Molecular mechanism of glucocorticoid action

Ghassem Attarzadeh Yazdi

Doctor of Philosophy

The University of Edinburgh

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Declaration

I certify that the work presented in this thesis was performed by myself in the division of Neuroscience, University of Edinburgh.

GHASSEM ATTARZADEH YAZDI
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Finally, I am grateful for financial support by Bandar Abbas University, IRAN.
Dedication

I would like to dedicate the present work to my wife, Farina and my parents for their encouragement and assistance.
Abstract

Adrenal corticosteroids are involved in multiple aspects of homeostatic control. The early delayed (<2h) action of glucocorticoids is mediated by rapid induction of mRNA and protein synthesis. Within this time-frame, glucocorticoids potently modify the electrical excitability of target cells through regulation of ion channels. Increasing evidence suggests that in neurones and endocrine cells, large conductance calcium- and voltage-activated potassium channels (BK channels) are important targets for glucocorticoid action. The aim of this thesis project was to investigate the mechanisms by which glucocorticoid hormones regulate the activity of BK channels in human embryonic kidney 293 (HEK 293) cells as the model system for glucocorticoid-action. It was shown that glucocorticoids act via endogenously expressed type II receptors in a concentration- and time-dependent manner in these cells. Dexamethasone (100 nM) had no significant effect on Dexras1 mRNA but significantly increased serum- and glucocorticoid-induced protein kinase 1 (SGK-1) mRNA. Biochemical analysis showed that SGK-1 protein is increased by dexamethasone in a Triton X-100 insoluble fraction. Further work was directed toward analysing the possible association of SGK-1 and protein phosphatases with two BK channel α-subunit variants: ZERO-BK and STREX-BK, the latter contains the 59 amino-acid splice insert encoded by the stress hormone induced exon (STREX). HEK 293 cells stably expressing the respective channel subunits were analysed. Immunoprecipitations with antisera directed against the BK channel α-subunits showed that protein phosphatase 2A (PP2A) but not SGK-1 is constitutively associated with the STREX as well as the ZERO variant BK channel. Furthermore, the cytoplasmic C-terminal segment of the STREX-BK channel was necessary for
cell-surface expression of the channel and the association of the channel with PP2A. Dexamethasone failed to change the apparent amount of immunoreactive PP2A co-immunoprecipitating with the channel. In conclusion: SGK-1 but not Dexras1 is a protein rapidly induced by dexamethasone in HEK 293 cells. PP2A but not SGK-1 is in complex with both ZERO and STREX-BK channels, and dexamethasone does not alter this association. The cytoplasmic tail of the BK channels is essential for PP2A interaction.
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<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate Cyclase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AF</td>
<td>Aivation function</td>
</tr>
<tr>
<td>AGS-1</td>
<td>Activator of G-protein signalling-1</td>
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ triphosphate</td>
</tr>
<tr>
<td>BK channel</td>
<td>Large conductance calcium- and voltage-activated potassium channel (Maxi-K or Slo channel)</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3’, 5’-cyclic monophosphate (cyclic AMP)</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>CRF-41</td>
<td>41 amino acid residue corticotrophin–releasing factor</td>
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<td>C-terminal</td>
<td>Carboxyl-terminal</td>
</tr>
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<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>Dexras1</td>
<td>Dexamethasone-inducible Ras protein 1</td>
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<tr>
<td>dH2O</td>
<td>Distilled water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotides triphosphate namely dATP, dCTP, dGTP, dTTP</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<td>G</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylyl cyclase</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GIP</td>
<td>Glucocorticoid-induced proteins</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GRE</td>
<td>Glucocorticoid response elements</td>
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<tr>
<td>Gs</td>
<td>Guanine nucleotide binding protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
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<tr>
<td>GYG</td>
<td>Glycine-tyrosine-glycine</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
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<td>HEK 293</td>
<td>human embryonic kidney cell line 293</td>
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<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]</td>
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<td>HPA axis</td>
<td>hypothalamic-pituitary-adrenal axis</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>hsp90</td>
<td>90 kDa heat shock protein</td>
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<td>IbTX</td>
<td>Iberiotoxin</td>
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<td>IgG</td>
<td>antibody</td>
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<td>IK channel</td>
<td>Intermediate conductance $K_{Ca}$ channel</td>
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<tr>
<td>IP</td>
<td>immunoprecipitate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl $\beta$-D-thiogalactopyranoside</td>
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<tr>
<td>ITS</td>
<td>Insulin-transferrin-sodium selenite</td>
</tr>
<tr>
<td>$K_{Ca}$ channel</td>
<td>Calcium-activated potassium channels</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>$K_v$ channel</td>
<td>Voltage-gated potassium channel</td>
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<tr>
<td>LB</td>
<td>Luria Berani-broth/agar</td>
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<td>LBD</td>
<td>ligand-binding domain</td>
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<td>LDS-LB</td>
<td>lithium dodecyl sulphate-loading buffer</td>
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<td>LZ</td>
<td>Leucine zipper</td>
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<td>MBG</td>
<td>Multiple cloning site</td>
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<td>MMTV-LTR</td>
<td>Mouse mammary tumor virus- long terminal repeat</td>
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<td>MOPS</td>
<td>Morpho-linepropane sulphonic acid</td>
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<td>Mr</td>
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<td>N-methyl-D-aspartate</td>
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<td>Amino-terminal</td>
</tr>
<tr>
<td>NTD</td>
<td>Amino-terminal domain</td>
</tr>
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<td>Nuc</td>
<td>Nucleosome</td>
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<td>Definition</td>
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<tr>
<td>OA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>O.D</td>
<td>Optical density</td>
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<tr>
<td>P</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBS-T</td>
<td>PBS+ 0.1% Tween 20</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PGS</td>
<td>Protein-G-Sepharose; protein from group C streptococci that binds Fc portion of IgG bound to sepharose</td>
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<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PK</td>
<td>Protein kinase</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A or cAMP-dependent protein kinase</td>
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<td>PKAc</td>
<td>PKA catalytic subunit</td>
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<tr>
<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PKG</td>
<td>Protein kinase G</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>Po</td>
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<td>PP</td>
<td>Protein phosphatase</td>
</tr>
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<td>Protein phosphatase 1</td>
</tr>
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<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>pS</td>
<td>picoSiemens</td>
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<tr>
<td>PSD</td>
<td>Phosphate SDS-DTT</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Explanation</td>
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<tr>
<td>RCK</td>
<td>Regulator of potassium conductance</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<td>S869</td>
<td>Serine-869</td>
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<td>SDS</td>
<td>sodium dodecylsulphate (sodium Lauryl Sulphate)</td>
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<td>SDS-loading buffer</td>
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<td>SGK-1</td>
<td>Serum and glucocorticoid-inducible kinase-1</td>
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<tr>
<td>SK channel</td>
<td>Small conductance K&lt;sub&gt;Ca&lt;/sub&gt; channels</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium Saline Citrate</td>
</tr>
<tr>
<td>SSPE</td>
<td>Sodium Chloride- Sodium Phosphate-EDTA</td>
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<tr>
<td>STREX</td>
<td>Stress regulated, or stress axis related exon, or a cysteine-rich 59 amino acid inserts at splice site 2</td>
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<tr>
<td>STREX channel</td>
<td>BK channel variant that expresses the STREX insert</td>
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<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris, Boric acid, EDTA</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>ZERO channel</td>
<td>BK channel splice variant lacking STREX inserts</td>
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<tr>
<td>α</td>
<td>Alpha</td>
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<tr>
<td>β</td>
<td>Beta</td>
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Dexras1

SGK-1

Interaction of PP2A with BK channel α-subunit

Cytoplasmic tail of the STREX variant BK channel α-subunit is necessary for its cell surface expression

Regulation of PP2A by glucocorticoid hormones

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Chapter One

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1.1: Introduction to glucocorticoids

1.1.1: Generic mechanisms of glucocorticoid action

Glucocorticoids (stress hormones) are synthesized and secreted by the adrenal cortex. Glucocorticoid hormones exert their effects through the glucocorticoid receptors (GRs), which belong to the superfamily of nuclear receptors that function as ligand-dependent transcription factors (Encio and Detera-Wadleigh., 1991; Carson-Jurica et al., 1990; Evans., 1988). The GRs mediate their effects in the body by altering gene expression in various tissues, e.g., lung, liver, bone marrow, immune system, and brain (De Kloet et al., 1998; Evans-Storms and Cidlowski., 1995; Schmid et al., 1995).

In addition to nuclear glucocorticoid receptors, there is recurring evidence for fast, non-genomic actions of glucocorticoids in several systems (Falkenstein et al., 2000; Chen et al., 1999a; Sackey et al., 1997; Gametchu et al., 1993; Orchinnik et al., 1991), however, a receptor molecule that mediates such actions has not been characterised.

1.1.2: The human glucocorticoid receptor gene and protein

The human glucocorticoid receptor (hGR) gene is located on chromosome 5 and consists of 9 exons. Alternative splicing of the hGR gene in exon 9 gives rise to hGRα and hGRβ mRNA. Both mRNAs contain exon 1-8 but different versions of exon 9 (9α and 9β) that translate into highly homologous receptor isoforms hGRα and hGRβ.
respectively. The two isoforms of hGR are thus identical through to amino acid 727, but then diverge, with hGRα having an additional 50 amino acids and hGRβ having an additional, nonhomologous 15 amino acids at their carboxyl-terminal (figure 1.1) (Oakley et al., 1996; Hollenberg et al., 1985). hGRα is expressed in almost all human tissues and cells and represents the classic GR (a 777 amino acid protein) that functions as a ligand-dependent transcription factor, whereas hGRβ does not bind glucocorticoids and inhibits the transcriptional activity of hGRα in a dose-dependent manner (Oakley et al., 1999; Oakley et al., 1996; Bamberger et al., 1996).

The hGRα is composed of three functional domains: 1) An amino-terminal domain (NTD), which consists of amino acids 1-420 and contains a major transactivation domain, termed activation function (AF)-1. 2) A central cysteine-rich DNA-binding domain (DBD), which contains two zinc finger motifs through which it binds to a specific DNA sequence in the promoter region of target genes (glucocorticoid response elements, GREs). 3) A carboxyl-terminal, ligand-binding domain (LBD), which contains amino acids 527-777 (figure 1.1) (Oakley et al., 1996; Dalman et al., 1991; Carson-Jurica et al., 1990; Picard and Yamamoto, 1987; Beato et al., 1987).

hGRα forms a complex with two molecules of 90 kDa heat shock protein (hsp90) and several other proteins (figure 1.2) that act as negative transcriptional regulators preventing DNA binding of the receptor (Rajapandi et al., 2000; Bamberger et al., 1996; Pratt W. B., 1993; Dalman et al., 1991; Picard et al., 1990; Scherrer et al., 1990).
Figure 1.1
Schematic representation of the structure of the human glucocorticoid receptor (hGR) gene and protein

Alternative splicing of the hGR primary transcript gives rise to the two mRNA and protein isoforms, hGRα and hGRβ. hGRα contains an additional 50 amino acids after amino acid 727 while hGRβ has an additional 15 amino acids after amino acid 727. The functional protein domains of hGR are DNA-binding domain (DBD); ligand-binding domain (LBD); amino-terminal domain (NTD). The numbered boxes represent the number of exons of the respective genes the size of the boxes is an indication of the relative size of the exons.
1.1.3: Mechanisms of glucocorticoid receptor action

Entry of glucocorticoids into the cell and subsequent binding to the LBD of glucocorticoid receptor (GR) leads to a series of steps including: 1) Dissociation of hsp90 and other proteins from GR complex. 2) Dimerization of GR in the cytoplasm. 3) Translocation of the hormone-bound receptor to the nucleus. 4) High-affinity binding of the GR to glucocorticoid response element (GRE) which is found in the regulatory region of target genes. 5) The interaction of GRE-bound receptors with elements of the transcription machinery, resulting in the activation of gene transcription and induction of glucocorticoid-induced proteins (GIPs) (figure 1.2) (McNally et al., 2000; Bamberger et al., 1996; Becker et al., 1986).

In contrast to genomic glucocorticoid action, nongenomic glucocorticoid effects (less than 15 min) are mediated by modulation of protein function reflecting changes in membrane physicochemical properties (Falkenstein et al., 2000; Chen et al., 1999a; Sackey et al., 1997), but the receptor molecular has not been characterised.

The mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter has been used extensively as a useful model for studying the effects of GR on gene regulation since the beginning of the 1980s (Zhang et al., 2003; Hager et al., 2000; Leckie et al., 1995). The MMTV-LTR has six well-defined positioned nucleosome families, designated nucleosomes (Nuc A–F). Nuc A overlaps the TATA region and the transcription start site, and Nuc F is the farthest 5' to the transcription start site. Long terminal repeat in the MMTV genome is one of the prominent examples of GRE-
containing promoters (Cole et al., 1995; Beato et al., 1995; Hynes et al., 1981) which contains four GREs and is regulated by glucocorticoid hormones (Hynes et al., 1981).

When MMTV integrates into the genome of a mammalian cell, it adopts a specific chromatin organization (Fragoso et al., 1995; Richard-Foy and Hager., 1987).

The MMTV promoter is silent in the absence of hormone (i.e. agonist) and is activated by glucocorticoid hormones as well as progestins and androgens (McNally et al., 2000; Becker et al., 1986).
Figure 1.2
Schematic representation of glucocorticoid action

Figure 1.2: Schematic representation of glucocorticoid action

Genomic action of glucocorticoid hormones is mediated by passing the glucocorticoid hormone (G) through the plasma membrane and binding to the ligand-binding domain of the specific glucocorticoid receptor (GR). Upon glucocorticoid hormone binding, the glucocorticoid receptor undergoes an allosteric change, which results in dissociation from 90 kDa heat shock proteins (hsp90) and other proteins, leading to activation of GR. The activated receptor forms a homodimer and is translocated to the nucleus through the nucleopore. Inside the nucleus, the receptor complex binds to specific DNA response elements (glucocorticoid response elements, GREs) to activate gene transcription and result in increased mRNA and protein synthesis. In contrast, nongenomic action of glucocorticoid is mediated by altering the membrane physicochemical properties (Based on SIGMA-ALDRICH).
1.1.4 Biological effects of glucocorticoids

It has been indicated that glucocorticoids regulate a variety of biologic processes and exert profound influences on many physiologic functions, including basal and stress-related homeostasis, growth and development, reproduction, the immune system including anti-inflammatory and immunosuppressive actions (Tsai et al., 1994; Munck et al., 1990; Munck et al., 1984). Glucocorticoids can also inhibit a few features of sympathetic function. For example, Glucocorticoids inhibit catecholamine release in response to some stressors (Komesaroff and Funder., 1994; Kvetnansky et al., 1993) and decrease cardiac norepinephrine turnover (Westfall and Osada., 1969). It has been determined that release of glucocorticoid into the peripheral circulation on response to stress is mediated through activation of the hypothalamic-pituitary-adrenal (HPA) axis. Increasing evidence suggests that these homeostatic mechanisms, such as the immune response, in turn directly modulate the activity of the HPA axis (Bateman et al., 1989; Munck et al., 1984).

1.2 Introduction to the HPA and glucocorticoid inhibition of ACTH secretion

Glucocorticoids are largely under the control of the HPA axis (De Kloet et al., 1998; Dallman et al., 1992). Activity of the HPA axis is regulated by many stimuli such as inflammation, pain, infection and stress. These stimuli cause excitation of the hypothalamus (paraventricular nucleus), which responds by releasing 41 amino acid
residue corticotrophin-releasing factor (CRF-41) and arginine vasopressin (AVP), to increase the secretion of adrenocorticotropic hormone (ACTH, a 39-amino acid peptide) from the anterior pituitary gland (Antoni., 1996; Antoni et al., 1992; Antoni, 1986).

ACTH enters the systemic circulation and binds to specific high-affinity receptors, a seven transmembrane domain protein, located on the surface of cells of the middle layer of the adrenal cortex (z. fasciculata). ACTH binding to its receptor in the adrenal cortex activates a guanine nucleotide binding protein (Gs protein) resulting in an increase of intracellular cyclic adenosine monophosphate (cAMP) and finally enhances the production of cortisol (corticosterone in rodents), the main glucocorticoid hormone in the body (Newton R., 2000). Glucocorticoids regulate their own production by negative feedback at several sites such as the hypothalamus and anterior pituitary corticotroph cell (figure 1.3) (De Kloet et al., 1998; Dallman et al., 1992). The anterior pituitary corticotroph cell is central to the integration of HPA axis function, and is a target for HPA axis feedback regulation by glucocorticoids (Jones and Gillham, 1988; Keller-Wood et al., 1988).
Many stimuli including inflammation, pain, infection and stress cause excitation of the hypothalamus. The hypothalamus secretes 41 amino acid residue corticotrophin-releasing factor (CRF-41) and arginine vasopressin (AVP), which stimulate the release of ACTH from anterior pituitary corticotroph cells. ACTH drives the release of glucocorticoids from the adrenal glands and glucocorticoids regulate their own production by feedback inhibition of hypothalamic-pituitary-adrenal (HPA) activity at several feedback sites, including the hypothalamus and anterior pituitary corticotroph.
1.2.1 Mechanism of 41 amino acid residue corticotrophin–releasing factor (CRF-41) and arginine vasopressin (AVP) stimulated ACTH secretion

At the level of anterior pituitary corticotroph cells, CRF-41 binds to a G-protein coupled receptor (GPCR) and activates G₉ protein (King and Baertschi., 1990; Taylor et al., 1990), leading to elevation of cAMP which activates cAMP-dependent protein kinase (PKA) (King and Baertschi., 1990; Taylor et al., 1990; Reisine and Jensen., 1986; Reisine et al., 1985).

It has been shown that in anterior pituitary corticotroph AtT20 cells, PKA inhibits the large conductance calcium- and voltage-activated potassium (BK) channels which results in sustained depolarization and enhanced secretion of ACTH (Shipston et al., 1996). In addition, Ca²⁺ influx through voltage dependent L-type calcium channels is an essential component of CRF-41-stimulated ACTH release in AtT20 cells (figure 1.4) (Childs and Burke., 1987; Luini et al., 1985).

In addition to PKA, ACTH secretion is regulated by protein kinase C (PKC). In anterior pituitary corticotroph cells, PKC mediates the sustained phase of ACTH secretion stimulated by activation of the phospholipase C pathway by the hypothalamic secretagogue vasopressin (Oki et al., 1990; Carvallo and Aguilera., 1989). Vasopressin elicits a biphasic elevation of intracellular free calcium (Tse and Lee., 1998; Corcuff et al., 1993) and during the sustained phase of calcium influx stimulates PKC translocation and enhances PKC activity at the plasma membrane, an effect that is mimicked by the cell-permeant PKC-activating phorbol esters (Carvallo and Aguilera., 1989).
Figure 1.4: Schematic representation of CRF-41 action in AtT20 cells

In anterior pituitary corticotroph AtT20 cells, CRF-41 activates adenylyl cyclase (AC) through stimulatory guanine nucleotide binding protein (Gs). AC produces cyclic adenosine monophosphate (cAMP) and activation of cAMP-dependent protein kinase (PKA). PKA inhibits large conductance calcium- and voltage-activated potassium (BK) channel, which results in sustained depolarization and enhanced exocytosis. On the other, PKA activates the voltage-gated calcium channels (L-type) and increases intracellular calcium and increases ACTH secretion (Modified from Antoni., 2000).
In AtT20 mouse corticotroph cells, phorbol-ester (an analogue of diacyl glycerol)-mediated activation of PKC has been proposed to exert effects both distal and proximal on voltage-dependent calcium influx, which may result from activation of different PKC isoforms (McFerran et al., 1995) to elicit ACTH release (McFerran et al., 1995; Clark and Kemppainen., 1994; Woods et al., 1992; Phillips and Tashjian., 1982). Intracellular free calcium measurements in AtT20 cells suggest that PKC-induced calcium influx results, at least in part, from inhibition of tetraethylammonium (TEA)-sensitive potassium conductances. This inhibition results in membrane depolarization and subsequent, indirect, enhancement of voltage-gated calcium influx (Reisine T., 1989; Reisine and Guild., 1987).

In addition, glucocorticoids inhibit ACTH secretion evoked by activation of the PKA (Shipston et al., 1996) or the PKC (McFerran et al., 1995; Clark and Kemppainen., 1994; Woods et al., 1992) signaling pathways.

1.2.2 Glucocorticoid inhibition of CRF-stimulated ACTH secretion

Corticosteroid feedback inhibition of the HPA axis is a key process in the maintenance of body homeostasis (De Kloet et al., 1998; Dallman et al., 1992) and is mediated at several sites such as the hippocampus, the hypothalamus and anterior pituitary corticotroph cells (figure 1.3) (De Kloet et al., 1998; Dallman et al., 1992). At the level of anterior pituitary corticotroph cells, glucocorticoid inhibition of CRF-stimulated ACTH secretion is best described as developing in two phases that involve distinct changes of gene expression (Dayanithi and Antoni., 1989).
Early phase: Occurring between 15 min to less than 3 h is a potentially generic mechanism of glucocorticoid action involving induction of de novo protein and mRNA synthesis.

Late phase: occurring more than 3 h to days and involves suppression of differentiated cell function, including metabolism or ACTH synthesis and down regulation of CRF signaling pathways (Bilezikjian and Vale., 1983).

1.2.3 Mechanism of early glucocorticoid inhibition of CRF-stimulated ACTH secretion

It has been demonstrated that glucocorticoid hormones inhibit CRF-stimulated ACTH secretion directly at the pituitary level (Shipston et al., 1996; Oki et al., 1991). Shipston and co-workers demonstrated that BK channels are important targets of early glucocorticoid-mediated inhibition of CRF-stimulated ACTH secretion in anterior pituitary corticotroph AtT20 cells (Shipston et al., 1996). Patch-clamp analysis in AtT20 cells revealed a significant and selective inhibition of BK channels upon activation of PKA by CRF. The synthetic glucocorticoid dexamethasone prevented PKA-mediated inhibition of BK currents and iberiotoxin (a highly specific blocker of BK channels) (Galvez et al., 1990) abolished early glucocorticoid-induced inhibition of CRF-stimulated ACTH release (Shipston et al., 1996). Thus in anterior pituitary corticotroph AtT20 cells, glucocorticoids inhibit CRF-stimulated ACTH secretion, by blocking PKA-mediated inhibition of BK channels.

In primary cultures of rat anterior pituitary cells, the secretion of ACTH induced by CRF was markedly inhibited upon a 2 h exposure to 100 nM corticosterone. Application of
the broad specificity K⁺ channel blockers clofilium and astemizole produced no significant enhancement of CRF-induced ACTH release (Lim et al., 2002), but blocked the inhibitory action of corticosterone. In contrast, blockers of BK-channels such as TEA and charybdotoxin failed to influence the inhibition by corticosterone. In contrast to AtT20 cells, BK channels appear to have no pivotal role in glucocorticoid action in normal (rat) corticotroph cells. Thus, the unraveling of the glucocorticoid signaling pathway to BK channels as observed in AtT20 cells (Tian et al., 1998; Shipston et al., 1996) and recapitulated in human embryonic kidney 293 (HEK 293) cells (Tian et al., 2001b) should provide novel and valuable information as to the mechanism of action of glucocorticoid hormones.

**Summary:** cAMP-dependent phosphorylations of L-type calcium channels as well as BK-type potassium channels are required for triggering the ACTH secretory response to CRF (Shipston et al., 1996; Luini et al., 1985). Proteins rapidly (within 2 h) induced by glucocorticoids acting via the type II GR block PKA-mediated inhibition of BK channels in pituitary corticotroph tumour cells. The action of the steroid is pivotal for its early inhibitory effect on the secretion of ACTH (Shipston et al., 1996); however, the mechanisms of this regulation are poorly understood.

1.3 Introduction to BK channels

The large super-family of potassium channels is a wide range of functionally and structurally diverse transmembrane ion channels responsible for conducting the
physiologically important cation, potassium (Coetzee et al., 1999; Brown A. M., 1993). Within this family, the calcium-activated potassium (K\textsubscript{Ca}) channels are distinct due to their activation by elevation in intracellular free calcium with the subsequent potassium ion efflux contributing to cellular hyperpolarisation (Coetzee et al., 1999; Vergara et al., 1998). K\textsubscript{Ca} channels are sub-divided according to the relative potassium conductance into three sub-families (Coetzee et al., 1999); large (BK), intermediate (IK) and small (SK) conductance of K\textsubscript{Ca} channels (Table 1.1).

**Table 1.1**

**Relative conductances of the three K\textsubscript{Ca} channel families**

<table>
<thead>
<tr>
<th>KCa channel sub-family</th>
<th>Abbreviation</th>
<th>Conductance range (picoSiemens, pS)</th>
<th>gated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small conductance</td>
<td>SK</td>
<td>2-20</td>
<td>internal Ca\textsuperscript{2+}</td>
</tr>
<tr>
<td>Intermediated conductance</td>
<td>IK</td>
<td>20-80</td>
<td>internal Ca\textsuperscript{2+}</td>
</tr>
<tr>
<td>Large conductance</td>
<td>BK</td>
<td>150-350</td>
<td>internal Ca\textsuperscript{2+} and membrane potential</td>
</tr>
</tbody>
</table>

**Table 1.1:** At least three types of calcium-activated potassium (K\textsubscript{Ca}) channels have been described including large conductance (BK) channels with conductance 150-350 pS, small conductance (SK) channels with conductance 2-20 pS, and intermediate conductance (IK) channels with conductance 20-80 pS.
The BK (Maxi-K or Slo) channels have an unusually large potassium conductance, approximately ten-fold greater than other potassium channels, and are activated by both transmembrane voltage and intracellular free calcium levels (Sah and Faber., 2002; Weiger et al., 2002; Gribkoff et al., 2001; Hu et al., 2001; Shi and Cui., 2001; Zhang et al., 2001; Toro et al., 1998). In contrast, other K\textsubscript{ca} channels (IK and SK channels) are activated by calcium and not depolarization (Vergara et al., 1998) and K\textsubscript{v} channels are sensitive to voltage but not calcium (Hong et al., 2001).

