Cloning and Functional Co-expression of Cardiovascular Receptors and Ion Channels

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract

There is an expanding family of cyclic nucleotide-gated cation channels (CNGCs) with expression of family members reported in rod and cone photoreceptor cells, olfactory epithelium, heart, kidney, sperm and aorta. Although functions have been assigned to CNGCs in sensory cells, the function of such channels in non-sensory cells is unknown. A PCR-based screen showed that a CNGC is expressed throughout bovine heart tissue and also in bovine aorta, a bovine aorta endothelial cell line and a human umbilical vein endothelial cell line. Sequence data from these amplified products showed that the CNGC expressed in bovine heart and vasculature is highly related to the bovine rod photoreceptor channel. This was also the case for PCR-generated clones spanning the entire coding sequence of the CNGC from porcine coronary artery smooth muscle tissue. Cyclic GMP is an important messenger in vascular smooth muscle relaxation and therefore this CNGC may play a key role in this process. The second messenger pathways which may be involved in the gating of cardiovascular CNGCs have also been studied by attempting to heterologously co-express a cGMP-generating receptor, ANP-RA, with a CNGC in HEK293 cells.

The inward rectifier K⁺ channel subunits Kir 3.1 and Kir 3.4 have been heterologously co-expressed in MEL cells using a mammalian expression vector which incorporates the β-globin LCR and promoter allowing high levels of gene expression in a 'position independent' manner. Electrophysiological analysis of these co-expressing cell lines shows that Kir 3.1 and Kir 3.4 form a heteromultimeric ion channel complex which displays the features of the native G-protein activated atrial muscarinic K⁺ channel, KACh, and it is probable that these two inward rectifier channel subunits are major components of KACh.
Acknowledgements

Thank you to Dr. S. M. Duffy, Dr. J. M. Quayle and Dr. N. Davies for carrying out the electrophysiological experiments described in this thesis. I am also grateful to Dr. K. Groschner for his assistance and for the provision of electrophysiological data for this thesis.

Many thanks to Prof. W. Brammar and Dr. E. Conley for their advice throughout my Ph.D. studies.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(0-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalo virus</td>
</tr>
<tr>
<td>CNGC</td>
<td>cyclic nucleotide gated channel</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-deoxyribonucleotide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiotreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>equilibrium potential</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-O,O'-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GTP-γ-S</td>
<td>guanosine 5'-O-(3-thiotri-phosphate)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>I</td>
<td>current</td>
</tr>
<tr>
<td>IBMX</td>
<td>3'-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulphonic acid</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>phenol/chloroform/isoamyl alcohol</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pp</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>pS</td>
<td>picosiemens</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SSC</td>
<td>standard sodium citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate/EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris/EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
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Introduction
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1.0 Introduction

1.1 Cyclic-Nucleotide-Gated Cation Channels

1.1.1 The Family of cGMP-Gated Cation Channels

The cyclic nucleotide cGMP is a second messenger which is synthesised from GTP by both soluble and particulate guanylate cyclases. cGMP performs its second messenger functions by regulating protein kinases, protein phosphatases and ion channels. The effect of cGMP on ion channels has been studied intensely in vertebrate rod photoreceptors which respond to light by closing a cation channel in the plasma membrane. This channel is controlled by cGMP which activates the channel directly without involving any other signalling components. The photoreceptor channel carries a mixed inward cationic current under physiological conditions and in the dark state Na\(^+\) and Ca\(^{2+}\) ions enter the rod cell through open cGMP-gated channels. In the light, a cGMP phosphodiesterase is activated which causes cGMP hydrolysis and hence channel closure, resulting in membrane hyperpolarisation and a decrease in synaptic neurotransmitter release.

The cDNA for the bovine rod photoreceptor cGMP-gated channel has been isolated, cloned, sequenced and functionally expressed in *Xenopus* oocytes (Kaupp et al., 1989). Shortly after this a cDNA sequence encoding a cAMP-gated channel was isolated from olfactory epithelium (Dhallan et al., 1990; Ludwig et al., 1990) where the channel was shown to play a crucial role in olfactory signal transduction. Although this olfactory channel can be gated by cGMP, cAMP appears to be physiologically more important, as odorant-stimulated second messenger pathways result in adenylate cyclase, and not guanylate cyclase, activation. Recently, sequences encoding cyclic nucleotide-gated channels (CNGCs) have also been cloned from non-sensory tissues such as bovine sperm, rabbit aorta, bovine kidney and rabbit sinoatrial node (see table 1.1 for references), where their function is less clear. The CNGC proteins described so far are encoded by three different genes: CNG-1, CNG-2 and CNG-3 (refer to table 1.1) which share approximately 60% amino acid sequence homology.

