hyperpolarized potentials open-times
distributions could only be fitted by a single exponential.

Values for the forward blocking-rate constant, k[B,] at
−50 mV, was 2.06 × 10⁻⁸ M⁻¹ s⁻¹; at −75 mV it was 3.82
× 10⁻¹⁰ M⁻¹ s⁻¹; and at −100 mV it was 6.58 × 10⁻¹⁰ M⁻¹ s⁻¹.
As predicted by the simple channel block model, k[B,]
increased with membrane hyperpolarization. The voltage
sensitivity of k[B,] was described by the equation:
\[ k[B,] = k[B,0] \exp(\alpha [E - V_k]) \]
where \( k[B,0] \) is the forward blocking rate constant
at the membrane potential E, \( k[B,0] \) is the value for \( k[B,] \)
at 0 mV and \( V_k \) is a constant. The value for \( k[B,0] \) was
0.83 × 10⁻¹⁰ M⁻¹ s⁻¹ and \( V_k \) was −35.5 mV. The correlation
coefficient (r) was 0.97. Therefore \( k[B,] \) showed an e-fold change
every 36 mV. It was also possible to estimate the
closing rate constants (\( k_C \)) for the long openings. This was
the intercept of the plot of 1/T2 versus concentration. At
−750 mV the value for \( k_C \) was 447 s⁻¹; at −75 mV it was
396 s⁻¹; and at −100 mV it was 143 s⁻¹. As can be observed
the closing rate decreases with membrane hyperpolarization,
this supports earlier observations where open durations were
longer at hyperpolarized potentials.

Figure 2 Levamisole (3 µM)-activated channels from a cell-attached
patch illustrating the voltage-sensitivity of open-time durations. C
denotes the closed state. O denotes the open state. (a) Individual
channels recorded at −75 mV and histogram of open-times at this
potential. The open-times were fitted by two exponentials (not shown)
with time constants of 0.92 ms and 2.14 ms. The corrected
mean open-time was 1.59 ms. (b) Individual channels recorded at
+75 mV and histogram of open-times at this potential. The
open-times were fitted by two exponentials (not shown) with time
constants of 0.53 ms and 1.45 ms. The corrected mean open-time
was 0.91 ms.

Figure 3 Graph showing P vs levamisole concentration; (■)−75 mV membrane potential; (O) +75 mV membrane potential.
All values are mean (with vertical bars showing s.e.), n = 4 to n = 9.
LEVAMISOLE-ACTIVATED CHANNELS

Figure 4 Levamisole-(30 μM) activated channels and open-time histograms from an isolated inside-out patch. (a) Membrane potential -75 mV; (b) membrane potential +75 mV. The open-times in this patch were fitted with two exponentials (not shown). (a) T1 = 0.14 ms T2 = 0.69 ms; (b) T1 = 0.41 ms T2 = 1.91 ms. Note the presence of flickering open channel-block in (a).

Table 4 The effect of membrane potential on mean block time

<table>
<thead>
<tr>
<th>Levamisole conc. (μM)</th>
<th>Membrane potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-35 mV</td>
</tr>
<tr>
<td>30</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>30 + 90</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.

Determination of \( K_B \)

The values for \( k_{+B} \) and \( k_{-B} \) determined above were used to calculate the channel-block dissociation constant, \( K_B \). At -50 mV, \( K_B \) was \( 2.55 \times 10^4 \text{s}^{-1}/2.06 \times 10^4 \text{M}^{-1} \text{s}^{-1} \) or 123 μM; at -75 mV, \( K_B \) was \( 1.75 \times 10^4 \text{s}^{-1}/3.82 \times 10^4 \text{M}^{-1} \text{s}^{-1} \) or 46 μM; and at -100 mV, \( K_B \) was \( 0.62 \times 10^4 \text{s}^{-1}/6.58 \times 10^4 \text{M}^{-1} \text{s}^{-1} \) or 9.4 μM. The semi-log plot of \( K_B \) versus membrane potential is shown in Figure 7, this shows an e-fold change in \( K_B \) every 19.4 mV.