1.3.1 BK channel \textit{\alpha}-subunit

BK channels are assembled as tetramers (Quirk and Reinhart, 2001; Shen et al., 1994) of pore-forming alpha (\textit{\alpha})-subunits encoded by a single gene, KCNMA1 (also referred to as Slo1) that undergoes extensive alternative splicing (TsengCrank et al., 1994). Slo1 homologues have been identified and described in numerous species such as mouse (Butler et al., 1993), chicken (Jiang et al., 1997), cow (Moss et al., 1996) and the human gene mapped on chromosome 10 (Pallanck and Ganetzky, 1994).

1.3.1.1 Structure of BK channel \textit{\alpha}-subunit

BK channel \textit{\alpha}-subunits contain sequence similarities to voltage-gated potassium (K\textsubscript{v}) channels in having six transmembrane helices (S1-S6). The BK channel \textit{\alpha}-subunits have an additional transmembrane segment (S0) at the amino-terminal (N-terminal). The S5-S6 transmembrane segments constitute the P-loop, which acts as the lining of the pore (figure 1.6) (Choe S., 2002; Doyle et al., 1998; Meera et al., 1997). The P-domain contains the sequence glycine-tyrosine-glycine (GYG), which has been termed
the K⁺ selectivity sequence (figure 1.5) (Heginbotham et al., 1994; Yellen G., 1987). The BK channel pore is suggested to exhibit distinct electrostatic and conformational pore properties resulting from unique aromatic and associated residues that provide proximal cation binding sites to maximise the ionic repulsion between fluxing potassium ion concentration for faster reactivity (Lagrutta et al., 1998). The P-loop shares sequence homology with other potassium channels and is the site of action of wide-spectrum potassium channel blockers such as TEA (Lagrutta et al., 1998). The S4 transmembrane segment of the BK channel α-subunit contains positively charged amino acids that are conserved among voltage-dependent channels (Cui and Aldrich., 2000; Diaz et al., 1998) and is likely to function as the voltage sensor.

**Carboxyl-terminal (C-terminal) domain**

The cytoplasmic C-terminal domain of the BK channel α-subunit protein constitutes ~70% of the total protein mass. This domain is suggested to be soluble, cytoplasmic and to include four putative hydrophobic α-helices (S7-S10) (Tanaka et al., 1997; Meera et al., 1997). The C-terminus contains two calcium-binding sites, the calcium bowl as well as the regulatory potassium conductance (RCK) segment, that are involved in activation of the BK channel by Ca²⁺ (figures 1.5 and 1.6) (Jiang et al., 2002). The cytoplasmic C-terminal tail of the BK channel α-subunit also contains five sites of alternative splicing (figures 1.5 and 1.6) (Coetzee et al., 1999).
The amino acid sequence of BK channel STREX splice variant α-subunit

The murine STREX channel α (α)-subunit sequence (NCBI accession number: AAD49225). The yellow boxes show the alpha helices, S0-S6 transmembrane helices and putative cytoplasmic helices S7-S10. The triangles show sites of alternative splicing (1 to 5). PKA phosphorylation sites are showed by pink and the 59 residue STREX insert at splice site 2 is showed by green box. Calcium bowl and regulatory potassium conductance (RCK) domain are shown with gray box.
Figure 1.6
Schematic representation of functional domains of the BK channel α-subunit

The BK channel α-subunit contains 7 putative transmembrane segments S0-S6. The pore-forming P-loop, containing the K⁺ ion selectivity signature, lies between segments S5 and S6 while the S4 segment is the predominant voltage sensing domain. The C-terminal contains additional four hydrophobic segments (S7-S10), two Ca²⁺ regulatory sites (RCK and Ca²⁺ bowl) and five established sites of alternative splicing (1 to 5).
Furthermore, the C-terminal of the α-subunit also contains a structural motif (Leucine zipper, LZ) that was originally described in classes of DNA-binding proteins (Landschulz et al., 1988). It has been reported that LZ plays an important role in coordinating both the assembly of ion channels as well as their interaction with protein kinase and protein phosphatase signaling complexes. Tian and co-workers demonstrated that Leucine zipper 1 (LZ1) in the C terminus of mammalian BK channels (residues 513-543) is required for the association and regulation of the channels by PKA, and other putative leucine zippers in the BK channel protein may provide anchoring for other regulatory enzyme complexes (Tian et al., 2003).

In addition, several accessory proteins and modulatory factors (section 1.3.4) like protein kinases and protein phosphatase, lipid, redox reagents and hormones directly or indirectly associate with, or exert influence through, the C-terminal domain (Lu et al., 2006).

Calcium bowl and RCK

Each α-subunit of the BK channel has a calcium bowl, which consists of 28-amino acid residues and is located in the hyper-conserved region between helices S9 and S10 (figures 1.5 and 1.6). The calcium bowl, a negatively charged, aspartate-rich motif was identified by mutagenesis (Wei et al., 1994) and exhibits homology with calcium-binding proteases (Moss et al., 1996) and plasma membrane calcium pumps (Schreiber and Salkoff., 1997). The calcium bowl is involved in the modulation of the BK channel by the ubiquitous second messenger, Ca²⁺ (Schreiber and Salkoff., 1997; Wei et al., 1994; Schreiber., 1992).
The C-terminal structure contains the regulator of RCK domain, a second potential regulatory element (figures 1.5 and 1.6) (Jiang et al., 2002). The RCK domain is implicated in calcium sensing by its analogy to homologous regions in other calcium-gated channels (Shi and Cui., 2001).

It has been shown that most of the negatively charged residues of the Ca\(^{2+}\) bowl and the RCK domain are aspartic acid, a key residue for coordinating Ca\(^{2+}\) in Ca\(^{2+}\) binding proteins (McPhalen et al., 1991). Mutation of five consecutive aspartate residues located in the Ca\(^{2+}\) bowl (mSlo1 residues 897-901) reduced the calcium sensitivity of the BK channels (Schreiber and Salkoff., 1997).

The RCK domain contains three aspartate residues (D362, D367 and D369 in mSlo1) conserved in Slo1 forms. It has been shown that aspartate 367 is more sensitive to Ca\(^{2+}\) as the mutations D362A and D369A each resulted in currents with Ca\(^{2+}\) sensitivity similar to wild-type currents. In contrast, D367A produced a marked reduction in the ability of Ca\(^{2+}\) to shift gating (Xia et al., 2002).

It has been shown that BK channels can function as purely voltage-gated in the absence of intracellular free Ca\(^{2+}\), but this requires high-voltage depolarization (Horrrigan and Aldrich., 1999; Meera et al., 1997). In the presence of physiological intracellular free Ca\(^{2+}\) levels, the voltage range of activation is shifted to more negative potentials that are physiologically relevant (Zhang et al., 2001; Jacquin and Gruol., 1999; Moss et al., 1999; Meera et al., 1997).

1.3.1.2 N-terminal domain

As noted in section 1.3.1.1, the BK channels posses an additional transmembrane helix
at the N-terminus – S0 –which is not found in other potassium channel proteins (figure 1.6) (Toro et al., 1998). The N-terminal domain of the pore-forming α-subunit is crucial for interaction with modulatory beta (β)-subunits of BK channels (Wallner et al., 1996). The study of Xenopus oocytes transfected with N-terminally truncated or C-terminally truncated forms of mouse Slo showed that open-time, conductance, and voltage dependence of BK channels are determined by the N-terminal core, whereas Ca\textsuperscript{2+}-sensitivity of the channel appears to involve a region of several negatively charged residues at the C-terminal core (Wei et al., 1994).

1.3.1.3 Splicing of the BK channel α-subunit gene

Alternative pre-RNA splicing is a mechanism that enables the generation of multiple proteins with divergent functional and regulatory properties from a single gene by the processing of the nuclear pre-mRNA into several discrete isoforms with distinct exon combinations (Grabowski and Black., 2001; Dredge et al., 2001). Alternative pre-mRNA splicing enables the generation of BK channel variants with distinct properties including, differences in calcium and voltage-dependence (Liu et al., 2002; Ha et al., 2000; Shipston et al., 1999; Saito et al., 1997; TsengCrank et al., 1994), regulation by protein phosphorylation (Zhang et al., 2004; Zhou et al., 2001; Tian et al., 2001a), and other intracellular signaling cascades (Erxleben et al., 2002; Tian et al., 2001b) as well as cell surface expression (Kwon and Guggino., 2004; Zarei et al., 2004).

The cytoplasmic C-terminal tail of the BK channel α-subunit is the region of the greatest diversity among BK channel isoforms due to the presence of five sites of alternative
splicing throughout the domain (figures 1.5 and 1.6) (Coetzee et al., 1999; TsengCrank et al., 1994).

Distinct α-subunit splice-variant mRNAs may be expressed in the same cell, differentially expressed between tissues, or even neighboring cells (Rosenblatt et al., 1997; Navaratnam D. S., 1997), and dynamic modification of splice-variant mRNA expression (Xie and Black., 2001; Xie and McCobb., 1998) may result in altered BK channel phenotype and cellular regulation (Lovell and McCobb., 2001).

The STREX insert

Stress hormones including glucocorticoids and adrenal androgens are suggested to induce complex alternative splicing of BK channel mRNA to fine-tune chromaffin cell excitability and secretory activity (Lai and McCobb., 2002; Lovell and McCobb., 2001; Xie and McCobb., 1998) leading to the naming of the insert, STREX, for stress-regulated, or stress axis-related-exon (Xie and McCobb., 1998). STREX (a cysteine-rich 59 amino acid) inserts at splice site 2 in the C-terminal of the BK channel α-subunit (figures 1.5 and 1.7) (Saito et al., 1997; Shipston et al., 1999). Channels containing STREX (STREX variant BK channels) are highly expressed in endocrine tissues such as the adrenal and pituitary gland, whereas ZERO variant BK channels (lacking a 59 cysteine-rich amino acid splice inserts) are the predominant brain variant (Chen et al., 2005; Shipston et al., 1999; Saito et al., 1997). STREX channels are more sensitive to the intracellular free calcium level than channels composed of the ZERO variant. Also cAMP-dependent PKA activates the ZERO variant channels, whereas it inhibits STREX variant channels (figure 1.7) (Chen et al., 2005; Tian et al., 2001a).
Figure 1.7
Schematic representation of STREX and ZERO BK channel α-subunit splice variants

59 amino acids
STREX

N-terminal

C-terminal

STREX BK channel α-subunit splice variant

STREX insert: PKMSIYKRMRACFDCGRSERDCSCMGRVGRNVDITERTFPISSVS VNDCSTSFRAF

N-terminal

C-terminal

ZERO BK channel α-subunit splice variant

Figure 1.7: Schematic representation of STREX and ZERO BK channel α-subunits splice variants

Full-length channel protein from the STREX BK channel c-subunit splice variant is identical to the ZERO variant protein except for the inclusion of the 59 amino acids STREX insert at splice site 2. PKA phosphorylation site in STREX variant BK channels is located in STREX insert at serine-4 (S4strex) which shown with color box.
1.3.2 The beta (β)-subunit

Biochemical purification (Garcia-Calvo et al., 1994) and co-immunoprecipitation (Knaus et al., 1994) of the BK channel complex have identified four individual β-subunits that confer distinct regulatory influences upon BK channels activity. The BK channel β-subunits are encoded by four distinct genes, KCNMB1-4 (Uebele et al., 2000; Behrens et al., 2000; Brenner et al., 2000). The BK channel β-subunits do not form ion channels when expressed in the absence of the α-subunits, but alter the intrinsic properties of the BK channels, namely calcium sensitivity, gating kinetics and response to modulators like protein kinases (Ramanathan et al., 2000; Ramanathan et al., 1999; Chang et al., 1997; Meera et al., 1996; Dworetzky et al., 1996; McManus et al., 1995; Garcia-Calvo et al., 1994). All four β-subunits share a common topology of two transmembrane helices with intracellular termini, N-linked glycosylation and molecular weights between 25-31 kDa (Weiger et al., 2002; Weiger et al., 2000; Wanner et al., 1999).

The β1-subunit (KCNMB1) was identified as a protein associated with BK channels in smooth muscle membrane preparation and exhibits limited expression in the brain (Brenner et al., 2000; Jiang et al., 1999; Saito et al., 1997; Meera et al., 1996; Dworetzky et al., 1996; TsengCrank et al., 1996; McManus et al., 1995). Cloning and expression of α and β1-subunits revealed that while the α-subunit forms a functional channel, co-expression of the β1-subunit increases the sensitivity of the channel to Ca$^{2+}$ for a wide range of membrane voltages (Cox and Aldrich., 2000; Wallner et al., 1996; TsengCrank et al., 1996; McManus et al., 1995).
Co-expression of α and β2-subunit (KCNMB2) produces inactivating BK currents in several tissues including adrenal chromaffin cells (Weiger et al., 2002; Weiger et al., 2000) and pancreatic β-cells where they are observed to modulate neurosecretion patterns (Xia et al., 1999; Li et al., 1999).

The β3-subunit is in enriched in the testes, pancreas and spleen (Xia et al., 2000) and has slight influence to enhance BK channel activation rates (Behrens et al., 2000). In contrast β4-subunit (KCNMB4) exhibits low expression outwith the nervous system and is enriched in brain tissue where it slows BK channel gating kinetics and modulates calcium sensitivity in a calcium-dependent manner (Jin et al., 2002; Weiger et al., 2000).

1.3.3 The physiological roles of BK channels

When BK channels are open, K+ flows out of cells through the open channels, driving the repolarization and hyperpolarization of the membrane potential. This change in membrane potential allows BK channels to control diverse physiological processes including regulation of vascular tone (Sausbier et al., 2005; Brenner et al., 2000), micturition (Meredith et al., 2004), neuronal excitability (Sausbier et al., 2004; Shao et al., 1999), innate immunity (Ahuwalia et al., 2004) and hearing (Ruttiger et al., 2004; Pyott et al., 2004).

It has been indicated that BK channel activity is enhanced by increasing intracellular calcium and the incumbent hyperpolarisation is crucial to the negative feedback of calcium signaling, resulting in the termination of calcium entry by the closure of voltage-gated calcium channels (Robitaille and Charlton., 1992). Through this regulation, BK channels are critical to several secretory processes ranging from sweating to
neurotransmitter release. The BK channels of epithelial cells (Huang et al., 1999; James and Okada., 1994) are suggested to be integral to calcium-mediated transepithelial fluid secretion such as that of equine sweat glands (Huang et al., 1999). In endocrine tissues such as glomus and chromaffin cells, the blockade of the BK channels can elicit catecholamine secretion (Pardal et al., 2000). In AtT20 cells, BK channels are important targets of glucocorticoid-mediated inhibition of CRF-induced ACTH secretion (Shipston et al., 1996).

1.3.4 Modulation of BK channels

In addition to direct actions of Ca\(^{2+}\) and voltage, the BK channels are subject to regulation by many cellular factors including protein kinases, protein phosphatases (section 1.3.4.1), lipids, nitric oxide, redox reagents, and hormones (see section 1.3.4.2). These enable BK channels to react to and facilitate many physiological processes. Some of these regulatory mechanisms are described below.

1.3.4.1 Protein phosphorylation

Protein phosphorylation is one of the most common mechanisms of protein modulation. Numerous signal transduction pathways exert physiologically relevant actions by altering the activities of protein kinases and protein phosphatases thus controlling the reversible phosphorylation and subsequent functional regulation of target proteins (Levitan., 1999; Armstrong., 1989). Activated protein kinases induce the hydrolysis and transfer of the terminal phosphate of adenosine 5'-triphosphate (ATP) onto the hydroxyl
group of serine, threonine or tyrosine residue of specific, targeted consensus sequences. Protein phosphatases act in opposition, mediating the dephosphorylation of such residues. Although, some studies have indicated a direct influence of ATP on the activity of BK channels in the absence of protein kinase activation through mechanisms that is undetermined currently (Clark et al., 1999), most investigators concur that the use of non-hydrolysable ATP analogues such as AMP-ANP has no influence on channel activity (Esguerra et al., 1994) This suggests a requirement for ATP hydrolysis in the regulation BK channels involving phosphorylation by native protein kinases (Esguerra et al., 1994). The reversibility of this modulation implicates the activity of protein phosphatases and thus indicates that reversible protein phosphorylation has a significant role in the regulation of BK channels (Zhou et al., 2001; Tian et al., 1998; Esguerra et al., 1994).

**Intracellular signal transduction pathways that impinge on BK channels**

Several, distinct protein kinases are implicated as regulators of the activity of the BK channel including serine-threonine kinases such as cAMP-dependent protein kinase (Tian et al., 2004; Gong et al., 2002; Shipston et al., 1999; Klaerke et al., 1996; Shipston et al., 1996; Lee et al., 1995; Levitan., 1994; Esguerra et al., 1994; White et al., 1991), protein kinase C (Hall and Armstrong., 2000; Tian et al., 1999; Shipston and Armstrong., 1996), protein kinase G (White et al., 1993) and tyrosine-phosphorylating protein kinases (Ling et al., 2000; Wang et al., 1999). Equally, protein phosphatases (PP) such as PP1 and PP2A are implicated as modulators of BK channels (Widmer et al., 2003; Shipston et al., 1996; White et al., 1991). As individual protein kinases and
phosphatases are regulated by distinct signal-transduction pathways, these findings demonstrate that several factors of physiological control converge on these ubiquitous ion channels.

PKC is stimulated by phospholipids and calcium generated and released through inositol phospholipid hydrolysis via activation of phospholipase C (PLC) (Zhou et al., 2001; Chik et al., 2001; Dekker and Parker, 1994). In many neurons (Doerner et al., 1988) and endocrine cells (Hall and Armstrong, 2000; Tian et al., 1999; Shipston and Armstrong, 1996), PKC is a potent inhibitor of BK channel activity. In rat pituitary cell line (GH4C1 cells), PKC has been proposed to limit the maximal activity of BK channels, irrespective of the prevailing voltage and calcium conditions (Hall and Armstrong, 2000).

Moreover, the BK channels are regulated by protein kinase G (PKG), which is stimulated by elevation of intracellular cyclic guanosine monophosphate (cGMP) following activation of soluble guanylyl cyclase (GC) isoforms by nitric oxide (NO) or activation of plasma membrane guanylyl cyclase receptors for peptides including atrial natriuretic peptide (Fukao et al., 1999; Alioua et al., 1998; White et al., 1993).

Finally, PKA regulates the BK channels through intracellular cAMP concentration influenced by indirect hormonal modulation of membranous adenylate cyclase by several physiological processes and responses (Taylor et al., 1990; Flockhart and Corbin, 1982). Sequence analysis in combination with site directed mutagenesis has identified several putative phosphorylation target sequences within the α-subunit, particularly in the C-terminal domain (Toro et al., 1998; Alicua et al., 1998). Serine-942
(S942) of the drosophila BK channel was identified as a putative site of direct phosphorylation by PKA following the inhibition of the PKA-mediated response (Nara et al., 1998; Esguerra et al., 1994). In mSlo, serine 869 (S869 which is the same of S942 in drosophila) and serine4STREX (S4STREX) were identified as targets of phosphorylation by PKA (Tian et al., 2001a). It has been shown that splice variants of BK channels provide the diversity and plasticity of BK channel sensitivities to PKA, as cAMP-dependent PKA activates the ZERO variant channels by phosphorylation of the C-terminal PKA consensus site (S869) while cAMP-dependent PKA inhibits STREX variant channels by phosphorylation of (STREX)-specific PKA consensus site (S4STREX) (figure 1.7) (Tian et al., 2001a). Furthermore, cAMP-dependent PKA phosphorylation of S869 site in all four α-subunits is required for channel activation while, inhibition of BK channel activity requires phosphorylation of only a single α-subunit at S4STREX site (Tian et al., 2004).

PKA and protein phosphatases

BK channel proteins are regulated by PKA and protein phosphatases (PP2A/PP1) which are associated as a regulatory complex at the plasma membrane of excitable cells (Widmer et al., 2003; Shipston., 2001; Tian et al., 2001b; Hall and Armstrong., 2000; Levitan., 1999; Wang et al., 1999; Reinhart and Levitan., 1995; Bielefeldt and Jackson., 1994; White et al., 1991). For example in mammalian pituitary tumour cells, somatostatin (an agonist of Gi-coupled receptor) increases the activity of BK channels through dephosphorylation of a cAMP-dependent phosphorylation site in the BK
channel protein or a closely associated regulatory molecule by one or more protein phosphatases (White et al., 1991).

In rat cerebellar Purkinje cells, single-channel analysis from inside-out patches demonstrated that the activity of BK channels is regulated by multiple endogenous protein kinases and protein phosphatases present in the membrane patch (Widmer et al., 2003). The low activity phenotype BK channels in these cells were activated by PKA associated with the patch and the extent of PKA activation was limited by an opposing endogenous PP2A. Furthermore, PKA activation was dependent upon the prior phosphorylation status of the BK channel complex controlled by PP1 (Widmer et al., 2003).

BK channels purified from rat brain and reconstituted in lipid bilayers showed two different biophysical and regulatory phenotypes: so-called type 1 and type 2 channels. Type 1 channels (fast gating, high sensitivity to charybdotoxin, activated by low Ca$^{2+}$) are stimulated by PKA and this effect was reversed by PP2A but not by PP1. Type 2 channels (slower open and closed times, less sensitive to charybdotoxin and Ca$^{2+}$) are down regulated by PKA, an effect also specifically reversed by PP2A (Reinhart et al., 1991; Chung et al., 1991).

In many neurons and smooth muscle cells, PKA activates BK channels (Schubert and Nelson., 2001; Levitan., 1999; Lee et al., 1995; Reinhart et al., 1991), whereas in endocrine cells of the anterior pituitary gland, BK channels are inhibited (Shipston et al., 1996; White et al., 1991; Sikdar et al., 1989). It has been demonstrated that in AtT20 cells (Tian et al., 1998) and HEK 293 cells (Tian et al., 2001b), the STREX BK channel
splice variant is potently inhibited by PKA activity intimately associated with the channel complex and the extent of PKA inhibition is limited by PP2A.

Although BK channels are potently regulated by dephosphorylation by multiple protein phosphatases (Widmer et al., 2003; Tian et al., 2001b; White et al., 1991), how these enzymes are targeted to BK channels is unknown. Dynamic interaction of protein phosphatases with the channel complex would provide an important mechanism to tune BK channel function and behavior. Thus, to further examine the association of protein phosphatase with BK channels, we examined the association of BK channels with protein phosphatase in HEK 293 cells.

1.3.4.2 Other modes of regulation

In addition to protein phosphorylation, many other factors influence BK channel activity such as Nitric oxide and glucocorticoid hormones.

**Nitric oxide (NO)**

The free radical gas NO is now recognized as a major messenger molecule that has diverse functions throughout the body (Hobbs and Ignarro., 1996; Furchgott and Zawadzki., 1980). Activation of cellular NO synthase generates production of endothelium-derived relaxing factor (Hofmann et al., 2000). This diffusible gas activates soluble guanylate cyclase (GC) inducing cGMP production and subsequent activation of PKG and regulates BK channels through phosphorylation (Hofmann et al., 2000). Inhibition of NO synthase reduces the BK channel open probability (Po) in rat smooth muscle cells (Ye et al., 2000).
Glucocorticoid hormones

Increasing evidence suggests that glucocorticoids, via induction of de novo protein synthesis, can rapidly modulate multiple protein phosphorylation/ dephosphorylation cascades. For example, in hepatocytes glucocorticoids rapidly induce the p21 cyclin-dependent kinase (CDK) inhibitor that blocks CDK2 phosphorylation pathways (Cha et al., 1998); in rat mammary epithelial cells, glucocorticoids upregulate the inducible serine/threonine kinase SGK-1 (Webster et al., 1993a) and glucocorticoids block PKC signaling in osteoblasts via a putative tyrosine phosphatase pathway (Hulley et al., 1998).

In neuroendocrine anterior pituitary corticotroph AtT20 cells, proteins rapidly (within 2h) induced by glucocorticoids block PKA-mediated inhibition of BK channel activity (Tian et al., 1998; Shipston et al., 1996). This action of the steroid was abolished by blockers of protein phosphatase 1/2A and mimicked by the catalytic subunit of PP2A (figure 1.8) (Tian et al., 2001b; Tian et al., 1998).

Characterisation of the expression of BK channel subunits in AtT20 cells showed no evidence for β-subunits and the predominance of the STREX splice variant α-subunit (Shipston et al., 1999). Importantly, when the STREX variant α-subunit was transfected into HEK 293 cells, the regulation by glucocorticoids was identical to that seen in AtT20 cells (Tian et al., 2001b; Tian et al., 1998; Shipston et al., 1996). This indicates that the same pool of genes is induced by glucocorticoids in mouse corticotroph tumour cells as well as HEK 293 cells. In addition, as mentioned above, increasing evidence suggests that the activity of BK channels is dynamically regulated by the interaction of protein kinases and phosphatases intimately associated with the channel complex (Reinhart
Figure 1.8
Control of STREX BK channel splice variant by CRF-41 and glucocorticoid induced protein(s)

In AtT20 corticotroph cells, CRF-41 binds to its receptor and activates Gs protein. Gs activates adenylyl cyclase (AC) which results in enhanced production of cAMP which activates PKA. PKA phosphorylation inhibits the STREX variant BK channel. Synthetic glucocorticoid hormone, dexamethasone, binds to intracellular type II glucocorticoid receptor that regulates gene transcription from target gene and induces glucocorticoid-induced protein(s) (GIPs). The blockade of PKA-mediated inhibition of BK channel activity by GIPs presumably is a result of modified PP2A-like activity closely associated with the BK channel complex.
and Levitan., 1995; Levitan., 1994; White et al., 1993; White et al., 1991). As these phenomena are apparent in isolated patches of membrane, it is reasonable to hypothesize that they involve protein-protein interactions within tightly coupled signaling complexes including BK channel subunits. Identification of the BK channel complex as a target for glucocorticoid action should allow us to characterize glucocorticoid-induced proteins involved in ion channel regulation and provide further insights into the mechanism and role of rapid glucocorticoid regulation of excitability in neuroendocrine and neuronal cells.