A further CNGC cDNA, recently cloned from mouse kidney inner medullary collecting ducts, specifies a protein which differs by only five amino acids from the cDNA encoding the mouse rod photoreceptor channel (Karlson et al., 1995). Kidney CNGC cDNA sequences from several mouse strains were compared and shown to be identical, demonstrating that the differences in the mouse photoreceptor cDNA and kidney DNA are tissue-specific and the mouse kidney CNGC sequence is novel. It is possible that cGMP actually inhibits this particular channel preventing the reabsorption of sodium in the inner medullary collecting duct although, as yet, there are no data to confirm such speculation.
Table 1.1  Tissue Distribution of CNG-1, CNG-2 and CNG-3

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>GENE</th>
<th>Peptide Length (aa)</th>
<th>BIOLOGICAL FUNCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal Rod</td>
<td>CNG-1</td>
<td>690</td>
<td>Involved in phototransduction; channel closure results in hyperpolarisation of the rods altering transmitter release.</td>
<td>Kaupp et al., 1989</td>
</tr>
<tr>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfactory</td>
<td>CNG-2</td>
<td>664</td>
<td>Odorants bind receptors on the olfactory epithelium resulting in elevation of cAMP and channel opening.</td>
<td>Dhallan et al., 1990</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rat)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinal Cone</td>
<td>CNG-3</td>
<td>735</td>
<td>Phototransduction</td>
<td>Bonigk et al., 1993</td>
</tr>
<tr>
<td>(chick)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td>CNG-2</td>
<td>732</td>
<td>Function unknown</td>
<td>Biel et al., 1993</td>
</tr>
<tr>
<td>(rabbit)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm</td>
<td>CNG-3</td>
<td>706</td>
<td>Function unknown but may be involved in the chemotaxis of sperm by controlling Ca^{2+} entry.</td>
<td>Weyand et al., 1994</td>
</tr>
<tr>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>CNG-3</td>
<td>706</td>
<td>Function unknown but may be involved in the regulation of cellular Ca^{2+}.</td>
<td>Biel et al., 1994</td>
</tr>
<tr>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinoatrial node</td>
<td>CNG-1</td>
<td>689</td>
<td>Function unknown.</td>
<td>Hundal et al., 1993</td>
</tr>
<tr>
<td>(rabbit)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CNGCs have been shown to exist as heteromultimers and β-subunits to the rod photoreceptor CNGC (Chen et al., 1993) and the olfactory CNGC (Liman et al., 1994) have been cloned. The photoreceptor β-subunit shares 30% amino acid identity to the photoreceptor α-subunit and the olfactory β-subunit shares 52% amino acid identity with the olfactory α-subunit. The two β-subunits share only 30% homology with each other, which suggests that the β-subunit is important in determining the physiological function of CNGCs in different tissues.

Northern blot experiments have shown the expression of CNG-1 in rat kidney as well as heart and retinal tissue (Ahmad et al., 1990) and PCR-based screens have revealed the presence of CNGC sequences in several tissue-types other than those already described including pancreas, colon, adrenal gland and pineal gland (Distler et al., 1994). It therefore appears that the CNGCs are a growing family of versatile proteins adapted to provide various biological functions in a wide variety of tissues, although the distinct role of these channels in non-sensory tissues is still under investigation.
1.1.2 The Structure and Function of Cyclic Nucleotide-Gated Channels