Burst durations

As stated earlier, the burst durations exhibit a degree of voltage-sensitivity. The bursts consisted of two components; the brief bursts (mean T1) and the longer bursts (mean T2). Values for T1 and T2, with their respective proportions A1 and A2 are listed in Table 3. The simple channel-block model predicts that the mean burst-length should increase with concentration. In order to examine this prediction the corrected mean burst lengths were determined at different levamisole concentrations. For example at -75 mV with 2–10 μM levamisole the corrected mean burst length was \( 2.50 \pm 0.28 \text{ ms (n = 16)} \); with 30 μM levamisole it was \( 1.14 \pm 0.32 \text{ ms (n = 6)} \); and with 90 μM levamisole it was \( 1.40 \pm 0.21 \text{ ms (n = 9)} \). Thus there was no significant increase in the corrected mean burst duration. Similar results were obtained at -100 mV. It was concluded that the mean burst durations did not increase with increasing levamisole concentration, a result not predicted by the simple channel-block model.
Figure 5 (a) Illustration of channel activity with 30 μM levamisole; isolated inside-out patch, -75 mV. Fast flickering channel-block can be clearly observed. C: denotes the closed state. O: denotes the open state. The mean blocked-times were calculated from the measurements of blocked durations (indicated by the line between the arrows). In this experiment the mean blocked-time was 0.43 ms. (b) Semi-log plot of \( K_{-B} \) against membrane potential, all values are mean ± s.e.. The voltage sensitivity of \( K_{-B} \) was described by the equation: \( K_{-B} = K_{-B0} \exp(E/\nu_k) \). Where \( K_{-B} \) is the unblocking rate constant at the membrane potential \( E \), \( K_{-B0} \) is the value for \( K_{-B} \) at 0 mV and \( \nu_k \) is a constant. The value for \( K_{-B0} \) was 11.99 ms\(^{-1} \) and \( \nu_k \) was 35.02 mV.

Figure 6 Reciprocal plot of open-time constant T2 against concentration, at -50 mV (△), -75 mV (O) and -100 mV (■). The intercept represents the closing rate constant (\( \zeta_2 \)) and the slope represents the forward blocking rate (\( K_{+B} \)). Each point is the mean ± s.e. (vertical bars). Lines were fitted to 2–30 μM by least square regression without including the results at 90 μM. Values for \( K_{+B} \) were determined as -50 mV, 2.06 × 10\(^{-7} \) M\(^{-1}\) s\(^{-1} \); -75 mV, 3.82 × 10\(^{-7} \) M\(^{-1}\) s\(^{-1} \); and -100 mV, 6.58 × 10\(^{-7} \) M\(^{-1}\) s\(^{-1} \). The closing rate constant \( \zeta_2 \) was also determined: -50 mV, 447 s\(^{-1} \); -75 mV, 396 s\(^{-1} \); and -100 mV, 143 s\(^{-1} \) from the intercept on the ordinate scale.

Figure 7 Semi-log plot of \( K_B \) versus membrane potential. The voltage-sensitivity of \( K_B \) was described by the equation: \( K_B = K_{B0} \exp(E/\nu_k) \). Where \( K_B \) is the dissociation rate constant at the membrane potential \( E \), \( K_{B0} \) is the value for \( K_B \) at 0 mV and \( \nu_k \) is a constant. The value for \( K_{B0} \) was 1780 μM and \( \nu_k \) was 19.4 mV.

Desensitization

At higher concentrations of levamisole (30 μM and 90 μM) long closed-times were seen in some patches between clusters of channel opening (Figure 8). These closed-times lasted many seconds and were observed at both depolarized and hyperpolarized potentials. The details of the closed-time components observed at higher concentrations are summarized in Table 2. These very long closed periods appear to correspond to a slow desensitization process (Cachelin & Colquhoun, 1989) and are similar to the very long closed-times observed with high concentrations of acetylcholine in Ascaris (Pennington & Martin, 1990). In addition to the long closed durations, shorter closed times (between 10 and 30 ms) were detected at high levamisole concentrations. These might also be part of a desensitized state as they occur at both depolarized and hyperpolarized potentials but only with high concentrations.