Thus taken together, we used HEK 293 cells as a model for a generic action of glucocorticoids involving protein phosphatase(s), which can influence the activity BK channels.

1.4 Glucocorticoid-inducible proteins

The rapid induction of serum and glucocorticoid-inducible kinase-1, SGK-1 (Chen et al., 1999b; Webster et al., 1993a) and glucocorticoid-inducible Ras protein1, Dexras1 (Kemppainen and Behrend, 1998) in several system raises the possibility that they may be glucocorticoid-induced proteins in HEK 293 cells and involved in regulation of BK channels.

1.4.1 Dexras1

Dexras1 was identified as a dexamethasone-inducible member of the Ras superfamily of G proteins in AtT20 mouse corticotroph cells (Kemppainen and Behrend., 1998). Treatment of AtT-20 cells with dexamethasone for 30 min resulted in increased mRNA
for Dexras1; the highest expression level appeared after 2 h of treatment (Kemppainen and Behrend., 1998). Dexras1 mRNA is expressed in a broad tissue distribution including brain, anterior pituitary, heart, kidney, and liver (Brogan et al., 2001; Tu and Wu., 1999; Kemppainen and Behrend., 1998).

Dexras1 shares structural similarity with the Ras family of small molecular weight GTPases (Graham et al., 2001). It has molecular masses ranging from 30.2 to 33.4 kDa, which is significantly larger than other Ras family members that are 20 to 24 kDa. The increased molecular mass can be accounted for by the presence of an extended carboxyl terminus variable region (Kemppainen and Behrend., 1998; Malumbres and Pellicer., 1998; Bourne et al., 1991).

Orthologues of mouse Dexras1 have been reported, in human (GenBank Accession No. AF069506; (Cismowski et al., 1999) and rat (GenBank Accession No. AF239157), and share 98% homology with mouse Dexras1. Human Dexras1 is located on chromosome 2q32. The most closely related paralogue of Dexras1 is human Dexras2 and rat Dexras2 (GenBank Accession Nos. HS569D19 and AF134409) which share approximately 80% homology with human and mouse Dexras1. Human Dexras2 is located at chromosome 22q13.1, and was identified as tumor endothelial marker-2, a potential regulator of tumor angiogenesis and revascularization (St Croix et al., 2000). Rat Dexras2 is also known as the ras homolog enriched in striatum due to a particularly high level of expression in that tissue (Graham et al., 2001; Falk et al., 1999).
The Dexras1 protein is activated by S-nitrosylation induced by neuronal nitric oxide (NO) synthase in brain (Fang et al., 2000). It has been shown that Dexras1 activation by neuronal NO synthase is enhanced in the presence of CAPON, neuronal NO synthase adaptor protein, (Fang et al., 2000). As mentioned in section 1.3.4.2, NO activates soluble guanylate cyclase (GC) induction cGMP production and subsequent activation of PKG regulates BK channels by phosphorylation (Hofmann et al., 2000). Inhibition of NO synthase reduces the BK channels open probability (P_o) in rat smooth muscle cells (Ye et al., 2000) and it has been suggested that NO modulates BK channels via direct chemical modification of the α-subunit of the channel or an associated protein (Ahern et al., 1999). These findings raise the possibility that the regulation of BK channel by NO involves Dexras1.

Human Dexras1 has been also identified as a receptor-independent activator of the Gi/Go family heterotrimeric G-proteins (hence its alternative name is activator of G-protein signaling-1, AGS-1) (Cismowski et al., 2000; Cismowski et al., 1999). Expression of AGS-1 in yeast and in mammalian cells results in the activation of Gi/Go. In addition, the in vivo and in vitro properties of AGS-1 are consistent with it functioning as a direct guanine nucleotide exchange factor (GEF) for Gi/Go (Graham et al., 2004; Cismowski et al., 2000).

The induction of Dexras1 by glucocorticoids suggests that Dexras1 may represent a nexus between Gi- and glucocorticoid-dependent signaling pathways.

It has been shown that Dexras1 inhibits adenylyl cyclase activity through ligand/receptor independent activation of Gi (Graham et al., 2004) and it also inhibits
cAMP-dependent secretion in AtT-20 corticotroph cells (Graham et al., 2001). Moreover, both glucocorticoids (Shipston et al., 1999; Shipston et al., 1996) and agonists of Gi-coupled receptors, such as somatostatin (Reisine et al., 1988; Luini et al., 1986) inhibit stimulus-coupled ACTH secretion. Somatostatin appears to inhibit secretagogue-stimulated release of ACTH by multiple mechanisms including inhibition of adenyl cyclase and voltage-dependent calcium current (Luini et al., 1986; Luini et al., 1985; Reisine et al., 1985), activation of a K+ channel (Pennefather et al., 1988), and direct blockage of secretion via a G protein (Luini and De Matteis, 1990).

It also known that BK channels are important targets of glucocorticoid-mediated inhibition of CRF-induced ACTH secretion in AtT20 cells (Shipston et al., 1996). Taken together, it is justified to hypothesize that Dexras1 could mediate the glucocorticoid-dependent inhibition of ACTH secretion (a model for glucocorticoid action) via BK channels.

1.4.2 SGK-1

Activation of the glucocorticoid receptor by ligand binding directly regulates the transcription of several mediators, including SGK-1 (Mikosz et al., 2001; Webster et al., 1993a). SGK-1 contains a catalytic domain that is approximately 45-55% homologous to the catalytic domains of several well characterized serine/threonine protein kinases that are constitutively expressed such as protein kinase B (Akt/PKB), PKA, PKC and the rat ribosomal S6 protein kinase (S6K) (Webster et al., 1993a; Webster et al., 1993b).
It has been reported that SGK-1 mRNA expression is increased by glucocorticoid hormones in various cells including rat mammary epithelial tumor cells (Webster et al., 1993a) and A6 cells (Chen et al., 1999b). In addition to glucocorticoids, SGK-1 transcript levels have been shown to be stimulated in a tissue-specific manner by serum (Webster et al., 1993a), mineralocorticoids (Pearce, 2001; Wang et al., 2001; Chen et al., 1999b), osmotic changes (Waldegger et al., 2000; Waldegger et al., 1997), cytokines (Cooper et al., 2001; Cowling and Birnboim, 2000), ischemic injury of the brain (Imaizumi et al., 1994), changes in cell volume (Warntges et al., 2002; Klingel et al., 2000), chronic viral hepatitis (Warntges et al., 2002) and insulin and IGF-1, insulin-like growth factor 1 (Perrotti et al., 2001; Kobayashi et al., 1999; Park et al., 1999). Furthermore, SGK-1 can be phosphorylated and activated by phosphatidylinositol 3 (PI3)-kinase (Gamper et al., 2002b; Park et al., 1999; Kobayashi et al., 1999).

Function of SGK-1

It has been proposed that SGK-1 participates in the regulation of epithelial transport (Wagner et al., 2001, Lang et al., 2000) cell volume (Bohmer et al., 2000), cell proliferation (Gamper et al., 2002a) and neuronal excitability (Warntges et al., 2002). SGK-1 has been linked to the function of ion channels, such as epithelial Na⁺ channel (Wagner et al., 2001) and voltage-gated potassium channels involved in neuronal excitability (Warntges et al., 2002). It has been demonstrated that SGK-1 is a physiological mediator of aldosterone action in kidney cells (McCormick et al., 2005; Volk et al., 2001; Wang et al., 2001; Shigaev et al., 2000; Chen et al., 1999b; Naray-
Fejes-Toth et al., 1999). In cells of the ascending limb of Henle, SGK-1 phosphorylates the ubiquitin ligase Nedd4-2, which causes a significant reduction in the interaction of this ligase with the epithelial sodium channel, resulting in the net mineralocorticoid-dependent elevation in epithelial sodium channel levels in the membrane of renal cells and increasing the epithelial Na⁺ absorption (Zhou and Snyder., 2005; Snyder et al., 2002; Kamynina and Staub., 2002).

Several observations point to a physiological role of SGK-1 in the regulation of potassium channels. Electrophysiological studies in HEK 293 cells (Gamper et al., 2002a) and in Xenopus oocytes (Henke et al., 2004) showed that transfected SGK-1 stimulates voltage-gated K⁺ channels. In a patch-clamp study, insulin-like growth factor-1 up-regulates outwardly rectifying whole-cell K⁺ current and this current is inhibited by potassium channel blocker (TEA), Kᵥ1 channel blocker (margatoxin) and inhibitors of PI3-kinase (Wortmannin and LY294002). Moreover, this whole-cell K⁺ current is mimicked by overexpression of human SGK-1 (Gamper et al., 2002b). SGK-1 also up-regulates the activity of ROMK, renal outer medullary potassium, channels (Yoo et al., 2003; Yun et al., 2002).

Therefore, the rapid induction of SGK-1 mRNA in response to dexamethasone in several cells and regulation of potassium channel by SGK-1 together raise the possibility that SGK-1 may be a glucocorticoid-inducible protein in HEK 293 cells and could contribute to the regulation of BK channels.
Aims of this thesis

Adrenal glucocorticoids are pivotal for adaptation to environmental challenge in a wide range of tissues including the central nervous and endocrine systems. In neurons and endocrine cells, potassium channels are important targets for glucocorticoid action. Proteins rapidly (within 2h) induced by glucocorticoids regulate the activity of BK channels in pituitary corticotroph tumour AtT20 cells, but the mechanisms of this regulation are largely not understood. The identification of the glucocorticoid signaling pathway to BK channels should provide novel and valuable information as to the actions of glucocorticoid hormones on ion channels.

The present study aims to investigate the molecular mechanism of glucocorticoid action in HEK 293 cells. Chapter 3 investigates whether Dexras1 and/or SGK-1 are glucocorticoid-induced protein(s) in HEK 293 cells and associate with BK channels. In chapter 4, the assembly of the BK channel complex is investigated, with focus on the association of protein phosphatases with the channel α-subunit.
Chapter Two

Materials and Methods
Section 2: Materials and Methods

2.1 Molecular biology solutions and reagents

General molecular biology reagents used throughout this study were obtained from Sigma Chemical Company, except where stated otherwise.

For autoclaving, solutions were autoclaved at 121°C for 20 min by ASTELL, model Number: ASA 240.

2.1.1 depc-treated distilled water (depc-dH₂O)

0.05% diethylpyrocarbonate (v/v; depc) in distilled water (dH₂O)

2.1.1.2 Luria Bertani (LB) - broth

20 g of LB broth (w/v, Sigma, L-3022) dissolved in dH₂O to reach 1 litre final volume with stirring and sterilized by autoclaving.

2.1.1.3 Luria Bertani (LB) - Agar

35 g of LB Agar (w/v, Sigma, L-2897) dissolved in dH₂O to reach 1 litre final volume with stirring and sterilized by autoclaving.

2.1.1.4 Isopropyl-beta-D-Thiogalactopyranoside (IPTG)

1 M IPTG (Calbiochem) prepared by dissolving 2.383 g in 10 ml of dH₂O and stored at -20°C.
2.1.1.5 DNA marker

0.5 µg of 100 bp DNA marker (Invitrogen 15628-019 or Biolab 3231S) or 1 Kb DNA marker (Biolab N3232S).

2.1.1.6 Tris, Boric acid, EDTA (TBE) buffer

10x TBE buffer: 0.44 mM Tris, 0.49 mM Boric acid, 16 mM EDTA (Ethylene Diamine Tetra Acetic acid, Gibco) in dH₂O. Diluted as required.

2.1.1.7 DNA loading buffer

6x stocks prepared with 720 µl 50x TBE buffer (section 2.1.1.6), 330 µl glycerol and bromophenol blue, diluted to 1x with dH₂O and DNA for use.

2.1.1.8 RNA marker

4 µl of RNA marker (Promega, G319A) was used in agarose-formaldehyde gel.

2.1.1.9 RNA gel buffer (MOPS/EDTA buffer)

10x MOPS/EDTA: 0.2 M 4-morpho- linepropanesulfonic acid (MOPS), 50 mM sodium acetate (NaAc), 10 mM EDTA. Adjusted pH to 7.0 with 10 M NaOH and autoclaved. Diluted as required.

2.1.1.10 Sodium Saline Citrate (SSC)

20x SSC: 3M NaCl and 0.3 M sodium citrate in dH₂O. Adjusted pH to 7.0 with 1 M HCL and autoclaved. Diluted as required.
2.1.1.11 Sodium Chloride-Sodium Phosphate-EDTA (SSPE)

150 mM NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA in dH₂O. Adjusted pH to 7.4 with 10M NaOH and autoclaved.

2.1.1.12 Northern blot hybridization buffer

50% v/v (50 ml) deionised formamide (Sigma F9037) + 5x SSPE (25 ml of 20x SSPE, section 2.1.1.11) + 0.1% w/v SDS (1 ml of 10% SDS, Sodium Dodecyl Sulfate) + 0.2 mg/ml carrier DNA (2 ml of DNA 10mg/ml) + 1 mM EDTA (400 μl of EDTA 0.25 M) + 10% w/v Sodium Dextran Sulfate (10 g) + 5x Denhardt’s (10 ml of 50x Denhardt’s: 5 g Ficoll + 5 g Polyvinylpyrrolidone (PVP) and 5% w/v BSA fractionV dissolved in 50 ml dH₂O and filtered through 22 μm sterile filter).

2.1.1.13 Protein marker

4 μl of perfect protein marker 10-225 kDa (Bioscience, 69079-3).

2.1.1.14 Phosphate Buffered Saline (PBS)

137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, adjusted to pH 7.4.

2.1.1.15 Phosphate Buffered Saline + 0.1% Tween-20 (PBS-T buffer)

PBS (section 2.1.1.14) supplemented with 0.1% (v/v) Tween-20.

2.1.1.16 Western blotting buffer

25 mM Tris, 192 mM glycine, 20% v/v Methanol. Adjusted pH to 8.3.
2.1.1.17 PSD (Phosphate SDS-DTT)

Total concentration in samples: 50 mM Phosphate, 1% w/v SDS, and 91 mM Dithiothreitol (DTT).

2.1.1.18 1% Triton X-100 lysis buffer

150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100

2.1.1.19 4% Paraformaldehyde- 0.1 M sodium phosphate buffer

For making 8% w/v paraformaldehyde, 8 g paraformaldehyde powder was dissolved in dH₂O to reach 99 ml and added 1 ml of 1 M NaOH to total volume 100 ml. Then the solution was stirred on a hot plate, less than 65°C, until the solution was virtually clear. Finally 50 ml of above solution was mixed with 20 ml of 0.5 M sodium-phosphate, pH 7.3 and 30 ml dH₂O.

The solution was filtered over Whatman No. 1 filter paper and kept in dark bottle at 4°C (maximum 1 week).

2.1.1.20 Ampicillin (Amp)

A stock solution of Amp 100 mg/ml in dH₂O was diluted to 100 μg/ml in LB broth (section 2.1.1.2) or agar (section 2.1.1.3) for selection of bacteria expressing vector.

2.1.1.21 Geneticin (G 418)

A stock solution of 0.8 g/ml of G 418 (Calbiochem, 345810) in sterile Dulbecco's Modified Eagle's Medium (DMEM, Gibco) was diluted to 100 ml in DMEM +10% v/v
FCS to reach total concentration 0.8 mg/ml. This solution was used to culture the HEK 293 cells expressed with STREX-HA or ZERO-HA BK channel α-subunits.

2.2 General molecular biology protocols

2.2.1 General DNA analysis protocols

2.2.1.2 Agarose gel electrophoresis

1% agarose gels were prepared by dissolving 0.25 g agarose (electrophoresis grade, w/v; Invitrogen) in 25 ml depc-dH₂O. Then 25 μl of 0.5 mg/ml ethidium bromide (dissolved in 1x TBE buffer) was added to 1% agarose gels. Gels were run on a Pharmacia gel electrophoresis apparatus (GNA-100) in 1x TBE buffer (2.1.1.6). Samples were separated with 40 mA current for 25 min.

2.2.1.3 Purification of DNA from agarose gels

DNA bands cut from agarose gels were purified using the QIAGEN QIAEX II purification kit as per manufacturer’s instructions. Briefly, the DNA band was cut from agarose gel and dissolved in QX1 buffer (3 volumes of buffer to 1 volume of gel) with 10 μl of QIAEX II. Then the mixture was heated at 50°C for 10 min to solubilise the agarose and bind DNA to QIAEXII resin. The solution was centrifuged for 30 sec. The pellet was washed with QX1 buffer (for removing residual agarose) and then with PE buffer (for removing salt contaminates). The pellet was air-dried for 10-15 min at room temperature and then resuspended with 20 μl depc-dH₂O (section 2.1.1) and incubating at room temperature for 5 min. The reaction was pelleted by centrifugation (Eppendorf centrifuge 5415 C) at
10,000 g for 30 sec and supernatant (contains purified DNA) was stored at -20°C until use.

2.2.1.4 Sub-cloning of DNA into PGEM-T Easy vector

The PCR product was ligated into PGEM-T Easy vector following the manufacturer's instructions (Promega, A1360). The ligations were transformed into 100 µL of XL-1 blue E. coli competent cells by heating at 42°C for 45 sec. The ligations were recovered on ice for 5 min. The samples were diluted with 0.9 ml LB broth (section 2.1.1.2) and incubated by shaking (150 rpm) at 37°C for 1.5 h. 100 µl of each transformation culture was plated in a LB/ampicillin/IPTG/X-Gal plates. The plates were incubated for 16 h at 37°C. Assessment of ligation transformation was determined by selection of white colonies and plating onto another LB/ampicillin/IPTG/X-Gal plates. The plates were incubated at 37°C for 16 h. Then white colonies were selected for purification by alkaline lysis (2.2.1.6).

2.2.1.5 Restriction digestion of DNA

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called restriction endonucleases. These special enzymes recognize specific sequences in the DNA molecule and cut the DNA to produce restriction fragments. For restriction digestion of DNA, ~1 µg of DNA was combined with endonuclease(s) in appropriate buffer (Promega). The mixture was incubated at an appropriate temperature and time for optimal endonuclease activity as recommended by the enzyme manufacturers. Samples were analysed by agarose gel electrophoresis.
2.2.1.6 Alkaline lysis: Plasmid DNA purification

**Mini-preparation**

Plasmid DNA was transferred in XL-1 blue E. coli competent cells in appropriate medium/ampicillin (100 µg/ml) at 37°C for 16 h. The solution was briefly spun and cellular pellet of bacterial was resuspended in 100 µl of resuspension solution (25 mM Tris/HCl pH 8.0, 10 mM EDTA, 50 mM Glucose and 20 mg Lysozyme) and incubated for 5 min at RT. Gently, 200 µl of lysis solution (0.2 M NaOH plus 1% w/v SDS) was added and incubated for 5 min at room temperature to lyse the bacterial cells. Then, 150 µl of neutralization solution (3M sodium acetate, pH 5.2) was added gently and left on ice for 5 min to precipitate the plasmid DNA. The insoluble fraction, pellet, was removed by centrifugation at 10,000g for 5 min. 800 µl of 100% ethanol (v/v) was added to supernatant, incubated on ice for 15 min and spun 5 min. The pellet was resuspended in 200 µl dH₂O and following phenol/chloroform (1/1) for separating aqueous plasmid DNA at room temperature for 5 min. The mixture was spun for 5 min and the supernatant retained. The phenol was removed by adding 200 µl chloroform and the supernatant was kept after spinning for 5 min. 20 µl of sodium acetate (3M) plus 500 µl of 100% ethanol (v/v) were added to the supernatant and precipitated at 4°C overnight. Following centrifugation (30 min at 10,000 g), the supernatant was aspirated and 70% ethanol (v/v) was added to the pellet for removing residual salt. The DNA was pelleted and dried at room temperature with 20 to 50 µl of dH₂O after centrifugation.
**Midi- and Maxi-preparation**

Midi- (<200 µg) or maxi- (>200 µg) preparation of plasmid DNA were performed using the QIAGEN Midi and Maxi plasmid preparation kit, following the manufacturer's instructions (QIAGEN).

2.2.1.7 DNA quantification and determination of concentration

Measurement of the optical density of a DNA solution at 260 nm, O.D$_{260}$, allows the nucleic acid concentration to be determined. The ratio of O.D$_{260}$/O.D$_{280}$ was used as a measure of protein and phenol contamination with a value less than 1.5 indicative of high contamination.

The total DNA was estimated from the absorbance of samples at 260 nm according to the following formula:

\[
[\text{DNA}] = A_{260} \times \text{dilution} \times 50 \mu\text{g/\text{ml}} \quad (1 \text{ A}_260 \text{ O.D. Unit for DNA } = 50 \mu\text{g/\text{ml}})
\]

2.2.2 General RNA analysis protocols

2.2.2.1 Total RNA extraction

Cells (~10$^7$) were washed 3 times with cold PBS (section 2.1.1.14) and cells removed from the flask using Hanks' Buffered Salt Solution (HBSS, GIBCO, 2007-3) + 0.1% EDTA. Cells were pelleted at 200 g for 10 min. The cells were incubated with 1 ml TRIzol ® Reagent (phenol/guanidine isothiocyanate, Invitrogen, 15596-026). The cells were incubated at room temperature for 5 min in order to lyse the cells and dissolve the DNA in phenol. 0.2 ml of chloroform + isoamylalcohol (24:1) was added and gently shaken for 15 sec and left at room temperature for 3 min. After spinning at 10,000 g for
15 min at 4°C, the supernatant (total RNA) was separated from DNA, proteins and lipids. The RNA was precipitated with 0.5 ml isopropanol and incubated at room temperature for 10 min. The pellet (RNA) was pelleted by centrifuging at 10,000 g for 10 min at 4°C. The pellet was washed with 75% ethanol by gently vortexing and spun at 10,000 g for 5 min. The pellet (RNA) was air-dried at room temperature for 10 min. The RNA was resuspended in an appropriate volume of depc-dH₂O (section 2.1.1) and incubated at 60°C for 10 min. The total RNA was estimated by calculation of the absorbance of samples at 260 nm according to the following formula:

\[ [RNA] = A_{260} \times \text{dilution} \times 40 \mu g/ml \] (1 A₂₆₀ O.D. Unit for RNA = 40 μg/ml)

The pure RNA was considered using A₂₆₀/A₂₈₀ with the ratio 1.8 to 2.0.

To the remaining RNA, the RNA was mixed with 350 μl depc-dH₂O + 40μl 3M sodium acetate pH 5.2 and 1 ml absolute (100%) alcohol. The mixture was centrifuged at 10,000 g for 15 min and stored at -70°C.

2.2.2.2 RT-PCR

For Reverse Transcription-Polymerase Chain Reaction (RT-PCR), HEK 293 cells were seeded into 75-cm² flask and allowed to reach 80-90% confluence. RNA was isolated using TRIzol ® Reagent (section 2.2.2.1). Total RNA from HEK 293 cells was used as template Reverse Transcription with 1x buffer RT, 0.5 mM dNTP, 1 μM Oligo-dT primer, 10 units RNase inhibitor, and 4 units Omniscript Reverse Transcriptase using Omniscript Reverse Transcriptase kit (Qiagen, Cat. No. 205111) in a total volume of 20 μl for 60 min incubation at 37°C. 0.25 μg of RT product were used as template in PCR.
with 0.2 μM of each forward and reverse primers (see table 2.1), 200 μM dNTP, 2.5 mM MgCl₂, 1 unit Taq DNA polymerase and 1x pfu DNA polymerase buffer (200mM Tris-HCl, pH 8.8 at 25°C, 100mM KCl, 100mM (NH₄)₂SO₄, 20mM MgSO₄, 1.0% Triton X-100 and 1mg/ml nuclease-free BSA). The PCR of full-length human Dexras1 was carried out by forward primer A and reverse primer B which amplified for full-length (table 2.1). Otherwise, two fragments of Dexras1 (section 3.2.2) were carried out by forward primer A and reverse primer D or forward primer C and reverse primer B (table 2.1). PCR was carried out with 1 cycle for initial denaturation (95°C for 1 min), following 45 cycles for denaturation phase (95°C for 0.5 min), annealing phase (56°C for 0.5 min), extension phase (72°C for 1 min), and 1 cycle for final extension (72°C for 10 min). Products were loaded on 1% agarose gel electrophoresis (section 2.2.1.2). Gels were viewed in an ultraviolet viewing box.
**Table 2.1**
Characteristics of RT-PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A:</strong> Full-length of Dexras1, forward</td>
<td>5'-CACATGAAACTGGCCGC-3'</td>
</tr>
<tr>
<td><strong>B:</strong> Full-length of Dexras1, reverse</td>
<td>5'-CTAGCTGATGACGCAGCG-3'</td>
</tr>
<tr>
<td><strong>C:</strong> Fragment of Dexras1, forward</td>
<td>5'-CTGCGACGTGCTGCAC-3'</td>
</tr>
<tr>
<td><strong>D:</strong> Fragment of Dexras1, reverse</td>
<td>5'-CTAGCTGATGACGCAGCG-3'</td>
</tr>
</tbody>
</table>
2.2.2.3 Northern blot analysis

1% agarose gel was prepared by dissolving 0.75 g agarose (electrophoresis grade, w/v, Invitrogen) in 75 ml depc-dH₂O using microwave with swirling. The gel was allowed to cool and 10 ml RNA gel buffer (section 2.1.1.9) and 5 ml of 37% v/v formaldehyde (in fume cupboard) were added to the gel. RNA was extracted with TRIzol Reagent (Invitrogen) as described in section 2.2.2.1. 15.5 µl RNA sample buffer (0.2ml deionised formamide, 40 µl RNA gel buffer (section 2.1.1.9) and 70 µl formaldehyde) were added to 20 µg RNA (in 4.5 µl dH₂O). The RNA was denatured at 65°C for 15 min. The denatured-RNA was immediately put on ice and 2 µl RNA loading buffer (1 mM EDTA, 0.4% w/v Bromophenol blue, 0.4% Xylene cyanol, 50% Glycerol) plus 1 µl of 10 mg/ml ethidium bromide were added to denatured-RNA. The samples were loaded on the 1% agarose formaldehyde gels.