Hydropathy analysis of CNGC primary sequence suggests that the protein has six transmembrane domains, named H1, H2, H3, S4, H4 and H5, with a putative pore-lining domain between H4 and H5 which bears some resemblance to the pore domain of voltage-gated channels (see fig 1.2). The S4 transmembrane domain is so named due to its strong homology to the analogous S4 'voltage sensor' domain of the voltage-gated K+ channel family, although CNGCs are only very weakly voltage-dependent (Kaupp et al., 1989). The lack of any amino terminal signal peptide leads to the prediction that the amino terminus and thus also the carboxyl terminus are intracellular. The carboxyl terminus of the CNGC protein shows homology to the cGMP-binding domain of cGMP-dependent protein kinases and to a lesser extent to the cAMP-binding domain of cAMP-dependent protein kinases and is therefore thought to contain the cGMP-binding domain of the channel (Eismann et al., 1993). There is also at least one site, situated on the extracellular loop between H4 and the putative pore, which is glycosylated (Ludwig et al., 1990; Wohlfart et al., 1992). Functional channels are thought to consist of multimers of four or five subunits and high resolution electron microscopy has shown that the purified α subunit from rod photoreceptors can form a homomeric complex with 5-fold symmetry (Eismann et al., 1993). The molecular structure and function of the rod photoreceptor CNGC (α-subunit) and the olfactory CNGC (α subunit) have been studied in most detail and these observations are described below.

Fig 1.1 Diagram showing the possible multimeric structures of CNGCs with either four-fold or five-fold symmetry
Generalised monomeric protein domain topology model for a cyclic-nucleotide-gated (CNG) non-selective cationic channel

Extracellular

(a) Monomeric domains

Intracellular

'Weak, residual voltage-sensor' homologue
ELNRLLLFSRMF
aa271-285 (BOVROD)

'Pore-lining' domain
β-hairpin: aa348-368
(monomeric, 'unfolded')

N-terminal cleavage
aa93 (BOVROD)
aa86-100 (CHICKCONE)

N glycosylation site
Asn98 (BOVROD)
Asn286 (CHICKCONE)

S

Cyclic nucleotide ligand-binding domain
(monomeric, 'unfolded')

cGMP-binding domain
aa489-577 (BOVROD)

COOH

aa990 (BOVROD)
aa644 (CHICKROD)
aa735 (CHICKCONE)

Fig 1.2 Structural model for CNGCs
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1.1.3 The Amino Terminal Domain

Labelling of native bovine rod CNGCs with anti-channel monoclonal antibodies by Western blotting indicates that the channel has a subunit with a molecular weight of 63kDa whereas in *Xenopus* oocytes the channel has an apparent molecular weight of 78kDa. A 63kDa protein was also detected in similar Western blots carried out on human, rat, mouse and pig rod protein. This discrepancy in size between the native and cloned protein appears to be due to the cleavage of the first 92 amino terminal amino acids, predicted from the cDNA sequence, from the mature protein (Molday et al., 1991). The CNGC from cone photoreceptors has also been reported to exist in a truncated form (Bonigk et al., 1993). It is possible that the N-terminus of the CNGC may be required for the correct assembly of subunits into the plasma membrane, as appears to be the case for voltage-gated K⁺ channels (Li, et al., 1992), after which it is cleaved from the mature protein.

1.1.4 Channel Gating

As mentioned previously, the carboxyl terminus of CNGCs has been shown to be the site of cyclic nucleotide binding. The rod photoreceptor CNGC and the olfactory CNGC respond differently to cAMP and cGMP. The wild-type bovine olfactory channel and the wild-type bovine rod photoreceptor channel are both more sensitive to cGMP than cAMP, while the olfactory channel is more sensitive to both ligands than the photoreceptor channel. A threonine residue in the carboxyl terminus and present in both the olfactory and photoreceptor channel appears to be important in determining the specificity of ligand binding. If, in the olfactory channel, this threonine residue, invariantly present in all cGMP-binding domains is mutated to an alanine, as in all cAMP-binding domains, sensitivity to cGMP is drastically reduced while cAMP binding is not affected. This is possibly because the hydroxyl group of threonine forms an additional H-bond with cGMP but not cAMP, allowing discrimination between ligands to occur (Altenhofen et al., 1991). Construction of chimaeras between the two channels has resulted in the identification of a putative α helix close to this threonine residue within the carboxyl-terminal cyclic nucleotide binding domain which also appears to determine the selectivity of the channel activation by cGMP or cAMP (Goulding, et al., 1994).