Discussion

The simple block model

The simple channel-block model was used in this paper in order to describe quantitatively some of the effects observed...
with levamisole. The predictions of this model, for a charged blocker, include the expectations that: the corrected mean open-times decrease with increasing levamisole concentration; burst durations increase with increasing drug concentration; the forward blocking rate constant \( (k_{+g}) \), the unblocking rate \( (k_{-g}) \), and the dissociation constant \( K_a \) all vary with membrane potential; \( K_a \) and \( k_{-g} \) should have equal but opposite voltage-dependences; and the mean block duration and therefore \( k_{-g} \) are independent of drug concentration. The data presented in this report are consistent with the above predictions except that burst durations did not increase with concentration. This implies that the model used is a useful approximation but not a completely sufficient model.

We did not observe the predicted increase in the average duration of bursts, although there were frequent examples of long flickering bursts (see Figure 5). One possible explanation for the absence of increased mean burst durations could be that the channel might enter a closed state directly from the blocked state without reopening, i.e. the channel closes trapping levamisole or the blocked channel enters into a desensitised state. Limitations of the simple channel-block model are discussed further by Neher (1983).

One further difficulty in using the simple channel-block model is the presence of two open-states (O1 and O2). In our analysis it seems probable that the closed state (O1) fell below the lower limit of resolution of the recording set up. Thus the values for \( K_a \) would be appropriate for the open-state O2 at 90 \( \mu \)M. Since the values for \( K_a \) were not significantly different from the values for \( K_a \) at 30 \( \mu \)M where the open-state O1 could still be resolved, it implies that the presence of O1 did not affect our estimation of \( K_a \) for the open-state O2. Since the values for \( K_a \) were determined from the values of the open-state O2 (see methods), our determination of \( K_a \) is likely to be an estimate of the dissociation constant for the block of the open-state O2.

Comparison with acetylcholine-activated channels in Ascaris

At the single-channel level both acetylcholine (Pennington & Martin, 1990) and levamisole activate channels in the same concentration range: acetylcholine 1–100 \( \mu \)M; levamisole 1–90 \( \mu \)M. The \( I/V \) relationship with both levamisole and acetylcholine are linear, both agonists showed evidence of desensitization with long closed periods appearing at higher concentrations.

Although acetylcholine and levamisole appear to activate the same channels there are some differences in the behaviour of the single-channel currents. Acetylcholine activates channels with two distinguishable conductance levels (25–35 pS and 40–50 pS) (Pennington & Martin, 1990); in contrast single-channel currents activated by levamisole had a range of detectable conductance levels between 19–46 pS with a mean of 32.9 ± 1.23 pS \((n = 26)\).

The open-, closed-, and burst-time durations for levamisole or acetylcholine activated channels in this preparation show that both compounds produced two open-states and two burst-states and at least three closed-states. However the mean duration of the apparent open-times and burst-times for levamisole were shorter than for acetylcholine. The mean duration of apparent burst and long openings produced by acetylcholine (1–10 \( \mu \)M) was 1.18 ms and 4.89 ms, which compares with 0.80 ms and 2.30 ms for 2–10 \( \mu \)M levamisole under similar conditions. The mean brief and long burst durations for 1–10 \( \mu \)M acetylcholine were 1.17 ms and 7.62 ms compared to 1.24 ms and 3.70 ms for 2–10 \( \mu \)M levamisole.

A further difference between the effects of acetylcholine and levamisole is related to channel-block. Pennington & Martin (1990) did not find significant evidence for channel-block with acetylcholine on this preparation, although they did observe a reduction in the mean open-time at higher concentrations (25 \( \mu \)M) which might be interpreted as signs of block. Our results clearly show that levamisole blocks the channel at hyperpolarized potentials.