The gel was run in the RNA electrophoresis buffer (1x RNA gel buffer (section 2.1.1.9) in depc-dH₂O (section 2.1.1) for 2 h at voltage 100. After electrophoresis, the formaldehyde was removed by soaking the gel in the 20x SSC (section 2.1.1.10) for 60 min with gentle shaking at RT. Nylon membrane (Amersham Life Science, Hybond-N, 0.45 micron, RPN203N) was pre-wetted by floating on dH₂O for < 5 min and following by soaking in 20x SSC (section 2.1.1.10) for 5 min.

RNA was transferred from gel to membrane using capillary action in RNA electrophoresis buffer overnight at 4°C using 3 MM paper bridge and 20 alternate stacked blue towels (500g weight on top). The gel and nylon membrane were photographed after transferring.

RNA was fixed in the blot using heating the blot at 80°C for 3 h between 3 MM paper.
Generation of Dexras1, SGK-1 and cyclophilin A cDNA probes

**Dexras1**

A single RT-PCR expected band (207 bp, nucleotide 639-846, NCBI accession number AF172846) corresponding to human Dexras1 cDNA was amplified by PCR from HEK 293 cells RNA using the forward primer E (5'-CTGCGACGTGCTGCAC-3') and reverse primer B (5'-CTAGCTGATGACGCAGCG-3'). This fragment was used as hybridization probe in the mRNA blot for checking the levels of Dexras1 mRNA in HEK 293 cells (figure 2.1).

**SGK-1**

A 238 bp segment (nucleotide 108-346, NCBI accession number: CAA71138) of SGK-1 cDNA was amplified by RT-PCR from HEK 293 cells RNA using forward primer F (5'-GAACGACTTTATTCAGAAGATTGC-3') and reverse primer G (5'-TAGAAGAACCTTTCCAAAAACTGCC-3') (figure 2.1).

**Cyclophilin A**

A 170 bp segment (nucleotide 295-465) of cyclophilin A cDNA was amplified by RT-PCR from HEK 293 cells RNA using forward primer H (5'-CCATG GCAAATGCTGGAC-3') and reverse primer K (5'-CTTCTTGCTGGTCTTGCC-3') (figure 2.1).

**Labelling the probe (using Ready To-Go DNA labelling beads and α\(^{32}\)P, dCTP)**

The DNA probes were denatured by heating for 2 min at 95-100°C. Denatured-probes were immediately placed on ice for 2 min and then briefly centrifuged.
About 50 ng (in 45 μl dH₂O) of denatured-probe plus 5 μl of [α-32P] dCTP (Amersham, AA0005) were added to bead (Amersham Bioscience, 27-9240-01). The probes were incubated at 37°C for 15 min. The probes were purified using G-50 column (Amersham Biosciences, 27-5335-01) following the manufacturer's instructions.

Pre-hybridization and hybridization

Pre-hybridization
The transferred membranes were soaked in dH₂O for 3 min following 20x SSC (2.1.1.10) for 3 min. The membranes were incubated at 55°C with 100 ml hybridization buffer (section 2.1.1.12) for 1.5 h in a sealed plastic box.

Hybridization
Labelled probes were incubated at 60°C for 10 min. The denatured-probes were incubated with membranes in 25 ml of hybridization buffer (section 2.1.1.12) at 55°C for 16 h. The membranes were rinsed twice with 30 ml of 2x SSC + 0.1% w/v SDS at room temperature and 15 min in 100 ml of 2x SSC + 0.1% SDS with gentle agitation. Then the membranes were washed twice with 100 ml of 0.1x SSC + 0.1% SDS for 15 min each time.

Exposure of the membrane with multi sensitive film
Autoradiograph was prepared by exposing the membranes with multi sensitive film (Cyclone storage phosphor screen, Packard) for 0.5-1 days. All bands on each image (membrane) were outlined and the optical density (O.D) in each band was determined by OptiQuant image analysis software.
2.3 Cell culture

HEK 293 cells stably expressing STREX-HA and ZERO-HA variants BK channel α-subunits were made by Tian et al (Tian et al., 2001b). Briefly, cDNA encoding STREX and ZERO mouse BK channel variant α-subunit with an epitope HA (Hemagglutinin) fusion partner at the C-terminus were sub-cloned into the mammalian expression vector pcDNA3 and transfected into HEK 293 cells using lipofectamine 2000 reagent following the manufacturer’s instructions (Invitrogen, 11668-019).

The pcDNA3 vector confers Geneticin-resistance to cells that express it (Shipston et al., 1999; Clark et al., 1999). Therefore, HEK 293 cells transfected with STREX-HA and ZERO-HA BK channel-pcDNA3 vectors were cultured in DMEM containing 10% Foetal Calf Serum (v/v, FCS, Harlan) and 0.8 mg/ml Geneticin (antibiotic G 418 Sulfate, section 2.1.1.21) in a fully-humidified incubator containing 5% CO$_2$ at 37°C. Wild type HEK 293 cells and AtT20 cells were grown in 75-cm$^2$ flasks containing 10 ml of DMEM supplemented with 10% v/v FCS in a fully-humidified incubator containing 5% CO$_2$ at 37°C. Cells were routinely passaged every 5-7 days (passage 9-30) using Hanks’ Buffered Salt Solution containing 0.1% EDTA.

2.3.1 Transient transfection

Wild type HEK 293 cells were cultured in a new flask to reach 40-60% confluence after 24 h in DMEM (Gibco) containing 10% FCS (Harlan). Cells were transfected with 1 μg/ml of the plasmid using Lipofectamine 2000 reagent (Invitrogen, 11668-019) in DMEM for 5 h following the manufacturer’s instruction. The solution was replaced with
DMEM supplemented with 10% FCS in a fully-humidified incubator containing 5% CO₂ at 37°C for 48 h.

2.3.2 Treating the cells with dexamethasone

The cells were maintained in DMEM supplemented with 1x ITS (insulin transferrin selenium, Sigma, 13146) for 24 h in a humidified incubator containing 5% CO₂ at 37°C (Leckie et al., 1995). The cells were pretreated with DMEM supplemented with 25 mM HEPES pH 7.4 and 0.25% w/v bovine serum albumin (BSA) for 2 h at 37°C. Then the cells were treated with dexamethasone or vehicle, equal volume (0.001%) of Dimethyl Sulfoxide (DMSO) in DMEM supplemented with 25 mM HEPES pH 7.4 and 0.25% w/v at 37°C.

2.4 Protein analysis

2.4.1 Cell lysate preparation with Triton X-100 lysis buffer

Cells in 75 cm² flasks at 80-90% confluence were quickly washed 3 times with cold PBS (section 2.1.1.14) and subsequent scraped with 0.5 ml 1% Triton X-100 lysis buffer (section 2.1.1.18) supplemented with 1/100 of protease inhibitor cocktail (Sigma, P 8340). The cells were sonicated on ice with five sonication cycles, each consisting of 10 sec pulses at 10 sec intervals with an ultrasonic Cole-Parmer sonicator (TORBEO, 36800) at setting 3. Cell lysate (Triton X-100 soluble fraction) was prepared by removing the supernatant after spinning at 10,000 g for 15 min at 4°C.
2.4.2 Cell lysate preparation with Nonidet P-40 (NP-40) lysis buffer

The cells in 75 cm² flasks at 80-90% confluence were quickly washed 3 times with cold PBS (section 2.1.1.14) and scraped with 0.5 ml 1% NP-40 lysis buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT and 1% NP-40) supplemented with 1/100 of protease inhibitor cocktail. The cells were sonicated on ice with five sonication cycles, each consisting of 10 sec pulses at 10 sec intervals with an ultrasonic Cole-Parmer sonicator at setting 3. Cell lysate (NP-40 soluble fraction) was prepared by removing the supernatant after spinning at 10,000 g for 15 min at 4°C.

2.4.3 Determination of protein concentration

**Bradford assay**

Analysis of protein concentration was determined by a Bradford Coomassie protein assay kit (PIERCE) following the manufacturer’s instruction. Briefly, BSA with concentrations of 9 to 576 μg/ml was used to generate standard curve. Proteins were diluted 30 times and 10 μl of diluted samples was pipetted into the appropriate microplate wells. 290 μl of Coomassie reagent was added to each well and mixed with a pipette. The plate was left for 10 min at room temperature and the absorbance was measured at 595 nm with a spectrophotometer reader (DYNATECH, MR 7000).

**Bio-Rad DC protein Assay**

The protein concentration of samples contained 1% Triton-X100, 1% NP-40, 1% SDS or 1mM DTT were performed using a Bio-Rad DC protein assay as recommended in the
instruction manual (Catalog Number 500-0116). The standard, BSA, was prepared in the same buffer as the samples.

2.4.4 Preparing the Triton X-100 insoluble and soluble fractions after 20,000 g

After lysing the cells with Triton X-100 lysis buffer (section 2.4.1), the cell lysate was centrifuged at 1000g for 5 min at 4°C. The resultant supernatant was pelleted with ultracentrifuge (BECKMAN, model TL-100) at 20,000 g for 30 min at 4°C to separate insoluble (pellet, P20K) and soluble (supernatant, S20K) fractions.

2.4.5 Immunoprecipitation

2.5 mg of 1% Triton X-100 cell lysates (in 500 µl) was pre-cleared by incubation with 10 µl of 50% PGS (protein-G-sepharose (Sigma p3296) supplemented with 1% Triton X-100 (section 2.1.1.18) and agitated for 1 h at 4°C. Beads were spun at 10,000 g for 1 min at 4°C and the pre-cleared cell lysates used for immunoprecipitation.

2.4.5.1 Immunoprecipitation of BK channel α-subunit using sheep antisera anti-STREX + anti-S869

5 µl of antisera raised in sheep against BK channels (5 µl of anti-STREX serum against amino acids 628-641 and 5 µl of anti-S869 serum against amino acids 919-933 of the mouse STREX channel α-subunits) was added to the pre-cleared cell lysates and incubated overnight at 4°C. 16.6 µl of 50% PGS was added and incubated for 2 h at 4°C. The immunoprecipitates (IPs) were pelleted at 10,000 g for 1 min at 4°C.
2.4.5.2 Immunoprecipitation of PP2A-A and PP1

5 μl of rabbit anti-PP2A-A antibody (Upstate, 07-250) or rabbit anti-PP1 antibody (Upstate, 06-221) was added to the pre-cleared cell lysates and incubated overnight at 4°C and subsequent 16.6 μl of 50% PGS was added for 2 h at 4°C. The lysates were pelleted at 10,000 g for 1 min at 4°C.

2.4.5.3 Immunoprecipitation of I322-HA channel

5 μl of rabbit anti-HA antibody (Zymed, 71-5500) was added to the pre-cleared cell lysates and incubated overnight at 4°C and then 16.6 μl of 50% PGS was added for 2 h at 4°C. The lysates were pelleted at 10,000 g for 1 min at 4°C.

The pellets (IPs of BK channel α-subunits, PP2A-A, PP1 or I322-HA channel) were washed 3 times with 1% Triton X-100 lysis buffer (section 2.1.1.18) and once with dH₂O. The IPs were denatured at 100°C for 5 min in PSD (section 2.1.1.17). IPs were loaded onto a SDS-PAGE gel and separated using PhastSystem (next section 2.4.6).

2.4.6 Western blot analysis

Western blot analysis was performed using wet transfer of protein in acrylamide gels onto Immobilon™-P transfer membranes. Lysates or IPs were mixed with PSD (2.1.1.17) and denatured at 100°C for 5 min. Proteins with large molecular weight (>70 kDa) were analysed by a 7.5% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis homogeneous gel (SDS-PAGE, Amersham Biosciences). Small molecular weight proteins (<70 kDa) were separated by a 12.5% SDS-PAGE gel using
a Pharmacia Biotech PhastSystem apparatus (Pharmacia Biotech, Uppsala, Sweden). 4 μl of each extract with 1 μg/μl concentration was electrophoresed onto a SDS-PAGE homogeneous gel. The electrophoresis was started with current 10 mA and Volthours 1 before loading the proteins on a gel. Then, the current was decreased to 1 mA during loading the proteins on the gel. After loading, current was increased to 10 mA and Volthours to 75 in a 12.5% SDS-PAGE gel and 10 mA and 60 Volthours in a 7.5% gel.

**ECL detection**

Immobilon™-P Polyvinylidene Fluoride (PVDF) transfer membranes (MILLIPORE, IPVH 20200) were activated by methanol and soaked in Western blotting buffer for 15 min (section 2.1.1.16). The proteins were transferred from SDS-PAGE homogeneous gels to the methanol-activated membranes in PhastSystem microelectrophoresis apparatus (Amersham) with current 25 mA and Volthours 5. Equivalent protein transfer in the blots was determined using Coomassie blue staining (50% methanol, 0.05% Coomassie blue R-250) for 5 min at RT. Then the blots were de-stained with 100% methanol. The methanol was removed by washing the blots for 5 times for 5 min each time with dH₂O. The transferred blots were blocked in ECL blocking buffer (PBS-T buffer, section 2.1.1.15, + 5% (w/v) low fat milk, marvel) for 1 h at RT. Then blots were incubated overnight in appropriate primary antibody (table 2.2) in ECL blocking buffer. Blots were washed 4 times for 4 min each time with PBS-T buffer (section 2.1.1.15) and incubated
Table 2.2
Characteristics of Western blotting primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Epitope sequence</th>
<th>Dilution for WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PP2A-A (purified IgG)</td>
<td>Rabbit</td>
<td>Upstate</td>
<td>C-MAAADGDDSLY</td>
<td>1/500</td>
</tr>
<tr>
<td>Anti-PP2A-C (purified IgG)</td>
<td>Mouse</td>
<td>Upstate</td>
<td>C-RGEPHVTRRTPD YFL</td>
<td>1/500</td>
</tr>
<tr>
<td>Anti-PP1 (purified IgG)</td>
<td>Rabbit</td>
<td>Upstate</td>
<td>GRPITPPRNSAKAKKK</td>
<td>1/500</td>
</tr>
<tr>
<td>Anti-HA (affinity purified from rabbit antiserum)</td>
<td>Rabbit</td>
<td>Zymed</td>
<td>YPYDVPDYA</td>
<td>1/1000</td>
</tr>
<tr>
<td>Anti-BK channel α-subunit (affinity purified IgG)</td>
<td>Rabbit</td>
<td>Chemicon</td>
<td>C-terminal (residues 1098-1196) of mouse α-subunit of BK channel</td>
<td>1/300</td>
</tr>
<tr>
<td>Anti-Cyclophilin A (antiserum)</td>
<td>Rabbit</td>
<td>Upstate</td>
<td>Recombinant full length protein corresponding to human Cyclophilin A</td>
<td>1/1000</td>
</tr>
<tr>
<td>Anti-SGK-1 (purified IgG)</td>
<td>Rabbit</td>
<td>Upstate</td>
<td>C-GKSPDSVLVTASVK</td>
<td>1/1000</td>
</tr>
</tbody>
</table>

**WB:** Western blotting  
**IgG:** antibody
for 1 h at room temperature with secondary antibody conjugated to horseradish peroxidase (HRP) (table 2.3) in PBS-T buffer (section 2.1.1.15). Following exposing the blots to secondary antibody, the blots were washed 4 times for 4 min each time with PBS-T buffer (2.1.1.15). Then, the blots were incubated with Amersham Pharmacia Biotech enhanced chemiluminuescence reagents (ECL, Amersham Biosciences, RPN 2209) according to the manufacturer’s protocol. ECL reactions were detected by high performance chemiluminuescence film (Hyper film ECL, Amersham Biosciences, RPN2103K). The blots were developed for 4 min in developer (P7042-1GA, Sigma Aldrich) and 1 minute in fixer (P7167-1GA, Sigma Aldrich). Bands were outlined and O.D of each band was measured by ImageJ software.

LI-COR detection

Western blotting analysis for two-colour detection was performed using the Odyssey Western blotting protocol (Bioscience, Doc# 988-07568) for detection of two different antigens simultaneously on the same blot using antibodies labelled which dyes specify (table 2.3) and visualized in different fluorescence channels (700 and 800 nm). Two different host species primary antibody were used in each blot. FL-PVDF (MILLIPORE) membranes were activated by methanol and rinsed 5 times with dH₂O for 5 min each time, following 3 times for 5 min each time with Western blotting buffer (section 2.1.1.16). The proteins were transferred from SDS-PAGE homogeneous gel to methanol-activated FL-PVDF membrane using current 25 mA and Volthours 5. Transferred membranes were washed 4 times for 4 min each time with dH₂O and blocked with 50% LI-COR blocking buffer (50% OBB: Odyssey Blocking Buffer, LI-COR
### Table 2.3
Characteristics of Western Blotting secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution for WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse-HRP antibody</td>
<td>Sheep</td>
<td>Amersham</td>
<td>1/3000</td>
</tr>
<tr>
<td>Anti-rabbit-HRP antibody</td>
<td>Donkey</td>
<td>Amersham</td>
<td>1/3000</td>
</tr>
<tr>
<td>Alexa680-conjugated anti-mouse antibody</td>
<td>Goat</td>
<td>Molecular probes</td>
<td>1/3000</td>
</tr>
<tr>
<td>IrDye800-conjugated anti-rabbit antibody</td>
<td>Goat</td>
<td>Rockland</td>
<td>1/3000</td>
</tr>
</tbody>
</table>

**WB** = Western blot
Biosciences, 927-40000, diluted 1:1 with PBS) at room temperature for 1 h. The blots were incubated with appropriate primary antibody for overnight at 4°C (table 2.2) in 50% OBB supplemented with 0.1% Tween-20. Following washing the blots 4 times for 4 min each time with dH2O. Secondary antibodies (table 2.3) were applied 1 h at room temperature in LI-COR blocking buffer supplemented with 0.1% Tween-20. The blots were washed 4 times for 4 min each time with PBS-T buffer (section 2.1.1.15) with gentle shaking. The membranes were rinsed with PBS (section 2.1.1.14) to remove residual Tween-20. The membranes were dried at 37°C for 10 min and scanned with an Odyssey Infrared Imager (LI-COR, model, 9120).

2.4.6.1 Immunoabsorption

13.5% rabbit anti-SGK-1 antibody (07-315, Upstate) was made in 5% marvel blocking buffer (PBS-T buffer, section 2.1.1.15, + 5% (w/v) low fat milk, marvel).

25 μg of SGK-1 peptide (12-482, Upstate) was dissolved in 25 μl of 50 mM HCL and added 345 μl of 13.5% SGK-1 antibody (immunoabsorbed antibody using ~10.5 M antigen) and incubated with rolling at 4°C for ON. For control 345 μl of 13.5% SGK-1 antibody was added to 25 μl of 50 mM HCL and rolled for over night (ON) at 4°C. The Mixtures were centrifuged at 10,000g for 15 min. The blots were incubated with the supernatants of antibody + antigen or control for ECL detection as described in section 2.4.6.
2.4.7 Western blot membrane stripping

To enable re-probing of Western blot membranes, antibodies were removed by incubating the blots at 50°C for 30 min in Western blot stripping buffer (2% w/v SDS; 100 mM Mercaptoethanol, 62.5 mM Tris, pH 6.7). Then the blots were washed 3 times 5 min each time with PBS-T buffer (section 2.1.1.15) to remove the stripping buffer. The blots were incubated with primary and secondary antibodies as described in ECL detection section 2.4.6.

2.4.8 Protein visualization in the gel

Proteins in SDS-PAGE gels were visualized using Sypro Ruby gel stain (Molecular Probes, S-12001) using the protocol recommended by the manufacturer for gels. Briefly, gels were incubated with undiluted Sypro Ruby gel stain for 3 h at RT. Gels were incubated for 30 min in 10% v/v methanol, 7% v/v acetic acid to reduce the background fluorescence and increase sensitivity. The gels were viewed and photographed with an ultraviolet viewing box.

2.4.9 Immunocytochemistry

Six coverslips of 10 mm (VWR international) were put in each well of 6-well plates. To sterilize glass coverslips, 2 ml of 70% ethanol was put in each well of 6-well plates to cover the coverslips. Ethanol was washed off 3 times with sterile dH₂O. 2.5 ml of sterile 10 μg/ml poly L lysine solution was applied to each well for 10 min. The wells were washed with sterile dH₂O and then washed with DMEM. The cells were removed with trypsin supplemented with 0.1% EDTA. The cells were split to 6-well plates containing
coverslips with DMEM + 10% FCS and left at humidified incubator to reach 50-60% confluence for 24 h.

The DMEM + 10% FCS were removed and cells rinsed once with PBS at 4°C. Pre-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (2.1.1.19) was applied to the cells and left on ice for 20 min. The paraformaldehyde was removed immediately and 100% methanol was added at 20°C for 2 min. Methanol was replaced with 50 mM ammonium chloride (NH₄Cl) in 0.1 M sodium phosphate buffer (2.1.1.14) for 15 min at RT. The solution was changed to PBS + 0.1% NaN₃ (sodium azide) and stored at 4°C. Each coverslip (contains fixed cells) was placed into each well of a 24 well-plate with 1 ml PBS. PBS was replaced with antibody dilution solution (2% goat serum + 0.1% BSA + 0.1% saponin + 0.1% Sodium azide, NaN₃, in PBS) and incubated at room temperature for 5 min. The cells were incubated with 1/500 rabbit anti-HA antibody (Zymed, 71-5500) in dilution solution for 2-3 h at room temperature with no shaking. The solution was removed and washed 2 times with PBS. The coverslips were incubated with 1/500 goat Alex Fluor ® 488 anti-rabbit antibody (Nanoprobes) at room temperature for 30 min. Coverslips were washed with PBS and drain and mounted on superfrost slides on a drop (3 µl) of pre-warmed Permafluor. The slides were kept in dark and stored at 4°C. The sections were viewed under a Zeiss LSM 510 confocal microscope.

2.4.10 Luciferase assay

3x10⁵ cells per well of a six-well plates multiplate were grown overnight in DMEM +10% FCS in a humidified atmosphere at 37°C under 5% CO₂ until reach 40 to 60% confluence. HEK 293 cells were transiently transfected with 1 µg/ml of mouse mammary
tumor virus long terminal repeat (MMTV-LTR) luciferase reporter construct (Leckie et al., 1995), using lipofectamine 2000 reagent (Invitrogen, 11668-019) in DMEM for 5 h following the manufacturer's instruction. Then solution was changed to DMEM supplemented with 10% FCS and left in a humidified atmosphere at 37°C under 5% CO2 for 48 h.

Cells were treated with varying concentrations and times with the synthetic glucocorticoid agonist dexamethasone or DMSO (0.001%) at 37°C as described in section 2.3.2 Cells were quickly washed 3 times with cold PBS and lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 1% Triton X-100, 10 % glycerol, 2 mM 1,2-diaminocyclohexane-N,N,N,N(,-tetra acetic acid and 2.5 mg/ml BSA) supplemented with 1/100 of protease inhibitor cocktail (Sigma, P 8340). The cells were homogenized and briefly centrifuged at 10,000 g. Duplicate (20 µl) aliquots of the resulting supernatants were assayed for luciferase activity using 100 µl of Promega luciferase assay reagent (Promega, E1483) and emitted light detected with a T20/20 luminometer (Turner Designs, Sunnyvale, CA). Light emission was averaged over 10 sec with a 2 sec delay. Data from various experiments were compared with percentage of maximum light production (100%) in transfected HEK cells treated with 100 nM dexamethasone for 2 h.

2.5 Statistics

Data are expressed as mean ± standard error of mean (S.E.M), n=number of independent experiments. Statistical analysis for each group was performed by one-way
ANOVA after log transformation to achieve homogeneity of variances. Dunnett’s one-tailed post hoc test was used at a 95% confidence interval.

2.6 Supplier details

Amersham Biosciences: Amersham Biosciences UK Limited, Pollards Wood, Nightingales Lane, Chalfont St. Giles Bucks, UK, HP8 4SP

MWG Biotech Company: Anzinger Strasse 7a, 85560 Ebersberg, Germany

Upstate Biotechnology: Gemini Crescent, Dundee Technology Park, Dundee, Scotland, DD2 1SW.

Sigma: Fancy Road, Poole Dorset, England, BH12 4QH.

Promega: Promega UK Ltd, Delta House, Chilworth Science Park, Southampton UK, SO16 7NS.

Molecular Probes Inc: Poortgebouw, Rijnsburgerweg 10, 2333 AA Leiden, Netherlands.

Calbiochem: CN BIOSCIENCES (UK) LTD, Boulevard Industrial Park, Padge Road, Beeston, Nottingham, UK, NG9 2JR

Invitrogen Ltd: 3 Fountain Drive, Inchinnan Business Park, Paisley, UK, PA4 9RF.

QIAGEN Ltd: Boundary Court, Gatwick road, Crawley, west Sussex, RH10 2AX.

Roche Diagnostic: Roche Molecular Biochemicals, D-68298 Mannheim, Germany.

Turner Designs, Inc.: 845 W. Maude Avenue, Sunnyvale, CA 94085.

Rockland Immunochemicals, Inc.: P.O. Box 326, Gilbertsville, PA 19525.

CHEMICON International, Inc.: 28820 Single Oak Drive, Temecula, CA 92590.

Harlan: Dodgeford Lane, Loughborough • Leicestershire, LE12 9TE, England.
Biolabs: New England Biolabs, 240 County Road, Ipswich, MA 01938-2723.