Another domain in the amino terminus, extending from the middle of the cytoplasmic region of the amino terminus to the intracellular loop connecting the H2 and H3 transmembrane helices, also appears to be important in CNGC gating. It is proposed that this amino-terminal domain determines the ease of channel opening. As mentioned previously, the amino terminus of CNGC may be important in subunit assembly and therefore is a probable site of intersubunit interaction. Therefore, channel opening perhaps
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involves an allosteric change in inter-subunit association at the amino terminus following cyclic nucleotide binding at the carboxy terminus (Goulding, et al., 1994; Gordon, et al., 1995).

1.1.5 The β Subunit

The characteristics of cloned rod photoreceptor channels and cloned olfactory channels differ from those of the respective native channels. Both cloned channels exhibit distinct open-closed transitions, whereas the native channels display rapid, flickering channel opening (Toire et al., 1992). The native olfactory channel is also far more sensitive to cAMP and cGMP than the cloned channel (Dhallan et al., 1990). These differences indicated that a molecular component could be lacking from the channel and recently sequences encoding β-subunits for both channels have been isolated which, when co-expressed heterologously with sequences encoding their respective α subunit, yield channels that show characteristics far more similar to those of native channels than when the α subunit is expressed alone (Chen et al., 1993; Liman and Buck, 1994). Interestingly, neither β subunit could form functional channels when expression as a homomultimer was attempted. Two, alternatively spliced, β subunit clones were isolated from human retina named hRCNC2a and hRCNC2b. Both are derived from a single gene with the 5' end of hRCNC2b encoded from one or more exons upstream from those for hRCNC2a (Chen et al., 1993). Both of these alternatively spliced products are expressed in retinal rods (Chen et al., 1993; Chen, et al., 1994).

1.1.6 Modulation of CNGCs by Calmodulin

The β subunit of the rod photoreceptor CNGC has also been shown to be a site of interaction with calmodulin. Ca^{2+}-calmodulin has been shown to modulate the photoreceptor CNGC by binding to a 240kDa protein that is associated with the 63kDa channel subunit (α subunit) (Molday et al., 1990). The β subunit, hRCNC2b, has since been found to be a component of this 240kDa protein complex and it appears that it is hRCNC2b which is responsible for mediating the Ca^{2+}-calmodulin modulation of the channel (Chen et al., 1994). Binding of Ca^{2+}-calmodulin to the photoreceptor channel has been shown to have the effect of increasing the apparent Michaelis constant of the channel for cGMP, thus resulting in a decrease in the rate of cation influx into the rod cell outer segment. This regulatory mechanism is thought to increase the speed of recovery back to the dark state after phototransduction (Hsu and Molday, 1993 and 1994). The α subunit of the olfactory channel is also modulated by Ca^{2+}-calmodulin which, as in the photoreceptor channel, reduces the affinity of the channel for cyclic nucleotides (Liu et al., 1994). Chimaeric proteins consisting of the olfactory channel α subunit and the photoreceptor
channel α subunit, which does not bind calmodulin (Chen et al., 1994), allowed localisation of the calmodulin binding site to the amino terminus of the olfactory α subunit (Liu et al., 1994). It is possible that the photoreceptor β subunit also binds calmodulin at its amino terminus but this has yet to be studied. The interaction of calmodulin with the amino terminus of the olfactory channel once again implies the importance of this region in channel gating.

1.1.7 Modulation of CNCGs by Protein Phosphatases

Following patch excision the dose-response curve for cGMP activation of rod channels revealed an increase in apparent cGMP affinity over time. This increase in cGMP affinity could be blocked by specific serine/threonine phosphatase inhibitors indicating the involvement of an endogenous patch-associated protein phosphatase. The actions of this endogenous phosphatase could be mimicked by PP-1 but PP-2A appeared to have the reverse affect, suggesting that there may be two sites for protein phosphorylation on the rod CNGC (Gordon et al., 1992).

1.1.8 The S4 Domain

Although CNCGs display no voltage activation they contain a sequence motif that is thought to act as the voltage sensor in voltage-gated channels. This motif is named the S4 region in the voltage-activated channels and consists of an unusual stretch of hydrophobic and basic residues. The S4 domain of K+ channels is characterised by the presence of five to seven positively charged arginines or lysines positioned at every third residue interspersed with hydrophobic residues to form an amphiphatic α helix through the cell membrane. CNCGs have only four regularly spaced arginine or lysine residues in their S4 domain which are flanked by negatively charged residues on both sides of the motif. Such negatively charged residues are absent from the S4 domains of K+ channels and probably account for the relatively weak voltage dependence of CNCGs (Eismann et al., 1993).