Comparison with previous voltage-clamp experiments in Ascaris

Harrow & Graton (1985) examined the effects, in Ascaris, of both acetylcholine and levamisole using two-microelectrode current-clamp and voltage-clamp. They found that the \( I/V \) relationship obtained from two-microelectrode voltage-clamp experiments obtained by applying levamisole by microperfusion or iontophoresis to Ascaris muscle had both linear (0 to –30 mV) and non-linear sections (> –30 mV). They suggested that the non linearity was due to a voltage-sensitive channel-block and/or desensitization. In our experiments we observed some desensitization at higher concentrations of levamisole, but this appeared at both depolarized and hyperpolarized membrane potentials; levamisole did not just at membrane potentials greater than –30 mV. Channel-block was clearly observed in our experiments, the degree of block increased on membrane hyperpolarization. This is consistent with the voltage-sensitivity of the non linearity observed by Harrow & Graton (1985) and suggests that their observation reflects channel-block but not desensitization.

Harrow & Graton (1985) have shown that levamisole is an agonist at all concentrations (1 \( \mu \)M–10 mM) producing a sigmoid dose-response curve obtained under current-clamp conditions with a membrane potential of –30 mV. Our results clearly show that channel-block occurs at –35 mV and at this potential \( K_a \) was calculated at 285 \( \mu \)M. It is interesting to note that Harrow & Graton (1985) did not observe a reduction in the maximum conductance response with high concentrations (10 mM). This observation of Harrow & Graton (1985) is consistent with our observations at hyperpolarized potentials which showed that \( P_{opene} \) failed to decrease at higher concentrations, despite the presence of channel block.

Possible therapeutic significance

Receptor desensitization produced by levamisole may have significant effects therapeutically; some Ascaris parasites continuously treated with higher doses of levamisole recover from the levamisole-induced paralysis (Aceves et al., 1970; Coles et al., 1974; 1975). This recovery may be due to desensitization since like desensitization, recovery occurs only at higher doses. Consequently it is suggested that lower doses which do not produce desensitization, potentially have a greater therapeutic effect. Also the fact that \( P_{opene} \), in these experiments did not increase at hyperpolarized potentials when 2–90 \( \mu \)M levamisole was used suggests that high concentrations would not be therapeutically advantageous.

Therefore in conclusion, these experiments confirm that levamisole acts at the acetylcholine receptor on Ascaris suum muscle, both opening and blocking the channel.

We are grateful for the financial support of Merck Sharp & Dohme.

References


(Received May 18, 1992 Revised August 24, 1992 Accepted September 8, 1992)
Pyrantel activated single-channel currents in the nematode parasite *Ascaris suum*.

Dept of Preclinical Veterinary Science, Royal Dick School of Veterinary Studies, Summerhall, Edinburgh, EH9 1QH.

Several anthelmintic drugs, e.g. levamisole, pyrantel and morantel act as agonists at nicotinic acetylcholine receptors in the nematode parasite *Ascaris suum*, producing muscle depolarization and spastic paralysis (Aceves et al., 1970; Van Neuten, 1972; Coles et al., 1975; Harrow and Graton, 1985). A pharmacological profile of this receptor has been obtained (Natoff, 1969; Rozkova et al., 1980 and Colquhoun et al., 1991). The pharmacological differences between the nematode receptors and vertebrate nicotinic receptors are exploited for therapeutic purposes and permit the treatment of nematode infestations.

This present study has investigated the action of pyrantel (0.1-100μM) at the single-channel level using the patch-clamp technique on muscle vesicle membranes (Martin et al., 1991). Pyrantel activated a cation selective channel with conductance levels in the range 11-57pS. Subconductance states were detected. I/V plots were linear. The distribution of open-, closed- and burst-times showed that there were at least two open- and burst-states and three closed-states. At low concentrations (0.1μM) at -75mV the brief openings had a mean open duration of 0.52±0.19ms and the longer opening had a mean open duration of 2.40±0.48ms (Mean±s.e.,N=8). The open durations decreased on depolarization: at +75mV recordings with 0.1μM produced brief openings with mean open durations of 0.42ms±0.11ms and longer open times with mean durations of 1.63±0.13ms (Mean±s.e.,N=5). Occasionally at 10μM and mostly at 100μM only one open-state was resolved and channel events appeared as a sequence of rapid openings and closings characteristic of a fast flickering open-channel-block. With higher concentrations of pyrantel (100μM) in the pipette long closed periods separating clusters of openings thought to represent a desensitised closed state were detected at hyperpolarized and depolarized potentials.