Zymed: Now Invitrogen Corporation.

Gibco: Now part of Invitrogen.

Pharmacia Biotech: S-751 82 Uppsala, Sweden.

Calbiochem: EMD Biosciences, 10394 Pacific Centre Ct, San Diego, CA 92121.

Nanoprobes: 95 Horse Block road, Yaphank, NY 11980-9710. USA.
Chapter Three

Glucocorticoid-inducible proteins,

Dexras1 and SGK-1
Chapter Three

3.1 Introduction

Based on evidence summarised in chapter 1 section 1.4, the rapid induction of Dexras1 mRNA (Kemppainen and Behrend., 1998) and SGK-1 mRNA (Chen et al., 1999b) by glucocorticoids raised the possibility that Dexras1 and SGK-1 may be glucocorticoid-induced proteins in HEK 293 cells. There is currently no published information about the induction of endogenous SGK-1 or Dexras1 in response to dexamethasone in HEK 293 cell. Thus, the aims of the present study were to determine:

i) Are Dexras1 and/or SGK-1 glucocorticoid-inducible protein(s) in HEK 293 cells?

ii) If Dexras1 and/or SGK-1 are glucocorticoid-inducible protein(s) in HEK 293 cells, do they associate with BK channels?

3.2 Results

The presence of functional glucocorticoid receptors in HEK 293 cells was assessed upon transfection with a luciferase gene regulated by the glucocorticoid-inducible hormone response element that is found in the MMTV-LTR (Leckie et al., 1995).

3.2.1 MMTV-LTR luciferase activity in HEK 293 cells

The transcriptional behavior of the MMTV-LTR promoter was measured during exposure to the synthetic glucocorticoid dexamethasone.
Time-course of glucocorticoid action in HEK 293 cells

To characterise the time-course of glucocorticoid action in HEK 293 cells, the cells transiently transfected with MMTV-LTR luciferase reporter were incubated with 100 nM dexamethasone for 15 to 180 min. HEK 293 cells treated with DMSO (0.001%) for 120 min were assayed as the control vehicle group. Luciferase activity at dexamethasone 120 min was calculated and the corresponding ratio for vehicle and other dexamethasone time points were expressed as percentage of this value. All dexamethasone treatment time-points were compared with the vehicle control group and a difference with a p value less than 0.05 was considered statistically significant.

Monitoring of dexamethasone-stimulated MMTV-LTR luciferase activity revealed a time-dependent increase of luciferase expression by 100 nM dexamethasone. Luciferase activity was significantly increased following 30 min dexamethasone exposure compared with the vehicle group (figure 3.1). Maximal luciferase activity was observed at 120 min of dexamethasone administration (up to five-fold) and this activity was constant up to 180 min (Figure 3.1) (p<0.05, one-way ANOVA and Dunnett's test, results were typical of three similar experiments).

As a control for DMSO, HEK 293 cells transfected with MMTV-LTR luciferase reporter were treated with DMEM for 120 min. The results showed that DMSO (0.001%) had no significant effect on MMTV-regulated luciferase activity compared with DMEM (figure 3.1).
Figure 3.1
Time-course of the effect of dexamethasone on luciferase activity in HEK 293 cells

Figure 3.1: Time-course of the effect of dexamethasone on luciferase activity in HEK 293 cells

Human embryonic kidney 293 (HEK 293) cells transiently transfected with 1 µg of mouse mammary tumour virus long terminal (MMTV-LTR) luciferase reporter were treated with 100 nM dexamethasone for various times as indicated. The vehicle group received 0.001% of Dimethyl sulfoxide, DMSO, for 120 min. As a control group for vehicle, the cells were treated with Dulbecco's Modified Eagle's Medium (DMEM) for 120 min. 20 µl of whole cell lysate were assayed for luciferase activity as described under "Material and Methods" section 2.4.10. Data are expressed as a percentage of the luciferase activity measured in samples treated with dexamethasone for 120 min. The results shown are average of three independent experiments for each group. Data are Means ± S.E.M, * significant difference (p<0.05, one-way ANOVA and Dunnett's test, n=3/group) versus vehicle group.
Concentration-response of glucocorticoid action in HEK 293 cells

As 120 min was the maximally effective time-point for glucocorticoid receptor-induced luciferase activity, the effect of varying the concentration of dexamethasone was determined at this time point.

Exposure of HEK 293 cells transiently transfected with the MMTV-LTR plasmid to dexamethasone (0.1 to 1000 nM) for 120 min increased luciferase activity in a concentration-dependent manner (figure 3.2). The minimal effective concentration of dexamethasone was 1 nM and the maximal effect occurred at 100 nM (up to 5-fold above control level) (p<0.05, one-way ANOVA and Dunnett’s test, results were typical of three similar experiments).

Summary

The data showed that glucocorticoids act via endogenous glucocorticoid receptors in a concentration- and time-dependent manner in HEK 293 cells with maximal activity at 100 nM dexamethasone for 120 min exposure. In subsequent experiments, 100 nM dexamethasone was used to investigate the expression of Dexras1 and SGK-1 in HEK 293 cells.
Figure 3.2
Concentration dependence of the effect of dexamethasone on luciferase activity in HEK 293 cells

HEK 293 cells transiently transfected with 1 µg of MMTV-LTR luciferase reporter were treated with various concentrations of dexamethasone for 120 min as indicated. The control vehicle group received DMSO (0.001%) for 120 min. 20 µl of whole cell lysate was assayed for luciferase activity. Data are expressed as a percentage of the luciferase activity measured in samples treated with 100 nM dexamethasone for 120 min. The results shown are average of three independent experiments for each group. Data are Means ± S.E.M, * significant difference (p< 0.05, one-way ANOVA and Dunnett's test, n=3/group) versus vehicle group. Half-maximal effective concentration (EC50) was observed at 33.6 nM dexamethasone.
3.2.2 Effect of dexamethasone on the level of Dexras1 mRNA using RT-PCR

To address the effect of dexamethasone on the level of Dexras1 mRNA in HEK 293 cells, the cells were treated with 100 nM dexamethasone for various times (30 to 120 min). The cells treated with DMSO (0.001%) for 120 min were assayed as a control vehicle group. RT-PCR analysis of total RNA isolated from HEK 293 cells (described in "Material and Methods" section 2.2.2.1) failed to detect full-length human Dexras1 mRNA using forward primer A and reverse primer B, which were designed to amplify full-length human Dexras1 cDNA ("Materials and Methods" table 2.1). Furthermore, 100 nM dexamethasone (30 to 120 min exposure) failed to induce full-length human Dexras1 mRNA in HEK 293 cells (figure 3.3B) as determined by RT-PCR.

To address whether Dexras1 primers A and B are suitable to amplify full-length Dexras1 mRNA, the human Dexras1 pBluescript plasmid was used as template for PCR. The PCR analysis demonstrated that the full-length human Dexras1 is amplified from human Dexras1 pBluescript plasmid by primers A and B (figure 3.3C).
Figure 3.3
Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Dexras1 in HEK 293 cells

A:
1) Full length
Dexras1-846 bp

2) A 595 bp fragment
of Dexras1cDNA

3) A 577 bp fragment of
Dexras1 cDNA

B:

Dexamethasone (min)

Vehicle 30 60 120

Dexras1

D

C:

Dexamethasone (min)

Vehicle 30 60 120

Dexras1

D

D:

Marker
bp

A+D

C+B

Figure 3.3: Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Dexras1 in HEK 293 cells

A: Schematic representation of human Dexras1. 1) Schematic representation of full-length human Dexras1 amplified by forward primer A and reverse primer B ("Materials and Methods" table 2.1). 2) Schematic representation of a 595 bp human Dexras1 fragment (nucleotide 1-595) amplified by forward primer A and reverse primer D. 3) Schematic representation of a 577 bp human Dexras1 fragment (nucleotide 269-846) amplified by forward primer C and reverse primer B. B: HEK 293 cells were treated with 100 nM dexamethasone for different times (as shown above). The control vehicle group received DMSO (0.001%) for 120 min. RT-PCR was carried
out with total RNA isolated with TRIzol (described in "Materials and Methods" section 2.2.2.1) using forward primer A and reverse primer B, selected to amplify the entire 846-nucleotide open reading frame of human Dexras1. RT-PCR products were loaded onto a 1% agarose gel stained with ethidium bromide (section 2.2.1.2). The DNA molecular size marker is indicated in kilo base pairs (kbp) C: Forward primer A and reverse primer B were verified using human Dexras1 pBluescript plasmid as template. The Dexras1 sequence was checked by restriction enzymes analysis and DNA Sequencing. The DNA molecular size marker is indicated in base pairs (bp). D: Agarose gel electrophoresis of PCR of Dexras1 products amplified from HEK 293 cells. Reverse transcription was carried out with total RNA isolated from HEK 293 cells. PCR was carried out using RT template and specific Dexras1 primers that amplified 577 and 595 bp human Dexras1 cDNA fragments. The PCR products were loaded onto a 1% agarose gel. The sequences of two fragments were confirmed by restriction enzymes analysis and MWG DNA Sequencing. DNA molecular size marker is indicated in bp.
As full-length Dexras1 mRNA could not be detected, two sets of overlapping PCR primers were designed and used to amplify Dexras1 in the RT reaction mixture (figure 3.3A). RT-PCR analysis, using total RNA isolated from HEK 293 cells, showed a 595 bp fragment of Dexras1 cDNA using forward primer A and reverse primer D and a 577 bp Dexras1 cDNA fragment using forward primers C and reverse primer B (figure 3.3D). Thus full-length Dexras1 mRNA appears to be present in HEK 293 cells, but amplification of the full-length sequence from the RT-PCR reaction mixture was suboptimal under our conditions.

3.2.3 Northern blot analysis of Dexras1

As Dexras1 mRNA could not be detected by RT-PCR in vehicle-treated or dexamethasone-treated HEK 293 cells, the level of Dexras1 mRNA was examined by Northern blot analysis.

Effect of dexamethasone on the level of Dexras1 mRNA in HEK 293 cells stably expressing STREX-HA BK channel α-subunit (STREX-HA stable)

STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for 15 to 120 min and the cells treated with an equal volume of DMSO (0.001%) for 120 min served as the vehicle-treated control group. Northern blot analysis was performed on total RNA extracted with TRIzol (described in "Materials and Methods" section 2.2.2.1) from vehicle-treated or dexamethasone-treated cells. Total RNA (20 μg) was electrophoresed on a 1% agarose-formaldehyde gel and transferred to a nylon membrane as described under "Materials and Methods" section 2.2.2.3.
Dexras1 mRNA was detected by hybridizing the Northern blots with a $^{32}$P-labeled 207 bp human Dexras1 cDNA probe corresponding to bases 639 to 846 of the Dexras1 coding sequence (see "Materials and Methods" section 2.2.2.3). RNA loading was normalised for each lane, by stripping and rehybridizing the blots with a $^{32}$P-labeled 170 bp cyclophilin A cDNA probe, which is not regulated by glucocorticoid (Adkins et al., 1998), corresponding to sequence 295 to 465 of human cyclophilin A (described in "Materials and Methods" section 2.2.2.3). A 5 kbp Dexras1 mRNA (Tu and Wu., 1999) and a 0.74 kbp cyclophilin A mRNA (Adkins et al., 1998) were detected in the vehicle-treated as well as the dexamethasone-treated STREX-HA stable HEK 293 cells (figure 3.4A). Radioactive bands on each blot were outlined and the optical density (O.D) in each band was determined by OptiQuant image analysis software. O.D of the Dexras1 hybridized signal is divided by the O.D of the cyclophilin A signal of the same lane, to correct for different mRNA loading. The ratio of Dexras1 mRNA to cyclophilin A mRNA showed that 100 nM dexamethasone had no significant effect on the level of Dexras1 mRNA compared with control vehicle group in HEK 293 cells at any time points examined (15 to 120 min) (p<0.05, one-way ANOVA and Dunnett’s test, results were typical of five similar experiments) (figure 3.4B).
Figure 3.4
Lack of an effect of dexamethasone on Dexras1 mRNA in STREX-HA stable HEK 293 cells

A: Northern analysis for Dexras1 and cyclophilin A mRNA. HEK 293 cells stably expressing STREX-HA variant BK channel α-subunits (STREX-HA stable HEK 293 cells) were treated with 100 nM dexamethasone for various times as indicated. The vehicle group received DMSO (0.001%) for 120 min. The total RNA was extracted with TRIzol and 20 µg were loaded onto a 1% agarose-formaldehyde gel. The blots were incubated with a 32P-labeled 207 bp Dexras1 cDNA probe which hybridized with a single 5 kbp band (top row). To normalize RNA loading in each lane, the blot was stripped and reprobed with a 32P-labeled 170 bp cyclophilin A cDNA probe (bottom row). B: Average of the ratio of the optical densities (O.D) of Dexras1 to cyclophilin A mRNA from five independent experiments. The ratio of O.D of Dexras1 to cyclophilin A mRNA was calculated for the vehicle group and the corresponding ratio for dexamethasone time points (15 to 120 min) was expressed as percentage of this value. Data are Means ± S.E.M, (p<0.05, one-way ANOVA and Dunnett’s test, n=5/group) versus control vehicle group.
Expression of Dexras1 mRNA by dexamethasone in wild-type HEK 293 cells

To address whether the lack of an effect of dexamethasone on the level of Dexras1 mRNA in STREX-HA stable HEK 293 cells was due to the overexpression of BK channel α-subunit in these cells, the wild-type HEK 293 cells, were treated with 100 nM dexamethasone (15 to 120 min) or vehicle (0.001% DMSO) for 120 min under the same condition used for STREX-HA stable HEK 293 cells. Northern blot analysis of Dexras1 revealed that 100 nM dexamethasone had no effect on the level of Dexras1 mRNA compared with control vehicle group at any of the time points (15 to 120 min) tested (Data not shown).

Summary

1) Full-length Dexras1 mRNA could not be amplified by RT-PCR from STREX-stable HEK 293 cells, but experiments with overlapping primer pairs showed the presence of full-length Dexras1 mRNA. Indeed, the presence of full-length Dexras1 mRNA was also indicated by the results of Northern RNA analysis.

2) The levels of Dexras1 mRNA were readily detectable in HEK 293 cells but no change induced by dexamethasone could be discerned.

3.2.4 Northern blot analysis of SGK-1 in response to dexamethasone in HEK 293 cells

To address whether SGK-1 is a glucocorticoid-inducible protein in HEK 293 cells, and to verify the HEK 293 system for glucocorticoid induction of endogenous genes, possible
changes of SGK-1 mRNA in response to dexamethasone were examined using
Northern blot analysis.

STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for various
time intervals (15 to 120 min) or 0.001% DMSO (vehicle) for 120 min. Blots of
electrophoresed RNA were hybridized with a \(^{32}\)P-labeled 238 bp SGK-1 cDNA probe
(corresponding to bases 108 to 346 of the coding region of SGK-1 mRNA). Gel loading
was normalized by stripping and rehybridization of the blots with a \(^{32}\)P-labeled 170 bp
cyclophilin A cDNA probe, corresponding to bases 295 to 465 of the human cyclophilin
A mRNA coding region (described in "Materials and Methods" section 2.2.2.3).

A 2.6 kbp band corresponding to the expected size of human SGK-1 mRNA (Chen et
al., 1999b) reacted with the SGK-1 probe in both vehicle-treated and dexamethasone-
treated STREX-HA stable HEK 293 cells (figure 3.5). After standardisation with the
cyclophilin A signal, the data revealed that 100 nM dexamethasone increased SGK-1
mRNA in a time-dependent manner: statistically significant increase was found after 15
min which was maximal by 30 min and remained elevated until 120 min (results were
typical of five similar experiments, \(p<0.05\), one-way ANOVA and Dunnett's test) (figure
3.5). Similar data were obtained in wild-type HEK 293 cells (Data not shown).
Figure 3.5
Time-course of the effect of dexamethasone on SGK-1 mRNA in STREX-HA stable HEK 293 cells

A

Blot: 32P-labeled SGK-1 cDNA probe

Blot: 32P-labeled cyclophilin A cDNA probe

B

Ratio of SGK-1/cyclophilin A as 100% at vehicle group

Dexamethasone (min)

Vehicle 15 30 60 90 120

Figure 3.5: Time-course of the effect of dexamethasone on SGK-1 mRNA in STREX-HA stable HEK 293 cells

A: Northern analysis for SGK-1 and cyclophilin A mRNA. STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for the indicated times. The vehicle group received DMSO (0.001%) for 120 min. Total RNA was extracted with TRizol and 20 μg were loaded onto a 1% agarose-formaldehyde gel. The total RNA was transferred to a nylon membrane. The blots were hybridized with 32P-labeled 238 bp SGK-1 cDNA probe. SGK-1 message constituted a single major band on Northern blot with a size of 2.6 kbp (top row). Blots were stripped and rehybridized with a probe for cyclophilin A with a size of 0.74 kbp (bottom row). B: Average of the ratio of the O.D of SGK-1 to cyclophilin A mRNA from five independent experiments. The ratio of O.D of SGK-1 to cyclophilin A mRNA was calculated for vehicle group and the corresponding ratio for dexamethasone time points (15 to 120 min) was expressed as percentage of this value. Data are Means ± S.E.M, * significant difference (p<0.05, one-way ANOVA and Dunnett’s test, n=5/group) versus control vehicle group.
Summary, Northern blot analysis revealed that SGK-1 mRNA is induced in response to 100 nM dexamethasone (minimal induction at 15 min and maximal by 30 min) in both wild-type HEK 293 cells and HEK 293 cells stably expressing STREX-HA variant BK channel α-subunit.

3.2.5 Western blot analysis of SGK-1 in response to dexamethasone in HEK 293 cells
Northern blot analysis (section 3.2.4) revealed that SGK-1 mRNA is increased by 100 nM dexamethasone. Therefore it was of interest to investigate whether or not, the rapid induction of SGK-1 mRNA leads to an increase of the level of SGK-1 protein in HEK 293 cells.

3.2.5.1 Specificity of SGK-1 antibody to SGK-1 protein in HEK 293 cells
A purified rabbit anti-SGK-1 (C-terminal) antibody (Upstate, "Materials and Methods" table 2.2) was used to detect SGK-1 protein in HEK 293 cells. Cells were lysed and fractionated in 1% Triton X-100 lysis buffer (described in section 2.4.1). The Triton X-100 soluble fractions were separated on 12.5% polyacrylamide gels in SDS-PAGE and blots were probed with anti-SGK-1 antibody. The bands were detected by ECL chemiluminescence as described in "Materials and Methods" section 2.4.6. Anti-SGK-1 antibody reacted with a major ~50 K band, which is in line with the expected molecular weight of SGK-1 protein (Kobayashi et al., 1999), in both wild-type and STREX-HA stable HEK 293 cells (figure 3.6A). Some spurious bands of much lower intensity also evident but not in reproducible pattern.
Figure 3.6
Specificity of the anti-SGK-1 antibody in HEK 293 cells

HEK 293 cells and STREX-HA stable HEK 293 cells were homogenized in 1% Triton X-100 lysis buffer as described in "Materials and Methods" section 2.4.1. 4 μl of 1% Triton X-100 soluble fraction (1 μg/1μl) were loaded onto a 12.5% SDS-PAGE gel and electrotransferred to a PVDF membrane in Phastsystem apparatus (Amersham-Pharmacia, Uppsala, Sweden). A: The blot was probed with rabbit anti-SGK-1 antibody and immunoreactive bands were visualized at approximately 50 K by chemiluminescence (ECL) reagents. B and C: The specificity of SGK-1 antibody was determined by omitting the primary antibody or pre-incubation of the SGK-1 antibody with SGK-1 peptide antigen (described in "Materials and Methods" section 2.4.6.1).
The specificity of the SGK-1 detection method was determined by probing the blots with HRP-conjugated anti-rabbit secondary antibody alone (figure 3.6 B). Furthermore, pre-absorption of the SGK-1 antibody with SGK-1 peptide antigen (Upstate), described in "Materials and Methods" section 2.4.6.1, abolished the reaction with the ~50 K band (figure 3.6C). Thus, the commercially available anti-SGK-1 antibody appeared to produce specific staining of SGK-1 in immunoblots.

3.2.5.2 Effect of dexamethasone on the level of SGK-1 protein

In order to investigate the effect of dexamethasone on endogenous SGK-1 protein in STREX-HA stable HEK 293 cells, the cells were treated with 100 nM dexamethasone for 15 to 120 min. Controls were exposed to (0.001%) DMSO in DMEM for 120 min. Cell extracts prepared in 1% Triton X-100 lysis buffer (Triton X-100 soluble fraction, section 2.4.1) were processed for immunoblots as described in "Materials and Methods" section 2.4.6. The blots were incubated with anti-SGK-1 antibody and developed with ECL reagents (figure 3.7A). The level of SGK-1 protein was standardized in each lane upon staining for cyclophilin A as follows (figure 3.7B). The SGK-1 and cyclophilin A bands in each blot were outlined and optical density of bands were measured by ImageJ software. The ratio of optical density of SGK-1 to cyclophilin A protein at vehicle group was calculated and the corresponding ratio for dexamethasone time-points (15 to 120 min) was compared with this value. The results showed that 100 nM dexamethasone had no effect on the level of SGK-1 protein in the Triton X-100 soluble fraction when compared with the vehicle group in STREX-HA stable HEK 293 cells at any of the time-points examined (figure 3.7C) (results were typical of three similar
Figure 3.7
Effect of dexamethasone on the levels of SGK-1 protein in 1% Triton X-100 soluble fraction in STREX-HA stable HEK 293 cells

A
Blot: anti-SGK-1

B
Blot: anti-cyclophilin A

C

Figure 3.7: Effect of dexamethasone on the levels of SGK-1 protein in 1% Triton X-100 soluble fraction in STREX-HA stable HEK 293 cells

A and B: Western analysis for SGK-1 and cyclophilin A protein in 1% Triton X-100 soluble fraction. STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for the indicated times. The vehicle group received DMSO (0.001%) for 120 min. 4 μl of 1% Triton X-100 soluble proteins (1 μg/1 μl) were subjected to a 12.5 % SDS-PAGE gel and Western blotting by rabbit anti-SGK-1 antibody (A) in Phastsystem apparatus. The blots were probed with anti-cyclophilin A antibody (B) as loading control. C: Average of the ratio of O.D of SGK-1 to cyclophilin A protein in 1% Triton X-100 soluble fraction from three independent experiments. The ratio of O.D of SGK-1 to cyclophilin A protein was calculated for the vehicle group and the corresponding ratio for dexamethasone time points (15 to 120 min) was expressed as percentage of this value. Data are Means ± S.E.M, (p<0.05, one-way ANOVA and Dunnett's test, n=3/group) versus control vehicle group.
experiments). Similar data were obtained in wild-type HEK 293 cells (data not shown). Taken together, the results showed no changes in the levels of SGK-1 protein in the Triton X-100 soluble fraction in response to dexamethasone in HEK 293 cells.

It has been reported that the amount SGK-1 protein is rapidly and strongly enhanced by 100 nM dexamethasone after 30 min in A6 cells homogenized in NP-40 lysis buffer (Chen et al., 1999b). Thus, to determine the effect of NP-40 detergent on the expression of the SGK-1 protein, wild-type HEK 293 cells and STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for 15 to 120 min or 0.001% DMSO for 120 min (vehicle) and subsequently lysed with 1% NP-40 lysis buffer ("Materials and Methods" section 2.4.2). Once more, the results showed no change in the expression of SGK-1 protein in response to 100 nM dexamethasone in either STREX-HA stable HEK 293 cells (figure 3.8) or wild-type HEK 293 cells (Data not shown).

Taken together, application of 100 nM dexamethasone for 15 to 120 min does not change the level of endogenous SGK-1 protein in the 1% Triton X-100 or 1% NP-40 soluble fractions of either wild-type or STREX-HA stable HEK 293 cells.
Figure 3.8
Effect of dexamethasone on the levels of SGK-1 protein in 1% Nonidet-P40 soluble fraction in STREX-HA stable HEK 293 cells

A and B: Western analysis for SGK-1 and cyclophilin A protein in 1% Nonidet-P40 soluble fraction. STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone for indicated times and homogenized in 1% Nonidet-P40 lysis buffer. The cells treated with DMSO (0.001%) for 120 min were assayed as a control vehicle group. 4 μl of 1% Nonidet-P40 soluble fractions (1 μg/1μl) were analysed by Western blotting using a 12.5 % SDS-PAGE gel in Phastsystem apparatus. The blots were probed with anti-SGK-1 antibody (A) and SGK-1 proteins were visualized by ECL detection. Variations of gel loading were corrected against the corresponding cyclophilin A (B) blot values. C: Average of the ratio of O.D of SGK-1 to cyclophilin A protein in 1% Nonidet-P40 soluble fraction from three independent experiments. The ratio of O.D of SGK-1 to cyclophilin A protein was calculated for the vehicle group and the corresponding ratio for dexamethasone time points (15 to 120 min) was expressed as percentage of this value. Data are Means ± S.E.M, (p<0.05, one-way ANOVA and Dunnett’s test, n=3/group) versus control vehicle group.
3.2.5.3 Lack of an effect of proteasome inhibitors on SGK-1 protein expression

It has been reported that there is intensive degradation of SGK-1 cellular protein by the proteasome (Brickley et al., 2002). To address whether the lack of an effect of dexamethasone on SGK-1 protein was due to the rapid degradation of SGK-1 protein by the proteasome, STREX-HA stable HEK 293 cells and wild-type HEK 293 cells were pre-treated with 10 μM ALLN (N-Acetyl-Leu-Leu-Nle-CHO, calpain inhibitor 1 with weak proteasome inhibitory action) for 1 hour. The cells were subsequently exposed to 100 nM dexamethasone for various times (15 to 120 min) or 0.001% DMSO for 120 min (vehicle group) in the presence of the proteasome inhibitor. No changes on the levels of SGK-1 protein in Triton X-100 soluble fraction in response to dexamethasone were found in either wild-type HEK 293 cells or STRX-HA stable HEK 293 cells despite the presence of proteasome inhibitor (one-way ANOVA and Dunnett’s test, results were typical of three similar experiments) (data not shown).