1.1.9 The Pore Domain

The CNCGs also contain a pore-forming region between transmembrane domains H4 and H5 which contributes to the pore of the channel through which ions pass and closely resembles the equivalent region in voltage-activated K+ channels. Evidence that this pore is involved in ion permeation was provided by the formation of a chimaera in which the retinal pore region was replaced by the corresponding region of the olfactory domain. This chimaeric channel displays a conductance and ion permeation similar to that of the
Chapter 1 Introduction

olfactory channel which has a higher conductance and larger pore diameter than the
photoreceptor channel (Goulding et al., 1993).

An alignment of the pore region of the Shaker $K^+$ channel and the bovine retinal
cGMP-gated channel shows the sequences are highly similar despite their different
permeability properties. CNGCs do not particularly discriminate between monovalent
cations while potassium channels are highly selective for $K^+$. CNGCs are also permeable to
divalent cations and are also blocked by $Ca^{2+}$ and $Mg^{2+}$ at millimolar concentrations in a
complex voltage-dependent manner. CNGCs lack two amino acids (YG between G362 and
E363) that are present in the $K^+$ channel pore. Mutant $K^+$ channels lacking these two
amino acids were generated and they displayed the ion conduction properties of the
CNGCs showing little selection among monovalent cations (Heginbotham et al., 1992).
Divalent cation block of CNGCs was shown, in part, to be due to the presence of an acidic
residue in close proximity to the YG motif (E363 in CNGCs but D or N in $K^+$ channels). If
the aspartate residue in the YG mutation of the Shaker $K^+$ channel is replaced by a
glutamate $Ca^{2+}$ block is increased about six-fold (Heginbotham et al., 1992). Conversely,
when the acidic E363 residue in CNGCs is replaced with a neutral glutamine residue much
higher $Ca^{2+}$ concentrations are required to block the channel (Root and MacKinnon, 1993;
Eismann et al., 1994).

\begin{center}
\begin{tabular}{|l|}
\hline
| Fore | \hline
| Bovine RET CNG-1 | LARKYYGLYWSTLTTTIG...ETPPVROSEYTFWAD | \hline
| Bovine OLF CNG-2 | -S-E-I-C---------------...K-E-L--IP- | \hline
| Shaker $K^+$ channel | FPFSIPDAFW-AV-M--V-YGDMT-VGWGKIVGSLOA | \hline
\end{tabular}
\end{center}

Fig 1.3 Aligned amino acid sequences of the H5 domain for the bovine retinal channel, bovine olfactory
channel and the Shaker $K^+$ channel. Dashes represent amino acid identity and dots represent gaps between the
RET and OLF channels compared to the Shaker channel. Residues in bold are responsible for $K^+$ selectivity
in the Shaker channel

1.1.10 CNGCs and Voltage-Gated $K^+$ Channels belong to the same Superfamily of
Ion Channels

The close similarities between CNGCs and the voltage-gated $K^+$ channels, especially
in their S4 and pore-lining domains, have led to the conclusion that both channel families
belong to a superfamily of ion channels which also includes the voltage-gated $Na^+$ and
$Ca^{2+}$ channel families. Thus, each member of this superfamily shares core structures, such
as the S4 domain, and they are all descended from a common, ancestral ion channel (Jan
and Jan, 1990). This close relationship between members of the superfamily was