Desensitization has also been observed previously in this preparation with acetylcholine (>25μM) and levamisole (>30μM) (Pennington and Martin, 1990; Robertson and Martin, 1991); a flickering open-channel block has also been observed with levamisole (30-90μM) at hyperpolarized potentials (Robertson and Martin 1991). The therapeutic significance of these two phenomena for anthelmintic use remains to be fully evaluated.


We would like to thank Merck Sharp & Dohme, Wellcome Trust and S.E.R.C. for finanical support.
Cytoplasmic application of levamisole leads to activation of the nicotinic acetylcholine receptors in the parasite Ascaris suum.

Susan J. Robertson & Richard J. Martin
Dept of Preclinical Veterinary Sciences, R. (D.) S. of V. S., Summerhall, University of Edinburgh, Edinburgh, EH9 1QH.

Studies on the mode of action of various anthelmintics (including levamisole) indicate that they are selective agonists of nicotinic acetylcholine receptors present on the muscle cells of the parasitic nematode Ascaris suum.1-3 This action of levamisole results in depolarisation of muscle cells inducing spastic paralysis of the nematode.1-6 Analyses of levamisole-activated single-channel currents in Ascaris suum on application of levamisole to the extracellular surface of the patch has shown: (i) the channels are cationic with linear I/V plots; (ii) the channel conductance is approximately 35 pS; (iii) generally there are two open-states and three closed-states; and (iv) at high concentrations (>30 μM) desensitisation and open-channel-block occur. Desensitisation occurs at both depolarised and hyperpolarised potentials whereas channel-block is only observed at hyperpolarised potentials.7 The direction of voltage sensitivity of the channel-block was consistent with a cationic blocking agent (such as levamisole) approaching the channel from the extracellular side.

This study was performed to examine the effect of levamisole on channel kinetics when application was carried out via the bath solution. It was thought that levamisole may have access to a blocking site when entering the channel from the cytoplasmic side of the membrane. Initial experiments were performed where channels were activated with levamisole (2 μM) applied in the patch-pipette, isolated inside-out patches were used. In addition, levamisole (30-92 μM) was added to the cytoplasmic membrane surface via the bath solution. In 8 out of 9 experiments, addition of levamisole to the bath solution resulted in an increase in channel activity. There was a positive correlation between Popen and concentration, at both depolarised (correlation coefficient (r): 0.96; p<0.001) and hyperpolarised potentials (correlation coefficient (r): 0.94; p<0.005). This increase could be the result of one of two things: levamisole has an action on a different channel in this parasite or cytoplasmic application of levamisole has an effect on the original nicotinic channel. Current-Voltage relationships were determined before and after the addition of levamisole to the bath which showed no detectable change in conductance. With application of higher concentrations of levamisole to the bath solution desensitisation and open-channel-block were observed. Block only occurred at hyperpolarised potentials, an observation consistent with levamisole blocking the channel from the extracellular surface. Open channel-block was not observed at depolarised potentials suggesting channel asymmetry. One explanation for the increase in channel activity and alteration of channel kinetics with high concentrations of levamisole present in the bath solution is: levamisole in the uncharged form crosses the membrane, via the lipid phase, increasing the extracellular concentration of levamisole in the pipette. To determine the amount of unionised levamisole present in bath solutions, the pKa of levamisole was estimated from a simple titration of levamisole with molar NaOH. The pKa was calculated as 8.0, similar results were obtained in two other experiments. Using the Henderson Hasselbalch equation the ratio of the concentration of unionised to the concentration of ionised levamisole was 0.158 at pH 7.2.8 Therefore approximately 16% of the levamisole is in the unionised form which may be able to diffuse across the membrane. Thus it was concluded that levamisole crossed from the cytoplasmic side of the membrane increasing the extracellular concentration present in the patch pipette. The ability to cross lipid barriers like membrane or cuticle has an obvious therapeutic significance for the treatment of parasitic infestations.

References

This work will be reported more fully elsewhere.

We would like to thank Merck Sharp & Dohme (U.S.A.) for financial support.