Due to the weak effect of ALLN on the proteasome, the expression of SGK-1 protein was also examined in the presence of the more potent proteasome inhibitor, MG-132 (Carbobenzyo-L-leucyl-L-leucyl-L-leucinal) (Leithe and Rivedal., 2004; Coux et al., 1996; Rock et al., 1994). STREX-HA stable HEK 293 cells and wild-type HEK 293 cells were pre-treated for 1 hour with 10 μM MG-132 and subsequently exposed to 100 nM dexamethasone for various times (15 to 120 min) or 0.001% DMSO for 120 min (vehicle group) in the presence of MG-132. The Western blot analysis revealed that MG-132 had no effect on the level of SGK-1 protein expression at any time points examined with 100 nM dexamethasone in both wild-type HEK 293 cells (data not shown) and STREX-HA.
Figure 3.9
Effect of specific proteasome inhibitor MG-132 on the levels of SGK-1 protein in STREX-HA stable HEK 293 cells

A and B: Western analysis for SGK-1 and cyclophilin A protein in the presence of proteasome inhibitor. STREX-HA stable HEK 293 cells were pre-treated with 10 μM specific proteasome inhibitor MG-132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) for 1 h and then treated with 100 nM dexamethasone for different times (15 to 120 min) or 0.001% DMSO for 120 min (vehicle) in the presence of MG-132. 4 μl of 1% Triton X-100 soluble proteins (1 μg/μl) were separated by a 12.5% SDS-PAGE gel in Phastsystem apparatus. SGK-1 and cyclophilin A protein were evaluated by Western blot analysis using anti-SGK-1 antibody (A) and anti-cyclophilin A antibody (B). C: Average of the ratio of O.D of SGK-1 to cyclophilin A protein in the presence of MG-132 from three independent experiments. The ratio of O.D of SGK-1 to cyclophilin A protein was calculated for the vehicle group and the corresponding ratio for dexamethasone time points (15 to 120 min) was expressed as percentage of this value. Data are Means ± S.E.M, (p<0.05, one-way ANOVA and Dunnett’s test, n=3/group) versus control vehicle group.
3.2.5.4 Distribution of SGK-1 protein in HEK 293 cells

Previous data in HEK 293 cells (Brickley et al., 2002) showed that transfected SGK-1 is localized to the plasma membrane and it is only partially solubilised by non-denaturing detergents. Moreover, similar data were also reported for rat kidney tissue (Alvarez de la Rosa et al., 2003). These data raised the possibility that only a certain pool of SGK-1 protein is influenced by dexamethasone. To examine the distribution of endogenous SGK-1 protein in STREX-HA stable HEK 293 cells, the cells were homogenized in the absence of detergents and centrifuged at 20,000 g (described in "Materials and Methods" section 2.4.4). Western blot analysis of the pellet and supernatant fractions revealed that SGK-1 was only detectable in the particulate fraction (figure 3.10). When the same protocol was followed using Triton X-100 lysis buffer, Western blot analysis of the high-speed pellet and supernatant fractions indicated that over 50% of the total SGK-1 was found in the Triton X-100 soluble fraction (figure 3.10).

Taken together, the results showed that endogenous SGK-1 is largely in the particulate fraction in HEK 293 cells, and that a substantial proportion is solubilised by 1% Triton X-100.
Figure 3.10
Distribution of SGK-1 protein in STREX-HA stable HEK 293 cells

<table>
<thead>
<tr>
<th></th>
<th>Pellet (insoluble fraction)</th>
<th>Supernatant (soluble fraction)</th>
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<tr>
<td>SGK-1 protein</td>
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<tr>
<td>A</td>
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<tr>
<td>Lysis without detergent</td>
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<tr>
<td>Lysis with 1% Triton X-100</td>
<td>Mw KDa</td>
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</table>

Figure 3.10: Distribution of SGK-1 protein in STREX-HA stable HEK 293 cells

STREX-HA stable HEK 293 cells were homogenized in the absence of detergent or the presence of 1% Triton X-100. The homogenates were centrifuged at 20,000 g and equal amounts of protein from the pellet and the supernatant were separated by a 12.5% SDS-PAGE gel in Phastsystem apparatus. The proteins were transferred to a PVDF membrane and then analysed by Western blotting with anti-SGK-1 antibody. A: In the absence of detergents, SGK-1 was only detected in the pellet, particulate fraction. B: In the presence of 1% Triton X-100, SGK-1 was found in the pellet (insoluble fraction) and the supernatant (soluble fraction).
3.2.5.5 Dexamethasone increases the SGK-1 protein level in 1% Triton X-100 insoluble fractions

The expression of endogenous SGK-1 protein in Triton X-100 insoluble fraction (figure 3.10) raised the possibility that dexamethasone may selectively influence the Triton X-100 insoluble fraction of SGK-1.

As maximal glucocorticoid induced mRNA (SGK-1) level was obtained with 100 nM dexamethasone given for 120 min, STREX-HA stable HEK 293 cells were treated with 100 nM dexamethasone or vehicle (0.001% DMSO) for 120 min. The cells were homogenized in the presence of detergent (1% Triton X-100). The Triton X-100 insoluble fraction (pellet) and Triton X-100 soluble fraction (supernatant) separated at 20,000 g ("Materials and Methods" section 2.4.4) and subjected to SDS-PAGE and Western blot analysis using anti-SGK-1 antibody. The blots with the Triton X-100 soluble fraction were standardized by staining for cyclophilin A (figure 3.11B).

Most proteins are stained by Coomassie Blue to approximately the same extent. It is therefore convenient to estimate the amount of protein in the gel by Coomassie-stained band. Thus, the amount of protein in the Triton X-100 insoluble fraction was standardized by prominent Coomassie blue stained band in the blot (figure 3.11B).

The results showed that dexamethasone had no significant effect on the level of SGK-1 protein in the supernatant (figure 3.11C). In contrast, 100 nM dexamethasone for 120 min exposure increased SGK-1 protein level in the Triton X-100 insoluble pellet (figure 3.11C).
Figure 3.11: Effect of dexamethasone on the levels of SGK-1 protein in 1% Triton X-100 insoluble fraction

STREX-HA stable cells were treated with 0.001% DMSO for 120 min (control vehicle group) or 100 nM dexamethasone for 120 min (Dex120). The cells were homogenized in the present of 1% Triton X-100 lysis buffer. Equal amounts of proteins from supernatant (Triton X-100 soluble fraction) or pellet (Triton X-100 insoluble fraction) were loaded onto a 12.5% SDS-PAGE gel and stained with Coomassie blue.
transferred to a PVDF membrane in Phastsystem apparatus. A: ECL staining for SGK-1. B: Amount of loaded protein was determined by Coomassie blue staining (selected band is shown by arrow) for Triton X-100 insoluble soluble fraction and by cyclophilin A protein for Triton X-100 soluble fraction. C: The average of the ratio of O.D of SGK-1 to Coomassie blue or cyclophilin A from three independent experiments. The ratio of the O.D of SGK-1 to Coomassie blue in Triton X-100 insoluble fraction for the respective vehicle group was calculated and the corresponding ratio for dexamethasone 120 min was expressed as percentage of this value. The ratio of the O.D of SGK-1 to cyclophilin A in Triton X-100 soluble fraction for the respective vehicle group was calculated and the corresponding ratio for dexamethasone 120 min was expressed as percentage of this value. Values are reported as Means ± S.E.M,* significant difference (p<0.05, t test, n=3/group) vs. vehicle control group.
3.2.5.6 Lack of an association of SGK-1 protein with STREX-HA variant BK channels

The rapid induction of SGK-1 mRNA (section 3.2.4) by dexamethasone in HEK 293 cells raised the possibility that SGK-1 could be a mediator of the glucocorticoid control of BK channels. In order to probe for a potential physical interaction of SGK-1 with the BK channel complex, STREX-HA variant BK channel α-subunits were immunoprecipitated using sheep antisera against the BK channel α-subunits (anti-STREX + anti-S869) (described in section 2.4.5.1) from STREX-HA stable HEK 293 cells pretreated with 100 nM dexamethasone or 0.001% DMSO (vehicle) for 120 min. Immunoprecipitates were probed for the presence of SGK-1 and HA reactive protein bands in immunoblots using anti-SGK-1 antibody and anti-HA antibody ("Materials and Methods" table 2.2). No co-immunoprecipitation of endogenous SGK-1 protein was found in vehicle-treated or dexamethasone-treated STREX-HA stable HEK 293 cells (figure 3.12).
Figure 3.12
Lack of an association of SGK-1 with STREX-HA variant BK channel

HEK 293 cells stably expressing the STREX-HA variant BK channel α-subunits were treated with 0.001% DMSO for 120 min (control vehicle group) or 100 nM dexamethasone for 120 min. The cells were lysed in 1% Triton X-100 lysis buffer. STREX-HA BK channels were immunoprecipitated with sheep antisera against BK channel α-subunit (anti-STREX + anti-S869). Whole cell extracts and immunoprecipitates (IPs) were loaded onto a 12.5% SDS-PAGE gel and electrotransferred to a PVDF membrane in Phastsystem apparatus. A: an immunoreactive band of ~132 K molecular weight corresponding to the STREX-HA BK channel α-subunit was detected by rabbit anti-HA antibody. B: The blot was probed with rabbit anti-SGK-1 antibody.
3.3 Discussion

In an attempt to detect the glucocorticoid-inducible proteins in HEK 293 cells, we demonstrated that:

i) Glucocorticoids regulate the transcription of glucocorticoid responsive reporter plasmids via endogenous glucocorticoid receptors in a concentration- and time-dependent manner in HEK 293 cells.

ii) Levels of Dexras1 mRNA are readily detectable in HEK 293 cells and are not altered by exposure to dexamethasone. In contrast, dexamethasone rapidly augmented SGK-1 mRNA expression in HEK 293 cells.

iii) Glucocorticoids increased the level of SGK-1 protein in Triton X-100 insoluble fraction.

iv) SGK-1 was not associated with STREX-HA variant BK channel α-subunit and treatment with dexamethasone did not lead to the association of SGK-1 with the BK-channel α-subunit.

3.3.1 MMTV-LTR luciferase activity in HEK 293 cells

Previously Tian et al (Tian et al., 2001b) have shown the presence of type II glucocorticoid receptor immunoreactivity in HEK 293 cells as well as concentration dependent stimulation of luciferase activity driven by the MMTV-LTR promoter in transient transfections. As this work was restricted to 120 min exposure to dexamethasone, the present study set out to examine more detail the time-course and concentration-dependence of glucocorticoid action in HEK 293 cells. Measurements of MMTV-LTR luciferase activity confirmed that dexamethasone acts via endogenous
glucocorticoid receptors in HEK 293 cells in a concentration- and time-dependent manner with maximal activity at 120 min exposure to 100 nM as previously reported by Tian et al (Tian et al., 2001b).

Tian and co-workers (Tian et al., 2001b) reported that dexamethasone produced a concentration-dependent stimulation of luciferase activity with a $K_D$ of $4.5 \pm 3.6$ nM in HEK 293 cells, we found the EC50 of luciferase activity at 33.6 nM dexamethasone. The shifting of the concentration-response curve of luciferase activity to the right in this study compared with Tian's study may be due to several factors like different concentrations and conditions used for transient transfection of MMTV-LTR-luciferase reporter construct, different cell passages, different pre-treatment and dexamethasone treatment ways.

MMTV luciferase activity in different cells such as culture of transformed monkey kidney, COS-7 cells (Ray et al., 1999) and breast cancer cells, MDA-MB-453 (Wilson et al., 2002) showed the activation of the type II glucocorticoid receptor by dexamethasone. In COS-7 cells, significant MMTV luciferase activity was achieved at 0.1 nM by dexamethasone and maximal activity at 100 nM (Ray et al., 1999).

3.3.2 Effect of dexamethasone on the level of Dexras1 mRNA in HEK 293 cells

Kemppainen showed that Dexras1 mRNA is increased by dexamethasone in AtT-20 corticotroph cells (Kemppainen and Behrend., 1998). Presence of Dexras1 has been recently reported in pituitary tissue (Kemppainen et al., 2000) and human tissues like heart, liver, skeletal muscle, kidney and pancreas cells (Tu and Wu., 1999). Another
study has identified a putative glucocorticoid response element in the Dexras1 promoter (Kemppainen et al., 2000).

Our RT-PCR revealed no detection of full-length Dexras1 mRNA in HEK 293 cells, but two fragments of overlapping Dexras1 cDNA in the PCR indicated that full-length Dexras1 mRNA appears to be present in HEK 293 cells. This was confirmed by Northern mRNA analysis.

Many different things might have caused the failure to amplify full-length of Dexras1, for example; Insufficient template, insufficient primer, poor primer design, e.g. low melting temperature, contamination that inhibits the DNA polymerase (proteins, residual salts, residual organic chemicals, detergents).

DNA template quality and the amount of template are very important in order to obtain a PCR reaction and too little DNA fails to gives a PCR product (Chandler et al., 1998). One possible explanation of a lack of amplification of Dexras1 cDNA from HEK 293 cells may be the quality or low abundance of RT template in the PCR reaction. Further RT-PCR, with greater RT concentration (>0.25 µg) could resolve this issue.

Indeed it appeared that shorter products could be readily amplified from the Dexras1 mRNA in the same reactions indicating that Dexras1 mRNA is indeed present in HEK 293 cells. As these PCR reactions amplified across the entire Dexras1 coding region it appears likely that PCR reaction conditions as opposed to lack of sufficient full-length template were at fault.
Northern blot analysis of Dexras1

The present data showed the presence of a 5 kbp Dexras1 mRNA in HEK 293 cells which was similar to that reported in human fibrosarcoma HT-1080 cells (Tu and Wu., 1999). Although 100 nM dexamethasone induced Dexras1 mRNA within 30 min exposure the highest induction occurred at 120 min in AtT20 cells (Kemppainen and Behrend., 1998). In HEK 293 cells, we found no change of the level of Dexras1 mRNA in response to 100 nM dexamethasone up to 120 min. A possible explanation of the lack of induction of Dexras1 mRNA in HEK 293 cells is that the basal level of the Dexras1 mRNA was already high and readily detectable in HEK 293 cells. In contrast Dexras1 mRNA was undetectable by Northern analysis in AtT20 cells (Kemppainen and Behrend., 1998). Thus varying the conditions of the experiment to achieve low basal Dexras1 mRNA level may lead to a system where Dexras1 is induced. Indeed a glucocorticoid response element has been identified 3' of the Dexras1 polyadenylation site and shows complex, context dependent activity in reporter gene constructs (Kemppainen et al., 2003). Overall, the lack of induction of Dexras1 mRNA by dexamethasone in HEK 293 cells does not exclude Dexras1 as a potential mediator of dexamethasone action. Further experiments with Dexras1 gene knock-down technology could address this issue.

As a positive control for glucocorticoid mediated gene-induction, the expression of SGK-1 mRNA was assessed by Northern blot in HEK 293 cells. These studies showed that dexamethasone (100 nM) significantly increased SGK-1 mRNA in a time-dependent manner, a significant increase occurred within 15 min of steroid exposure and maximal
induction was found by 30 min. Similar results were reported in A6 cells by induction of SGK-1 mRNA in a time-dependent manner in response to 100 nM dexamethasone with maximal activity at 30 min (Chen et al., 1999b). Therefore, the rapid induction of SGK-1 mRNA in A6 cells (Chen et al., 1999b) and HEK 293 cells in the present study (section 3.2.4) raised the possibility that SGK-1 is a glucocorticoid-induced mediator in HEK 293 cells.

### 3.3.3 Western blot analysis of SGK-1

The present study showed that 100 nM dexamethasone had no effect on endogenous SGK-1 protein levels in Triton X-100 or NP-40 soluble fractions prepared from HEK 293 cells. In contrast, SGK-1 protein levels in the Triton X-100 insoluble fraction were increased by dexamethasone. Under condition used in the present study, SGK-1 is largely restricted to the particulate fraction in HEK 293 cells and a substantial proportion (over 50%) is solubilised by 1% Triton X-100 (figure 3.10). Dexamethasone appeared to increase the SGK-1 protein in a membrane domain which was not solubilised by Triton X-100. The lack of a detectable change in the amount of total SGK-1 protein is likely to be due to the relative amount of the Triton X-100 insoluble protein being low as a proportion of the total protein.

It has been shown that glucocorticoids increase phosphoinositide 3 (PI3) kinase (a subfamily of lipid kinases) activity by more than four-fold in a concentration-dependent manner, an effect which was blocked by RU486, LY294002 and wortmannin (Hafezi-Moghadam et al., 2002). Furthermore, SGK-1 is also regulated by the PI3-kinase signaling pathway (Gamper et al., 2002b; Park et al., 1999; Kobayashi et al., 1999).
has been shown that the key phosphorylation sites on SGK-1 are Thr\textsuperscript{256} and Ser\textsuperscript{422}, that are targeted by PI3 kinase (Kobayashi and Cohen., 1999). Furthermore, Western blot analysis revealed a direct interaction between PI3 kinase and the catalytic domain of SGK-1(Park et al., 1999). Therefore, it is hypothesize that the distribution of SGK-1 in Triton X-100 soluble and insoluble fraction (crude particular fraction) is regulated by phosphorylation via PI3 kinase. The identification of the upstream components between PI3-kinase and SGK-1 will be crucial for understanding this signaling pathway.

Lack of association of endogenous SGK-1 protein with BK channel

Although, our results revealed that endogenous SGK-1 is not associated with STREX-HA BK channel \( \alpha \)-subunit in vehicle-treated and dexamethasone-treated STREX-HA stable HEK 293 cells, the data do not rule out that dexamethasone regulates BK channels through SGK-1.

It has been demonstrated that SGK-1 regulates the epithelial sodium channels via binding and phosphorylation of Nedd4-2 protein (Snyder et al., 2002; Debonneville et al., 2001). A similar scenario is also possible for BK channels. In order to address this, gene knock-downs of SGK-1 could be carried out in combination with electrophysiological analysis of BK-channel activity. In case of a positive outcome, indicating an involvement of SGK-1 in the regulation of BK channels, immunoprecipitation of SGK-1 protein from vehicle-treated and dexamethasone–treated BK channel stable HEK 293 cells could be carried out followed by analysis of the

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immunoprecipitates for associated proteins by mass spectrometric methods. This should provide evidence as to the nature of SGK-1 target protein in HEK 293 cells.

In conclusion: SGK-1 but not Dexras1 is a protein rapidly induced by dexamethasone in HEK 293 cells. SGK-1 is not in complex with STREX-HA BK channels, and dexamethasone does not lead to the association of SGK-1 with the channel.
Chapter Four

Association of protein phosphatase 2A with BK channels
4.1 Introduction

Based on evidence summarised in chapter 1 section 1.3.4 it is justified to hypothesize glucocorticoids may exert their effects through protein phosphatase activity at the level of the BK channel complex. Identification of the BK channel complex as a target for glucocorticoid action should allow us to characterize glucocorticoid-induced proteins involved in ion channel regulation and provide further insights into the mechanism and role of rapid glucocorticoid regulation of excitability in neuroendocrine and neuronal cells.

Thus to address this hypothesis, we used biochemical approaches to investigate:

i) Does PP2A associate with the BK channel complex?

ii) Do glucocorticoids alter the amount of PP2A associated with the channel?

iii) Is PP2A association with the BK channel dependent on the presence of the STREX splice insert?

iv) Which region of the BK channel α-subunit is involved in the association with PP2A?

v) Is PP2A association region involved in trafficking of BK channels to the plasma membrane?

4.2 Results

4.2.1 Validation of anti-[HA] antibody in Western blot analysis of STREX-HA and ZERO-HA variant BK channels in extracts of HEK 293 cells

HEK 293 cells stably transfected with HA epitope tagged BK channel α-subunits (STREX and ZERO variants) (Tian et al., 2003) were lysed in 1% Triton X-100 lysis buffer (described in "Materials and Methods" section 2.4.1) and processed for
immunoblots. HA reactive protein bands were detected using rabbit anti-HA antibody ("Materials and Methods" table 2.2) which reacts specifically with HA-tagged fusion proteins (Tu and Wu., 1998). Western blot analysis revealed the expression of ~132 K and ~125 K protein bands which conform to the expected molecular masses of STREX and ZERO variant BK channel α-subunits, respectively (figures 4.1 and 4.2).

STREX-HA and ZERO-HA splice variant BK channel α-subunits stably expressed in HEK 293 cells were immunoprecipitated with antisera raised in sheep (anti-STREX serum against amino acids 628-641 and anti-S869 serum against amino acids 919-933 of the mouse STREX variant BK channel α-subunit) (described in "Materials and Methods" section 2.4.5.1). The immunoprecipitates (IPs) were separated by SDS-PAGE and blotted to PVDF membrane. An immunoreactive band migrating at ~132 K was observed in IPs from STREX-HA stable HEK 293 cells (figure 4.1) and a ~125 K immunoreactive band was observed in IPs from ZERO-HA stable HEK 293 cells (figure 4.2).

The specificity of the IPs was confirmed in three ways: i) The HA reactive bands were not detected in the IPs from non-transfected (wild-type) HEK 293 cells (figure 4.3) or pcDNA3 transfected HEK 293 cells (figure 4.5). ii) Immunoprecipitation with preimmune sera from the respective sheep in did not result in the pull-down of the ~132 K HA reactive bands in STREX-HA stable HEK 293 cells (figures 4.1 and 4.2). iii) The specificity of Western blot antibody (anti-HA antibody) was demonstrated with incubation of blots with HRP secondary antibody alone and no reaction was found (figure 4.1 and 4.2).
Figure 4.1
Immunoprecipitation of STREX-HA BK channel α-subunits from
HEK 293 cells stably transfected with STREX-HA BK channel α-subunits

HEK 293 cells stably transfected with hemagglutinin (HA) epitope-tagged STREX variant BK channel α-subunit pcDNA3 plasmid were lysed in 1% Triton X-100 lysis buffer (described in "Materials and Methods" section 2.4.1). The cell extracts were immunoprecipitated with antisera raised in sheep against BK channel α-subunits (anti-STREX serum against amino acids 628-641 and anti-S869 serum against amino acids 919-933 of the mouse STREX channel α-subunit) as described in section 2.4.5.1. The lysate and immunoprecipitates (IPs) were run on 7.5% homogeneous SDS-PAGE gel and the proteins were transferred to methanol-activated PVDF membrane in PhastSystem microelectrophoresis apparatus (Amersham-Pharmacia, Uppsala, Sweden) as described in "Materials and Methods" section 2.4.6. A: The transferred blots were incubated with rabbit anti-HA antibody, which reacts specifically with HA-tagged fusion proteins (Tu and Wu, 1998) and a ~132 K HA-reactive protein band (arrow), was found in whole cell extracts and IPs. The HA-immunoreactive band was absent from IPs with sheep non-immune serum, demonstrating specificity of IP antisera. B: The specificity of Western blot antibody (anti-HA antibody) was checked by incubating the blots with HRP-conjugated anti-rabbit antibody alone. Western blot analysis: Rabbit anti-HA antibody (1/1000). HRP-conjugated anti-rabbit antibody (1/3000); Detection was by ECL.
Figure 4.2
Immunoprecipitation of ZERO-HA BK channel subunits from HEK 293 cells stably transfected with ZERO-HA variant BK channel subunits

<table>
<thead>
<tr>
<th>Mw (kDa)</th>
<th>Cell lysate</th>
<th>anti-STREX</th>
<th>Non Immune + anti-S869 serum</th>
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<td>A</td>
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<td>Blot: anti-HA</td>
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B
Blot: No primary antibody

Figure 4.2: Immunoprecipitation of ZERO-HA BK channel subunits from HEK 293 cells stably transfected with ZERO-HA variant BK channel subunits

HEK 293 cells transfected with HA epitope-tagged ZERO variant BK channel subunits were lysed in 1% Triton X-100 lysis buffer (described in "Materials and Methods" section 2.4.1). The cell extracts were immunoprecipitated with sheep antisera (described in section 2.4.5.1). The extracts and IPs were run on 7.5% homogeneous SDS-PAGE gel and transferred to PVDF membrane in PhastSystem apparatus (section 2.4.6). A: The transferred blots were incubated with rabbit anti-HA antibody and a ~125 K HA-immunoreactive band (arrow) was found in the IPs and whole cell lysates. The specificity of IP antisera was demonstrated by sheep non-immune serum B: The specificity of Western blot antibody was determined by incubating the blots with HRP-conjugated anti-rabbit antibody alone. Western blot analysis: Rabbit anti-HA antibody (1/1000). HRP-conjugated anti-rabbit antibody (1/3000); Detection was by ECL.
4.2.2 Co-immunoprecipitation of endogenous protein phosphatases with BK channels in stably transfected HEK 293 cells

Electrophysiological studies demonstrated that dexamethasone blocks the PKA-mediated inhibition of STREX variant BK channel via regulation of PPase activity associated with the channel complex (Tian et al., 2001b; Tian et al., 1998). To probe a potential stable physical association between the BK channel protein and protein phosphatases, the BK channel α-subunits were immunoprecipitated with sheep antisera against the BK channel α-subunits (described in "Materials and Methods" section 2.4.5.1) from STREX-HA and ZERO-HA stable HEK 293 cells. The IPs were separated by 12.5% SDS-PAGE gel and blots were probed with anti-PP2A antibodies (rabbit anti-PP2A-A, regulatory subunit of PP2A, antibody and mouse anti-PP2A-C, catalytic subunit of PP2A, antibody). The results showed co-immunoprecipitation of the ~65 K regulatory subunit of PP2A (PP2A-A subunit) as well as the ~36 K catalytic subunit of PP2A (PP2A-C subunit) with both STREX-HA and ZERO-HA variant BK channel α-subunits (figure 4.3).

IPs from non-transfected HEK 293 cells and HEK 293 cells transfected with pcDNA3 vector were used in parallel as controls of specificity. The Western blot analysis showed no protein phosphatase subunit immunoreactivity in the IPs of wild-type HEK 293 cells (figure 4.3) or HEK 293 cells transfected with pcDNA3 vector (figure 4.5), indicating that the PP2A subunits are associated with the BK channels.
**Figure 4.3**

Co-immunoprecipitation of STREX-HA and ZERO-HA variant BK channel \(\alpha\)-subunits with PP2A

Wild-type HEK 293 cells and HEK 293 cells stably expressing STREX-HA and ZERO-HA variant BK channel \(\alpha\)-subunits were lysed in 1% Triton X-100 lysis buffer (section 2.4.1). The extracts were immunoprecipitated with sheep antisera anti-BK channel \(\alpha\)-subunits (section 2.4.5.1). The IPs were subjected to 12.5% SDS-PAGE and Western blot (section 2.4.6) A: A \(-132\) K HA-reactive protein band (corresponding to STREX-HA channel \(\alpha\)-subunits, top arrow) and a \(-125\) K HA-reactive protein band (corresponding to ZERO-HA channel \(\alpha\)-subunits, bottom arrow) were detected using 1/1000 rabbit anti-HA antibody. No HA-reactive band was found in wild-type HEK 293 cells. B: Co-immunoprecipitation of BK channel \(\alpha\)-subunits (STREX-HA and ZERO-HA variants) with regulatory subunit of PP2A, PP2A-A, subunit \(-65\) K, shown by arrow) were revealed using 1/500 rabbit anti-PP2A-A antibody (Upstate). C: The present of catalytic subunit of PP2A, PP2A-C, subunit \(-36\) K, shown by arrow) was revealed in the IPs of STREX-HA and ZERO-HA channel \(\alpha\)-subunits with 1/500 mouse anti-PP2A-C antibody (Upstate). Western blot analysis: HRP-conjugated anti-rabbit antibody (1/3000), HRP-conjugated anti-mouse antibody (1/3000); Detection was by ECL.
As a further test to verify the association of STREX variant BK channel α-subunits with PP2A, the PP2A-A subunits were immunoprecipitated using rabbit anti-PP2A-A antibody (Upstate) and the IPs were analysed for the presence of BK channel proteins. SDS-PAGE and subsequent immunoblotting of IPs showed an immunoreactive band of ~132 K molecular weight corresponding to the STREX-HA BK channel α-subunits in extracts prepared from cells stably expressing STREX-HA variant BK channel α-subunits. No comparable immunoreactive band was found when using protein G-sepharose (PGS, beads) alone (figure 4.4), and no reaction was seen in blots of the IPs where the primary antibody was omitted (figure 4.4).

Analogous experiments were carried out to address the association of STREX BK channel α-subunits with PP1 using rabbit anti-PP1 antibody (Upstate) to immunoprecipitate PP1 from STREX-HA stable HEK 293 cells. The IPs revealed an immunoreactive band of ~132 K molecular weight corresponds to the STREX-HA variant BK channel α-subunit and no reaction was found upon omission of the primary antibody (figure 4.4).
Figure 4.4: Co-immunoprecipitation of PP2A and PP1 with STREX variant BK channel α-subunits

HEK 293 cells stably expressing STREX-HA variant BK channel α-subunits were immunoprecipitated with rabbit anti-PP2A-A antibody and rabbit anti-PP1 antibody (described in "Materials and Methods" section 2.4.5.2) or protein-G sepharose (beads, Sigma) alone. A: The lysate and IPs were subjected to SDS-PAGE on a 7.5% gel and transblotted (section 2.4.6). The blots were incubated with 1/1000 rabbit anti-HA antibody ("Materials and Methods" table 2.2) and a ~132 K (arrow) HA-immunoreactive band (plausibly corresponding to the STREX-HA BK channel α-subunit) was found in the IPs of PP2A-A and PP1. The specificity of IPs was shown by the absence of HA protein immunoreactivity in precipitations with protein-G sepharose alone. B: The specificity of Western blot was determined by incubation of the blots with HRP secondary antibody alone. Western blot analysis: Rabbit anti-HA antibody (1/1000), HRP-conjugated anti-rabbit antibody (1/3000). Detection was by ECL.
4.2.3 Which region of C-terminal of BK channel $\alpha$-subunit is required for association to PP2A?

The data reported above revealed that PP2A constitutively associates with STREX and ZERO BK channels in HEK 293 cells stably transfected with the respective BK channel $\alpha$-subunits (section 4.2.2).

To address which region of the BK channel is required for association to PP2A, HEK 293 cells were transiently transfected with the following STREX variant BK channels:

i) Full-length of STREX-HA variant BK channel $\alpha$-subunits.

ii) STREX-HA variant BK channel $\alpha$-subunits truncated at Glycine 1020, (G1020-HA channel: lacks C-terminal 202 amino acid residues and contains domains such as S7-S10, RCK domain, calcium bowl and STREX splice insert, refer to figure 1.6).

iii) STREX-HA variant BK channel $\alpha$-subunits with no C-terminal, truncated at amino acid 322 of C-terminal (I322-HA channel, lacks C-terminal 900 amino acid residues after the transmembrane S6 helix, refer to figure 1.6).

Full-length STREX-HA BK channel $\alpha$-subunits and G1020-HA channels were immunoprecipitated with sheep antisera (section 2.4.5.1) and I322-HA channels with rabbit anti-HA antibody (section 2.4.5.3). The expression of HA-tagged channels and PP2A-C subunit was assessed by Western blot analysis using rabbit anti-HA antibody and mouse anti-PP2A-C antibody. A representative experiment is illustrated in figure 4.5. HA-tagged channels were detected in the IPs of STREX-HA BK channel $\alpha$-subunit (~132 K), G1020-HA channel (~106 K) and I322-HA channel (~35 K). PP2A-C protein band (~36 K) was detected in the IPs of STREX-HA BK channel and G1020-HA
Figure 4.5
Co-IP of PP2A-C subunit requires C-terminal of BK channel $\alpha$-subunit

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<td>G1020</td>
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**A**
Blot: anti-HA

**B**
Blot: anti-PP2A-C

STREX-HA channel $\alpha$-subunit

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~132 K

G1020-HA channel $\alpha$-subunit

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~106 K

I322-HA channel $\alpha$-subunit

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~35 K

**Figure 4.5: Co-IP of PP2A-C subunit requires C-terminal of BK channel $\alpha$-subunit**

HEK 293 cells were transiently transfected with expression plasmids for 1) STREX-HA BK channel $\alpha$-subunits 2) G1020-HA channel, STREX-HA BK channel $\alpha$-subunits truncated at Glycine 1020 of C-terminal 3) I322-HA channel, STREX-HA BK channel $\alpha$-subunits truncated at isoleucine 322 of C-terminal 4) pcDNA3 vector (as control cells) (section 2.3.1). The transfected cells were lysed in 1% Triton X-100 lysis buffer (section 2.4.1). STREX-HA channel, G1020-HA channel and pcDNA3 vector cell lysates were immunoprecipitated with sheep...
antisera anti-BK channel α-subunits (section 2.4.5.1). I322-HA channels were immunoprecipitated with rabbit anti-HA antibody (section 2.4.5.3). The IPs were run on 12.5% SDS-PAGE gel, transferred to FL-PVDF membrane and detected by LI-COR (section 2.4.6). A: The HA-reactive protein bands, indicated by black arrows, were detected at 1) a ~106 K in G1020-HA HEK 293 cells 2) a ~132 K in STREX-HA HEK 293 cells and 3) a ~35 K in I322-HA HEK 293 cells, using rabbit anti-HA antibody ("Materials and Methods" table 2.2). B: a ~36 K band corresponding to PP2A-C was revealed in the IPs of STREX-HA channel and G1020-HA channel, indicated by black arrows, using 1/500 mouse anti-PP2A-C antibody ("Materials and Methods" table 2.2). Two protein bands with molecular weights of 50 kDa and 25 kDa (red arrows) can be visualized, which correspond to the heavy and light chains, respectively, of IgG. Western blot analysis: First antibodies were detected by IrDye800-labelled anti-rabbit antibody (1/3000), Alexa680-conjugated anti-mouse antibody (1/3000), LI-COR detection.
Figure 4.6
Reciprocal Co-immunoprecipitation of PP2A-A with STREX BK channel α-subunit

HEK 293 cells were transiently transfected with STREX-HA channel α-subunits, G1020-HA channel, I322-HA channel constructs (in pcDNA3) and pcDNA3 alone as control (described in section 2.3.1). The transfected cells were lysed in 1% Triton X-100 lysis buffer (described in section 2.4.1). The cell extracts were immunoprecipitated with anti-PP2A-A antibody (described in section 2.4.5.2). The IPs were subjected to 7.5% SDS-PAGE gel, following transferred to PVDF membrane and detected using ECL (section 2.4.6). A ~132 K HA-reactive band (top arrow) corresponding to STREX-HA variant channel detected in the IPs from HEK 293 cells transiently transfected with STREX-HA BK channel α-subunits. Also a ~106 K HA-immunoreactive protein band corresponding to G1020-HA variant channel (bottom arrow) was detected in the IPs from HEK 293 cells transiently transfected with G1020-HA variant channel. In contrast, no HA band was found in the IPs from HEK 293 cells transiently transfected with I322-HA variant channel or pcDNA3 alone. Western blot analysis: Rabbit anti-HA antibody (1/1000), HRP-conjugated anti-rabbit antibody (1/3000); Detection was by ECL.
channel. However, no co-immunoprecipitation of PP2A-C with I322-HA was found (figure 4.5).

Subsequent reciprocal immunoprecipitations were performed using rabbit anti-PP2A-A antibody (section 2.4.5.2). IPs were subjected to SDS-PAGE and Western blot analysis using rabbit anti-HA antibody (Zymed). The data showed co-immunoprecipitation of PP2A-A with STREX variant BK channel α-subunit and G1020-HA, but not with I322-HA subunits (figure 4.6).

Taken together, the results suggest that the association of PP2A with STREX variant BK channel α-subunit is between residues I322 and G1020.

4.2.4 Subcellular localisation of C-terminal truncated STREX variant BK channel α-subunit

To verify which segment of STREX variant BK channel is involved in the targeting of the channel to the plasma membrane, HEK 293 cells were transiently transfected with: i) Full-length STREX variant BK channel α-subunits ii) G1020-HA channels iii) I322-HA channels. Cells fixed on glass cover slips were processed for indirect immunofluorescence using rabbit anti-HA as the primary antibody. The bulk of full-length STREX-HA channel α-subunits was localised at or very near the plasma membrane (figure 4.7). G1020-HA channels showed similar targeting to the plasma membrane (figure 4.7). In contrast, STREX-HA channel α-subunits truncated at I322 were diffusely distributed throughout the cell, plausibly reflecting retention in the endoplasmic reticulum (ER) (figure 4.7).
Figure 4.7
Immunocytochemical localisation of C-terminal STREX variant BK channel α-subunits

HEK 293 cells were transiently transfected with STREX-HA variant BK channel α-subunits, G1020-HA channel and I322-HA channel. Immunocytochemistry was performed using rabbit anti-HA antibody (Zymed) for 3 h at room temperature and 30 min with Alexa Fluor® 488 anti-rabbit antibody (described in "Materials and Methods" section 2.4.9). The sections were viewed under a Zeiss LSM 510 confocal microscope. The bar denotes 10 micrometers. A: Fluorescence image of HEK 293 cells transiently transfected with STREX-HA variant BK channel α-subunits was prominently localised at the plasma membrane. B: The G1020-HA channel that lacking C-terminal 202 amino acid residues of α-subunit was also observed at the plasma membrane C: A shorter STREX-HA channel α-subunit (I322-HA channel, lacking almost the entire C-terminal tail) was largely retained in the endoplasmic reticulum (ER).
Thus, these data demonstrated that STREX-HA BK channel α-subunits are localised at the plasma membrane and that in addition to the association with PP2A the C-terminal domain of the STREX-HA channel α-subunit between I322 and G1020 is critically involved in trafficking of the channel to the plasma membrane.

In summary, the results indicate that STREX BK channel α-subunits localised to the plasma membrane associate with PP2A, whereas the variant that showed retention in the cytoplasm does not interact with PP2A.

4.2.5 No effect of dexamethasone on the amount of immunoreactive PP2A associated with STREX BK channels

To address whether glucocorticoids modify the association of PP2A with the BK channel, STREX-HA and ZERO-HA stable HEK 293 cells were pretreated with 100 nM dexamethasone or vehicle (0.001% DMSO) for 120 min under the same conditions used for previous electrophysiological studies that showed dexamethasone induced blockade of PKA-mediated inhibition of STREX variant BK channels through PP2A (Tian et al., 2001b). STREX-HA and ZERO-HA variant BK channel α-subunits were immunoprecipitated with the sheep antisera (described in section 2.4.5.1). The presence of the PP2A-C subunit in the IPs was probed with mouse anti-PP2A-C antibody.

The PP2A-C and HA bands (corresponding to BK channel α-subunits) in each blot were outlined and the optical density of each band was determined by means of the ImageJ software.
Figure 4.8
Effect of dexamethasone on expression of PP2A-C associated with STREX variant BK channel α-subunits

HEK 293 cells stably expressing STREX-HA variant BK channel α-subunits were pretreated with 100 nM dexamethasone or vehicle (0.001% DMSO) for 120 min. The cells were lysed in 1% Triton X-100 lysis buffer (section 2.4.1) and cell extracts were immunoprecipitated using sheep antisera anti-BK channel α-subunits (anti-STREX + anti-S869) (section 2.4.5.1). IPs were run on 12.5% SDS-PAGE gel and transferred to FL-PVDF membrane (section 2.4.6). The blots were probed for HA-tagged channel (A) and PP2A-C subunit (B) by Western blotting. HA-tagged channels (~132 K, black arrow) and PP2A-C (~36 K, black arrow) were detected in IPs of STREX-HA stable HEK 293 cells pretreated with vehicle or dexamethasone. C: The ratio of O.D of PP2A-C to STREX-HA channel at vehicle group was calculated and the corresponding ratio for dexamethasone 120 min was expressed as percentage of this value. Data showed no change between vehicle and dexamethasone 120 min. Data are Means ± S.E.M, (p<0.05, t test, n=3/group). Two protein bands of IgG (50 kDa and 25 kDa) are showed with red arrows. Western blot analysis: Rabbit anti-HA antibody (1/1000), Mouse anti-PP2A-C antibody (1/500), IrDye 800-labelled anti-rabbit antibody (1/3000), Alexa680-conjugated anti-Mouse antibody (1/3000), LI-COR detection.
Figure 4.9
Effect of dexamethasone on expression of PP2A-C associated with ZERO variant BK channel α-subunits

HEK 293 cells stably expressing ZERO-HA variant BK channel α-subunits were pretreated with 100 nM dexamethasone or vehicle (0.001% DMSO) for 120 min. The treated cells were lysed in 1% Triton X-100 lysis buffer (section 2.4.1) and cell extracts were immunoprecipitated by sheep antisera (section 2.4.5.1). IPs were run on 12.5% SDS-PAGE gel and transferred to FL-PVDF membrane (section 2.4.6). The blots were probed for HA-tagged channels (A) and PP2A-C subunits (B) by Western blotting. HA-tagged channels (a ~125 K corresponding to ZERO-HA variant BK channel α-subunits, black arrow) and a ~36 K corresponding to PP2A-C (black arrow) were detected in IPs. C: The ratio of O.D of PP2A-C to ZERO-HA channel at vehicle was calculated and the corresponding ratio for dexamethasone 120 min was expressed as percentage of this value. Data showed no change between vehicle and dexamethasone 120 min. Data are Means ± S.E.M, (p<0.05, t test, n=3/group). Two protein bands of IgG (50 kDa and 25 kDa) are showed with red arrows. Western blot analysis: Rabbit anti-HA antibody (1/1000), Mouse anti-PP2A-C antibody (1/500), IrDye 800-labelled anti-rabbit antibody (1/3000), Alexa 680-conjugated anti-Mouse antibody (1/3000), LI-COR detection.
The data showed that 100 nM dexamethasone for 120 min had no effect on BK channel α-subunit protein expression in STREX-HA stable HEK 293 cells or ZERO-HA stable HEK 293 cells (equal protein loading was verified by Coomassie blue staining). To determine the effect of dexamethasone on the level of PP2A-C subunit associated with BK channels, the ratio of optical densities the PP2A-C and the HA bands were compared for the different treatment groups. The data indicated that dexamethasone treatment for 120 min did not change the amount of PP2A-C subunit associated with STREX-HA BK channel α-subunits (figure 4.8) or ZERO-HA BK channel α-subunits (figure 4.9).

4.2.6 Evidence for the association of PP2A-C subunits with native STREX variant BK channels in mouse corticotroph tumour (AtT20) cells

As in transfected HEK 293 cells the level of expression of the transfected protein can be over 20-times the physiological levels (Antoni et al., 1998), it was important to assess the association of endogenously expressed BK channels with endogenous PPase. Mouse AtT20 corticotroph cells express predominantly STREX variant BK channel mRNA and endogenous BK channels in these cells display characteristics similar to the STREX variant expressed in HEK 293 cells including the same regulation by PKA and PP2A (Tian et al., 2001b). Mouse AtT20 corticotroph cells were pretreated with 100 nM dexamethasone or vehicle (0.001% DMSO) for 120 min. The native BK channels were immunoprecipitated using sheep antisera against the BK channel α-subunits (anti-STREX + anti-S869) (described in section 2.4.5.1). Control, STREX-HA stable HEK 293
Figure 4.10
Endogenous co-IP of BK channel with PP2A-C subunit in AtT20 cells

AtT20 cells were pretreated with 0.001% DMSO (Vehicle) or 100 nM dexamethasone for 120 min. Native BK channels were immunoprecipitated with sheep antisera anti-BK channel α-subunits (anti-STREX + anti-S869) (section 2.4.5.1). STREX-HA stable HEK 293 cell lysates were used parallel as control. The specificity of IP antisera in AtT20 cells was checked using IP with sheep non-immune serum. The lysates and IPs were separated by electrophoresis (12.5% SDS-PAGE gel) and analysed by Western blotting (section 2.4.6). A: BK channel α-subunits (black arrow) were detected using rabbit anti-BK channel C-terminal antibody (Chemicon) ("Materials and Methods" table 2.2). No band was detected in IPs of non-immune serum in AtT20 cells. B: The association of PP2A-C subunit with BK channel α-subunits were detected by mouse anti-PP2A-C antibody ("Materials and Methods" table 2.2) as shown using black arrow. Two protein bands of IgG (50 kDa and 25 kDa) are showed with red arrows. Western blot analysis: Rabbit anti-BK channels C-terminal antibody (1/300), mouse anti-PP2A-C antibody (1/500), HRP-conjugated anti-rabbit antibody (1/1000) and HRP-conjugated anti-Mouse antibody (1/3000), Detection was by ECL.
cells were used as positive controls. The BK channel α-subunits were detected with an antibody against the C-terminus (CHEMICON) (Ling et al., 2003; Friis et al., 2003). The immunoreactive bands found were similar to those observed in STREX-HA stable HEK 293 cells. Furthermore, no immunoreactive band was found in the IPs with preimmune sera in AtT20 cells (Figure 4.10). The IPs contained a ~36 K band reactive with anti-PP2A-C subunit (figure 4.10). Once more, dexamethasone had no effect on the apparent amount of PP2A-C co-immunoprecipitating with the α-subunit (figure 4.10) at any of the time-points examined.

In summary, Single-channel analysis from AtT20 cells (Tian et al., 2001b) and rat cerebellar Purkinje neurons (Widmer et al., 2003) demonstrated that the activity of BK channels is regulated by multiple endogenous protein kinases and protein phosphatases in the membrane patch. These channels were regulated by PKA associated with the patch and the extent of PKA activation was limited by an opposing endogenous PP2A. Furthermore, it has been shown that in hippocampal CA1 neurons and AtT20 cells (Joels & de Kloet., 1989; Shipston et al., 1996; Tian et al., 1998), the activity of BK channels is modulated by glucocorticoid-induced signalling pathways. Single-channel recording in HEK 293 cells also revealed the same glucocorticoid-induced signalling pathway (Tian et al., 2001b). However, the underlying mechanism(s) are poorly understood. Our results showed that PP2A is constitutively associated with STREX variant as well as ZERO variant BK channel α-subunit in transfected HEK 293 cells. Similar data were also obtained in AtT20 cells, where the levels of STREX variant are in the
physiological range. Dexamethasone, which has marked PPase-dependent effects on the electrophysiological properties of the STREX variant BK channel failed to change the apparent amount of immunoreactive PP2A co-immunoprecipitated with the channel α-subunit. Thus, it is reasonable to hypothesize that dexamethasone may alter the enzymatic activity rather than the amount of the PP2A associated with the STREX BK channel.

4.3 Discussion

In an attempt to examine the association of PP2A with BK channels in HEK 293 cells, we demonstrated that i) Endogenous serine/threonine PP2A is constitutively associated with both STREX and ZERO variant BK channel α-subunit. ii) PP2A association requires amino acids 322-1020 from the cytoplasmic C-terminal domain of the STREX BK channel α-subunit. iii) The same segment of the STREX BK channel α-subunit is involved in trafficking of the channel protein to the plasma membrane. iv) Glucocorticoid action does not appear to involve the recruitment of the PP2A complex to the BK channel; rather it could entail stimulation of constitutively associated PPase activity.

4.3.1 Endogenous serine/threonine PP2A is constitutively associated with BK channel α-subunit

Electrophysiological studies have indicated that BK channels are regulated by PP2A and/or PP1 activity closely associated with the BK channel complex (Shipston., 2001; Tian et al., 2001b; Schubert and Nelson., 2001; Hall and Armstrong., 2000; Levitan., 1999; Wang et al., 1999). Although BK channels have been demonstrated to be
regulated by endogenous protein kinases and protein phosphatases in a number of systems (Tian et al., 2001b; Ling et al., 2000; Wang et al., 1999; White et al., 1991), biochemical demonstration of the association of BK channel with protein phosphatase(s) has not been previously reported.

In order to address this issue immunoprecipitations were carried out which is an established method for the analysis of protein/protein interactions. We immunoprecipitated the BK channel using antisera directed against the BK α-subunit and probed for the presence of co-immunoprecipitating PP2A protein subunits.

Our biochemical analysis revealed that PP2A is constitutively associated with both STREX and ZERO variant BK channels, as immunoblotting of cell lysates and IPs of STREX and ZERO variant BK channel α-subunits from HEK 293 cells stably expressing the STREX and ZERO variant BK channel α-subunits resulted in specific co-immunoprecipitation with PP2A-A and PP2A-C subunits. Furthermore, IPs of PP2A also confirmed the association of PP2A with STREX variant BK channel α-subunits.

The specificity of the co-IP was verified extensively.

1) Specificity of IPs of BK channel α-subunits were confirmed by no immunoreactive band in the i) IPs with preimmune sera from respective sheep ii) IPs with the same antisera from wild-type HEK 293 cells or HEK 293 cells transfected with pcDNA3. iii) IPs of beads (PGS) alone.

2) The specificity of Western blotting was also verified.
4.3.2 PP2A association requires the cytoplasmic C-terminal segment of the BK channel α-subunit

Which region of C-terminal of STREX variant channel is involved in association of with PP2A? To address this, we used truncated STREX variant BK channel α-subunits. We found STREX variant BK channel α-subunits truncated at Glycine 1020 (G1020 variant channel) have ability to interact with PP2A. Thus, the terminal 202 residues are not required for the association of the channel with PP2A.

In contrast, STREX variant BK channel α-subunit truncated at isoleucine 322 with no C-terminal tail (I322 variant channel) does not have the ability to interact with PP2A. Thus, the C-terminal tail of the STREX variant BK channel α-subunit is necessary for the interaction with PP2A.

Taken together the co-immunoprecipitation experiments with G1020 and I322 truncations suggest that residues 322-1020 of the cytoplasmic domain of the STREX BK channel α-subunit are necessary and adequate for the association of PP2A with the BK channel.

Finally, so far the results showed the interaction of A and C subunits of PP2A with BK channel α-subunit, therefore to check the association of β subunits of PP2A with BK channel, further biochemical study in HEK 293 cells using co-immunoprecipitation of β subunit of PP2A with BK channel α-subunit is required.

4.3.3 Cytoplasmic tail of STREX variant BK channel α-subunit is necessary for its cell surface expression
In polarized epithelial cells of the cortical collecting duct of the kidney, BK channels are located in the apical cell membrane (Wang et al., 2003; Woda et al., 2001). Moreover, in COS-7 cells (Kwon and Guggino., 2004) and Madin-Darby canine kidney cells (Bravo-Zehnder et al., 2000), it has been shown that the cytoplasmic C-terminal of the BK channel α-subunit is involved in BK channel expression to the cell surface.

To address which region of C-terminal of BK channel α-subunit is involved for trafficking of the channel to the plasma membrane, we focused on the residues 322-1020 in the C-terminal of the STREX BK channel α-subunit which were required for association with PP2A (section 4.3.2). Thus we immunostained STREX-HA variant BK channel α-subunits and STREX-HA variant BK channel α-subunits truncated at the C-terminus (G1020 variant and I322 variant channels).

The results demonstrated that i) STREX BK channel α-subunit expresses in the plasma membrane ii) G1020 variant channel expresses in the plasma membrane as well. Thus, the missing 202 residues of C-terminal in G1020 variant channel are not required for plasma membrane targeting. iii) STREX BK channel α-subunit truncated at I322, lacking almost the entire C-terminal tail, was largely retained in the endoplasmic reticulum and largely failed to reach the plasma membrane.

Taken together, the localisation studies revealed that the residues 322-1020 of cytoplasmic tail of STREX BK channel α-subunit is required for trafficking of the STREX BK channel to the plasma membrane.

It has been reported that the C-terminal of BK channel α-subunit after residue 1117 is essential for plasma membrane targeting in COS-7 cells (Kwon and Guggino., 2004).
This is in contrast with our study that shows that 202 residues of C-terminal after residue glycine 1020 are not critical for the targeting of STREX BK α-subunits to the plasma membrane. Many reasons may be involved for this different trafficking of C-terminal truncated BK channel in these two studies. i) Different trafficking may be mediated by different sensitivity of antibody used in two immunostainings (rabbit anti-HA antibody in present study and rabbit anti-myc antibody in Kwon and Guggino’s results). ii) Different cells used in these two studies also may be involved in different trafficking. To address this, further immunocytochemistry study in COS-7 cells transfected with G1020-HA variant BK channel could resolve this issue.

4.3.4 Glucocorticoid action does not appear to involve the recruitment of PP2A complex to the BK channel

This study demonstrates that; i) PP2A and PP1 are constitutively associated with BK channel. ii) Glucocorticoid does not involve the recruitment of PP2A to the BK channel complex in HEK 293 cells or in native BK channel cells, AtT20 cells.

It has been reported that the blockade of PKA-mediated inhibition of STREX BK channel activity by dexamethasone is mediated by PP2A-like activity intimately associated with the channels (Tian et al., 2001b). Analysis of IPs for PP1 in the present study suggested much lower abundance when compared with PP2A. Thus, taken together suggest that PP2A is main protein phosphatase for regulation of BK channels. Therefore, we have analyzed IPs of BK channel α-subunits pretreated with dexamethasone for PP2A immunoreactivity.
Our results with HEK 293 cells and AtT20 cells show that PP2A is constitutively associated with the STREX variant BK channel α-subunits and dexamethasone fails to change the apparent amount of immunoreactive PP2A associated with the channel.

Single-channel recording in AtT20 cells (Tian et al., 1998) and HEK 293 cells (Tian et al., 2001b) revealed that the STREX BK channel splice variant is potently inhibited by PKA activity intimately associated with the channel complex and the extent of PKA inhibition is limited by PP2A. Intriguingly the action of the PP2A is only evident in dexamethasone treated cells. Thus the results of electrophysiological work (Tian et al., 2001b; Tian et al., 1998) and our biochemical studies in AtT20 cells and HEK 293 cells suggest that the regulation of BK channels by glucocorticoid is likely to be due to changes in the enzymatic activity of the associated PP2A rather than the recruitment of more PP2A to BK channel.

In order to confirm this, we propose to analyse protein phosphatase activity (by using a malachite green phosphatase assay) of IPs of BK channels treated with dexamethasone for different times in HEK 293 cells (transfected with BK channels) and in AtT20 cells (as a native source of BK channels).
Chapter Five

General discussion & future work
Chapter Five

General discussion & future work

Steroid hormones are fundamentally important for body homeostasis by regulating the expression of a large number of genes. Glucocorticoid hormones, a class of steroid produced in the adrenal cortex, are pivotal for adaptation to environmental challenge in a wide range of tissues, but the understanding of the mode of action of these steroids is incomplete.

Identification of the BK channel complex as a target for glucocorticoid action should allow us to characterize glucocorticoid-induced proteins involved in the control of ion channel activity and provide further insights into the mechanisms of the regulation of cellular excitability by glucocorticoids.

Thus, the aim of the present project was to investigate the expression of glucocorticoid-induced proteins and the regulation of BK channels by glucocorticoid hormones using molecular and biochemical approaches in HEK 293.

HEK 293 cells

The characteristic of glucocorticoid modulation of the cAMP-dependent control of BK channels in HEK 293 cells were identical to those observed in AtT20 cells (Shipston et al., 1996; Tian et al., 2001a). Moreover, the same protein phosphatase dependent signaling cascades on BK channels are induced by glucocorticoids in AtT20 cells (Tian et al., 1998) and HEK 293 cells (Tian et al., 2001b).
As the level of endogenous BK channel in AtT20 cells are relatively low, an important advance for the biochemical analysis of glucocorticoid signalling to BK channels would be the reconstruction of glucocorticoid regulation in a model system that expresses high levels of transfected protein. Therefore, HEK 293 cells (which do not endogenously express BK channel subunits) are a suitable model for the analysis of glucocorticoid regulation of BK channels, and possibly other, ion channels.

In contrast, there are some disadvantages of using HEK 293 cells. i) HEK 293 cells express the glucocorticoid receptor (type II) and mineralocorticoid receptor, MR (Tian et al., 2001b; Kolla and Litwack, 2000). Glucocorticoids cortisol and corticosterone have affinity for binding to the MR (Myles and Funder, 1994) and MR binds specifically to GREs (Lombes et al., 1993). Thus, binding of glucocorticoids to MR and following binding of MR to GREs may alter the response of glucocorticoid receptors to glucocorticoids in HEK 293 cells. ii) High level of BK channels in transfected HEK 293 cells may lead to non specific protein-protein interaction and therefore, regulation of BK channels.

Therefore, to solve this issue, we checked the results of HEK 293 cells with AtT20 cells as native BK channels cells.

The main findings in this project were as follows:

i) In HEK 293 cells, dexamethasone exerts its actions via endogenous glucocorticoid receptors in a concentration- and time-dependent manner.
ii) High levels of Dexras1 mRNA are expressed by HEK 293 cells and exposure of the cells to dexamethasone has no effect on the levels of Dexras1 mRNA.

iii) In HEK 293 cells, SGK-1 mRNA levels are increased by dexamethasone in a time- and concentration-dependent manner.

iv) The amount of SGK-1 protein in a Triton X-100 insoluble fraction was increased by dexamethasone.

v) Endogenous SGK-1 is not associated with STREX BK channel α-subunits and dexamethasone does not lead to association of SGK-1 protein with the channel.

vi) Endogenous serine/threonine PP2A is constitutively associated with STREX and ZERO BK channel α-subunits in HEK 293 cells as well as AtT20 cells.

vii) PP2A association with STREX BK channel α-subunits requires the cytoplasmic C-terminal domain of the α-subunit (residues 322-1020). These residues are also involved in the trafficking of the α-subunit to the plasma membrane.

viii) In HEK 203 cells, treatment with dexamethasone did not alter the amount of PP2A-A or C subunits associated with the α-subunit of the BK channel.

There is consonant evidence from hippocampal CA1 neurons (Joëls and De Kloet., 1989) and neuroendocrine tumour (AtT20) cells (Shipston et al., 1996) which shows that glucocorticoid action is mediated by type II glucocorticoid receptors, occurs within 2 h
and requires de novo synthesis of mRNA as well as protein. In this study, the MMTV-LTR luciferase reporter plasmid data showed that HEK 293 cells contain endogenous glucocorticoid receptors and are maximally activated by 100 nM dexamethasone within 2 h. Therefore, 100 nM dexamethasone (up to 2 h exposure) was used in this study to investigate the expression of glucocorticoid-induced proteins and the regulation of BK channels by dexamethasone in HEK 293 cells.

The levels of various mRNAs such as SGK-1 (Webster et al., 1993a) and Dexras1 (Kemppainen and Behrend., 1998) are reportedly increased by glucocorticoids (within 2 h) but there is currently no published information whether or not the SGK-1 or Dexras1 are glucocorticoid-induced proteins in HEK 293 cells.

**Dexras1**

Dexras1, a newly identified member of the Ras superfamily of proteins, was discovered in AtT-20 corticotroph cells in 1998 as a gene rapidly induced by dexamethasone (Kemppainen and Behrend., 1998). Several nucleotide sequences predicting proteins with high degrees of homology to mouse Dexras1 have been reported, including human Dexras1 (GenBank Accession No. AF089506) and rat Dexras1 (GenBank Accession No. AF239157), which share 98% homology with mouse Dexras1 (Cismowski et al., 1999). Dexras1 is expressed in several tissues including heart, liver, skeletal muscle, kidney, pancreas cells (Tu and Wu., 1999) and the pituitary gland (Kemppainen et al., 2000). Recently, a glucocorticoid response element has been identified in the 3'-flanking region of the human Dexras1 gene (Kemppainen et al., 2003).
The fact that the Dexras1 gene is rapidly and positively responsive to glucocorticoids suggests that this molecule may serve a role mediating effects of these steroids. As the aim of this thesis project was to investigate the mechanisms by which glucocorticoid hormones regulate the activity of BK channels in HEK 293 cells, we addressed whether or not Dexras1 is a glucocorticoid-inducible protein in these cells. For this aim, Dexras1 mRNA was examined by RT-PCR and Northern blot analysis in HEK 293 cells.

Although full-length Dexras1 mRNA could not be detected by RT-PCR in HEK 293 cells, experiments with overlapping primer pairs clearly indicated the presence of full-length Dexras1 mRNA. The Northern mRNA analysis also confirmed the presence of full-length Dexras1 mRNA in HEK 293 cells. Several different factors might have caused the failure to amplify the full length mRNA in RT-PCR, for example; insufficient template, insufficient primer, poor primer design and contamination that inhibits the DNA polymerase.

Northern mRNA in AtT-20 corticotroph cells showed that dexamethasone increases the level of Dexras1 mRNA (40-fold) within 30 min, with levels declining sharply after 2 h of treatment (Kemppainen and Behrend., 1998), but Northern mRNA analysis in the present project revealed no change in the level of Dexras1 mRNA in response to 100 nM dexamethasone in HEK 293 cells. It is reasonable to suggest that the lack of a change of Dexras1 mRNA in response to dexamethasone may be due to the high basal level of Dexras1 mRNA expressed in these cells. Varying of the conditions of the experiment to achieve low basal Dexras1 mRNA level may lead to a system where Dexras1 is induced.
No induction of Dexras1 mRNA strongly suggests that under the same conditions in which dexamethasone regulates BK channel in HEK 293 cells, Dexras1 does not play a major role. However, experiments with Dexras1 gene knock-down technology should be used to address a possible involvement of Dexras1 in glucocorticoid action in HEK 293 cells.

SGK-1
Previous studies have reported the rapid induction of SGK-1 in response to dexamethasone in various cell types (Chen et al., 1999b; Webster et al., 1993a); however there was no evidence available for HEK 293 cells. In the present study, Northern mRNA and Western immunoblots of SGK-1 revealed that SGK-1 is a potential mediator of glucocorticoid action in HEK 293 cells. Because of constraints of time the issue as to whether SGK-1 is a genuine early gene-induction by dexamethasone or involves another mRNA and protein was not probed with blockers of protein synthesis. However, the significant rise of SGK-1 mRNA within the first 15 min of dexamethasone exposure strongly suggests that this is indeed a primary gene induction.

The potential functional role of SGK-1 as a mediator of glucocorticoid action in HEK 293 cells should be explored. As there are no specific pharmacological blockers of SGK-1 activity, a genetic knock-down approach such as anti-sense RNA or double stranded RNA interference would be required. Provided these manipulations bring about the desired reduction of SGK-1 protein levels, the effects of these manipulations on glucocorticoid induced changes in the characteristics of the BK channel could be analysed. A major challenge in this context would be to prove that it is indeed the
glucocorticoid regulated pool of Triton X100 insoluble SGK-1 protein that is contributing to the glucocorticoid effect. Virtually nothing is known about the intracellular targeting of this kinase to intracellular compartments. It is of note, that SGK-1 activity is controlled via the PI3-kinase signaling pathway (Gamper et al., 2002b; Park et al., 1999; Kobayashi et al., 1999), which is known to influence the membrane association of various signaling proteins. Thus, inhibition of PI-3 kinase activity by drugs such as wortmannin or LY294002 and monitoring of SGK-1 levels in the Triton X-100 insoluble fraction may shed light on this question. A further issue would be to prove that it is indeed the protein kinase activity of SGK-1 that is involved. For this purpose, dominant negative mutants lacking kinase activity (Park et al., 1999) could be transfected and the impact on dexamethasone mediated modulation of BK-channel activity analysed.

Should a role for SGK-1 in dexamethasone action on BK channels emerge from the experiments outlined above, it would be important to determine the target(s) of SGK-1 phosphorylation. The present project demonstrated no association of STREX BK channel ø-subunit with SGK-1 protein in HEK 293 cells. Furthermore, the STREX BK channel ø-subunit, which is sufficient for dexamethasone mediated regulation of BK channel activity, contains no consensus sites for phosphorylation by SGK-1. Thus, any involvement of SGK-1 in the regulation of the BK channel is likely to be indirect, i.e. through the phosphorylation of molecules distinct from channel subunits. Mass spectrometric identification of the proteins co-immunoprecipitating with BK channels may help to uncover the nature of proteins that contribute to the BK channel signaling complex and whether or not these might be substrates of SGK-1.
Interaction of PP2A with BK channel γ-subunit

Reversible protein phosphorylation represents a fundamental cellular regulatory mechanism to control the activity and function of plasma membrane ion channels (Levitan., 1999). The co-ordination, specificity, and compartmentalization of ion channel regulation by reversible protein phosphorylation is facilitated by assembly with signaling complexes comprising cognate protein kinases and protein phosphatases. BK channels have been widely exploited as models of ion channel regulation by reversible protein phosphorylation through the action of multiple protein kinases and phosphatases that are associated with the channel complex (Widmer et al., 2003; Shipston., 2001; Tian et al., 2001a; Hall and Armstrong., 2000; Levitan., 1999; Wang et al., 1999; Reinhart and Levitan., 1995; Bielefeldt and Jackson., 1994; White et al., 1991) For example, single-channel analysis from inside-out patches isolated from the soma of dissociated rat cerebellar Purkinje neurons demonstrated that the activity of BK channels is regulated by PKA and PP2A in the membrane patch (Widmer et al., 2003).

In mammalian pituitary tumour cells, somatostatin (an agonist of Gi-coupled receptor) increases the activity of BK channels through dephosphorylation of a cAMP-dependent phosphorylation site in the BK channel protein or a closely associated regulatory molecule by one or more protein phosphatases (White et al., 1991). In AtT20 cells (Tian et al., 1998) and HEK 293 cells (Tian et al., 2001b), the STREX BK channel α-subunit is potently regulated by PKA and protein phosphatase activity intimately associated with the channel complex.
Although BK channels are potently regulated by dephosphorylation by multiple protein phosphatases (Widmer *et al.*, 2003; Tian *et al.*, 2001b; Tian *et al.*, 1998; White *et al.*, 1991), the molecular identity of protein phosphatase(s) assembled with the BK channel complex is unknown.

The dynamic interactions of protein phosphatases with the BK channel complex provide an important mechanism to tune BK channel function and behaviour. Thus, in order to address the association of BK channel with protein phosphatase(s), immunoprecipitations, which is an established method for the analysis of protein/protein interactions, with antisera directed against the BK channel α-subunits were carried out in HEK 293 cells as the model system for glucocorticoid-action.

In the present study, the ability of both STREX and ZERO BK channel splice variants α-subunits to co-immunoprecipitate endogenous catalytic and regulatory subunits of PP2A demonstrated that endogenous PP2A is in constitutive association with both types of BK channel α-subunits tested. This result supports previous electrophysiological and pharmacological data showing that BK channel function is regulated via endogenous PP2A-like phosphatase activity associated with the channel (Tian *et al.*, 2001b). In this context two issues are of importance, firstly the mode of association of PP2A with the channel and secondly the regulation of PP2A activity associated with the channel.

BK channels show considerable diversity in their modulation by distinct kinase/phosphatase signaling pathways (Shipston., 2001; Levitan., 1999). For example, Serine-942 (S942) of the drosophila BK channel was identified as a putative site of
direct phosphorylation by PKA following the inhibition of the PKA-mediated response (Nara et al., 1998; Esguerra et al., 1994).

The alternative splicing at site of splicing C2 in the mouse BK channel C terminus generates five distinct splice variants: ZERO, e20, e21(STREX), e22, and e23. These splice variants are differentially sensitive to phosphorylation by endogenous cAMP-dependent protein kinase; ZERO, e20, and e22 variants are all activated, whereas e21 (STREX) is the only variant that is inhibited (Chen et al., 2005). The distinct response of the STREX and ZERO channel α-subunits to PKA is the result of the differential phosphorylation of specific PKA consensus sites (S4_{STREX} and S869) within the splice variants (Tian et al., 2001b). The activation of ZERO BK channel by cAMP-dependent protein kinase is dependent upon a conserved C-terminal PKA consensus motif (S869), whereas inhibition of STREX BK channels by cAMP-dependent protein kinase is dependent to a PKA consensus site in the STREX insert (S4_{STREX}) of the C-terminus (Tian et al., 2001a).

Throughout this study, the data demonstrated that the cytoplasmic C-terminal segment (residues 322-1020) of the STREX variant BK channel α-subunits, which contain S4_{STREX} and S869, was necessary for the association of the channel with PP2A. Thus, in order to address whether the PKA consensus sites (S4_{STREX} and S869) are or are not target for interaction of PP2A with STREX and ZERO BK channel α-subunits, further co-immunoprecipitations of PP2A with STREX and ZERO variants BK channel α-subunits in which the S4_{STREX} and S869 sites have been mutated to alanine (S869A and S4_{STREX}A) could be performed to clarify this issue.
Recently a structural motif, the leucine zipper (LZ) in the C-terminus of BK channels, originally described in classes of DNA-binding proteins (Landschulz et al., 1988), has been reported to play an important role in coordinating both the assembly of ion channels as well as their interaction with protein kinase and protein phosphatase signaling complexes (Tian et al., 2003; Marx et al., 2002; Hulme et al., 2002; Marx et al., 2001; Simmerman et al., 1996). It has been demonstrated that Leucine zipper 1 (LZ1, residues 513-543) is required for the association and regulation of the channels by PKA, and other putative leucine zippers in the BK channel protein may provide anchoring for further regulatory enzyme complexes (Tian et al., 2003). Thus, the use of LZ may be found to be a common mechanism of regulating and mediating protein-protein associations with profound implications, especially to the elucidation of the functioning and component identification of dynamic signaling complexes.

In this study, the data demonstrated that PP2A constitutively associated with the cytoplasmic C-terminal domain of STREX variant BK channel α-subunits, which contains LZ1 and LZ2 (Tian et al., 2003). Thus, to address whether LZ1 or LZ2 is required for interaction of PP2A with BK channels, Western immunoblots of co-immunoprecipitations of PP2A with BK channels from LZ1 or LZ2 mutant BK channel α-subunits could be carried out.

Protein kinases and protein phosphatases may associate with ion channels directly (Levitan., 1999; Wang et al., 1999) or through adaptor proteins, for example A kinase anchoring proteins (AKAPs) (Hulme et al., 2002; Marx et al., 2001; Fraser and Scott., 1999). In order to elucidate whether PP2A binds directly or through an intermediary
protein to BK channels, a blot-overlay assay using recombinantly expressed and purified STREX variant BK channel C-terminal α-subunit proteins could be devised to identify proteins that bind directly to the α-subunit, rather than through an intermediary protein.

Cytoplasmic tail of the STREX variant BK channel α-subunit is necessary for its cell surface expression

It has been demonstrated that the C-terminal of BK channel is necessary for the targeting of the channel to the plasma membrane (Chen et al., 2005; Kwon and Guggino., 2004; Bravo-Zehnder et al., 2000). However, the structural determinants that are involved in the trafficking of the channel to the plasma membrane are largely unknown.

Immunocytochemical studies of HEK 293 cells transiently expressing ZERO, STREX splice variants showed that ZERO and STREX splice variants of BK channel α-subunit are expressed at the cell surface (Chen et al., 2005). In addition, immunocytochemistry study of two isoforms of BK channel α-subunit (rbslo1 and rbslo2) from rabbit renal cells showed that rbslo1 (NCBI accession number AF201702), which is highly homologous to BK channels from other species such as mslo1 and hslol, expresses in plasma membrane. Whereas the rbslo2, which was truncated at position Gly781, was localised in the endoplasmic reticulum and Golgi region (Wang et al., 2003).

In Madin-Darby canine kidney cells (Bravo-Zehnder et al., 2000), it has been shown that the BK channel α-subunit truncated immediately after the S6 transmembrane region
(after residue 323) loses the capability for transport to the cell surface and is retained in the endoplasmic reticulum. Our results also demonstrated that STREX BK channel α-subunit truncated at I322, lacking almost the entire C-terminal tail, is largely retained in the endoplasmic reticulum and failed to reach the plasma membrane.

Furthermore, our results revealed that STREX BK channel α-subunit and STREX BK channel α-subunit truncated at glycine (G1020) is expressed in the plasma membrane. Therefore, the residues 322-1020 of cytoplasmic tail of STREX BK channel α-subunit, which is required for PP2A association with STREX BK channel α-subunit, are also required for trafficking of the STREX BK channel to the plasma membrane. The lack of association of I322 variant channel with PP2A may be due to not targeting of the channel to plasma membrane.

Scanning mutations could be carried out to investigate whether or not the PPase association and plasma membrane targeting could be dissociated. In order to address this, further immunocytochemical and co-immunoprecipitations studies with BK channel α-subunits mutated at C-terminal could resolve this issue.

Regulation of PP2A by glucocorticoid hormones

Our data showed that PP2A and PP1 are constitutively associated with the BK channel, the intensity of the reaction indicated that PP2A is much more abundant than PP1. Furthermore electrophysiological findings also pointed towards PP2A as the phosphatase involved in the action of glucocorticoid hormones on the BK channel.
Therefore, we have analyzed the IPs of BK channel α-subunits pretreated with dexamethasone for PP2A immunoreactivity.

Throughout this study, our data demonstrated that dexamethasone, which has marked PPase-dependent effects on the electrophysiological properties of the STREX variant BK channel (Tian et al., 2001b; Shipston et al., 1996) does not enhance the recruitment of the PP2A complex to the BK channel α-subunits in HEK 293 cells and in native BK channel expressing cells, AtT20 cells. Consequently, the regulation of BK channels by glucocorticoid is likely to be due to changes in the enzymatic activity of the associated PP2A rather than the recruitment of more PP2A catalytic subunits to BK channels. Therefore direct monitoring of the PPase activity associated with BK channel α-subunits pretreated is desirable to clarify the role of PP2A and/or PP1 in the action of glucocorticoids on the function of the STREX-BK channel.

Final thoughts

The data obtained indicate the association of PP2A with the BK channel. As the exact regulatory properties of PP2A depend on its subunit composition, it will be of importance to determine which additional components are present in the BK channel complex with the A and C subunits identified in the present study. This could be particularly relevant for the action of adrenal steroids on BK channel function. It would appear that the best plausible approach would be immunoaffinity isolation of the BK channel α-subunit and analysis of the associated proteins by mass–spectrometric techniques.
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No effect of dexamethasone on Dexras1 mRNA Human Embryonic Kidney (HEK 293) cells

Ghassem Attarzadeh Yazdi, Michael J Shipston and Ferenc Antoni, Department of Neuroscience, University of Edinburgh, EH8 9JZ, Membrane Biology Group, University of Edinburgh Medical School, Hugh Robson Building, Edinburgh, EH8 9XD. Scotland, United Kingdom

Previous work has indicated that Dexras1 is a glucocorticoid-induced protein that could mediate the effects of adrenal corticosteroids on the secretion of adrenocorticotrophic hormone. In the present study the effect of the synthetic glucocorticoid dexamethasone on Dexras1 expression was examined in HEK293 cells. A GR-reporter construct in which the expression of luciferase is under the control of the mammary tumor virus long-term repeat segment was transiently transfected into HEK 293 cells. The cells were exposed to dexamethasone 48h later. Analysis of dexamethasone action revealed dose- and time-dependent induction of luciferase activity. Analysis of the expression of endogenous mRNAs revealed expression of a ~5kb Dexras1 and a ~2.6kb glucocorticoid-induced protein kinase (SGK-1) mRNA species in HEK 293 cells.

Dexamethasone had no significant effect on Dexras1 mRNA following varying times of treatment (0 to 120 min) but significantly increased SGK-1 mRNA with maximum effect at 30 min. The results suggest that in HEK 293 cells; 1) dexamethasone acts via endogenously expressed GR; 2) Dexras1 is not a glucocorticoid-induced gene in this system, while SGK-1 mRNA is induced.
Ser/Thr protein phosphatases constitutively associated with large-conductance calcium-activated potassium (BK) channels

Ghassem Attarzadeh Yazdi, Michael J Shipston, Ferenc Antoni
Division of Neuroscience, University of Edinburgh, EH8 9JZ, Membrane Biology Group, University of Edinburgh Medical School, Hugh Robson Building, Edinburgh, EH8 9XD. Scotland, United Kingdom

Variants of BK-channels that arise through alternative exon splicing have distinct regulatory properties. Variants lacking splice inserts (ZERO) are activated by cAMP-dependent protein kinase (PKA) while those expressing STREX-1 at splice site 2 (STREX) are inhibited. Glucocorticoid hormones block PKA-mediated inhibition of STREX through a protein phosphatase (PP) but have no effect on ZERO. As under control conditions PP does not regulate STREX, we hypothesized that glucocorticoids could recruit PP to STREX. Therefore, we have analyzed immunoprecipitates (IPs) of BK-channel α-subunits for PP subunit immunoreactivity. The A and C subunits of PP2A were detected in IPs from transfected human embryonic kidney 293 cells (HEK 293) expressing STREX and similar results were obtained in cells expressing ZERO. IPs prepared with an antibody against the A subunit of PP2A contained STREX-BK channel. Analysis of IPs for PP1 C subunit indicated much lower abundance when compared with PP2A. Subunits of PP2A also coimmunoprecipitated with BK-channels in extracts of mouse corticotroph tumour cells that predominantly express STREX. Treatment with dexamethasone did not produce a change in the amount of PP2A subunits associated with STREX or ZERO. In summary, PP2A is constitutively associated with STREX and ZERO BK-channels and this is not altered by glucocorticoid treatment.