20
<table>
<thead>
<tr>
<th>GENETIC ELEMENT</th>
<th>TISSUE</th>
<th>$V_m$ (mV)</th>
<th>$K_{1/2}$ cGMP (µM)</th>
<th>$K_{1/2}$ cAMP (µM)</th>
<th>$n_c$ GMP</th>
<th>Single Channel Conductance</th>
<th>Further Characteristics</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>native rod (bovine)</td>
<td>diverse</td>
<td>10-50</td>
<td>1.500</td>
<td>1.5-3.1</td>
<td>32pS ($+120$mV) 25pS ($-120$mV) (no divalent cations)</td>
<td>Ca$^{2+}$ and Mg$^{2+}$ reduce the in situ single channel conductance to 0.1pS. Channel opening has flickering characteristics.</td>
<td>Nizzari et al., 1993; Eismann et al., 1993; Yau and Baylor, 1989</td>
<td></td>
</tr>
<tr>
<td>α subunit (bovine)</td>
<td>+50mV</td>
<td>52.3</td>
<td>no activity at 1mM</td>
<td>1.75</td>
<td>20pS ($+120$mV) (no divalent cations)</td>
<td>Channel openings are long and stable. Current is blocked by internal and external Ca$^{2+}$ (mM) in voltage-dependent manner.</td>
<td>Kaupp et al., 1989; Eismann et al., 1994</td>
<td></td>
</tr>
<tr>
<td>αβ subunits (human)</td>
<td>+60mV</td>
<td>60-80</td>
<td>2</td>
<td>no data</td>
<td>The β subunit confers wild-type properties onto the channel such as increased sensitivity to l-cis diltiazem, a CNGC blocker, decreased block by Ca$^{2+}$ and flickery opening characteristics.</td>
<td>Chen et al., 1993</td>
<td></td>
<td></td>
</tr>
<tr>
<td>native (rat olfactory)</td>
<td>+50mV</td>
<td>1</td>
<td>2.5</td>
<td>1.3</td>
<td>12-15pS</td>
<td>Olfactory channels have far greater sensitivity to cAMP than other family members. Flickering channels</td>
<td>Eismann et al., 1993; Frings et al., 1992</td>
<td></td>
</tr>
<tr>
<td>α subunit (rat olfactory)</td>
<td>+80mV</td>
<td>2.2</td>
<td>59</td>
<td>2.8</td>
<td>35pS</td>
<td>Cloned α subunit not so sensitive to cAMP and channel openings are long and stable.</td>
<td>Dhallan et al., 1990; Liman and Buck, 1994</td>
<td></td>
</tr>
<tr>
<td>αβ subunits (rat olfactory)</td>
<td>+80mV</td>
<td>3.8</td>
<td>11</td>
<td>1.3</td>
<td>Flickering characteristics prevented measurement</td>
<td>As with rod channels co-expression of α and β subunits results in a channel with properties more similar to the native channel such as flickering opening characteristics.</td>
<td>Liman and Buck, 1994</td>
<td></td>
</tr>
<tr>
<td>aorta (rabbit)</td>
<td>+80mV</td>
<td>1.7</td>
<td>59.5</td>
<td>2.2</td>
<td>no data</td>
<td>Sensitivity to cAMP and cGMP, cooperativity and ion selectivity is similar to olfactory channels, consistent with their close structural relationship</td>
<td>Biel et al., 1993</td>
<td></td>
</tr>
<tr>
<td>cone (cloned)</td>
<td>+80mV</td>
<td>34</td>
<td>&gt;1000</td>
<td>1.8</td>
<td>40-50pS (native fish)</td>
<td>Rod and cone channel properties are fairly similar except for the larger conductance of native cone channels compared to native rods.</td>
<td>Yau and Haynes, 1990; Bonigk et al., 1993</td>
<td></td>
</tr>
<tr>
<td>kidney (bovine)</td>
<td>+40mV</td>
<td>18</td>
<td>1900</td>
<td>2</td>
<td>no data</td>
<td>CNG 3 type channels appear to be more permeable to Ca$^{2+}$ than other family members.</td>
<td>Biel et al., 1994</td>
<td></td>
</tr>
<tr>
<td>CNG-3</td>
<td>sperm (bovine)</td>
<td>+80mV</td>
<td>8.3</td>
<td>1700</td>
<td>21pS (60mV)</td>
<td>The cloned channel has long stable openings while current recorded from sperm vesicle are flickery indicating that CNG-3 type channels also interact with a β subunit</td>
<td>Weyand et al., 1994</td>
<td></td>
</tr>
</tbody>
</table>
demonstrated convincingly when CNGCs were shown to be inactivated by a peptide consisting of the 'ball' domain of a Shaker K+ channel. Shaker K+ channels are rapidly inactivated by this 'ball' domain which is located at the amino terminus of the channel. The mechanism for CNGC inactivation was shown to be by binding of this 'ball' domain peptide to the conserved pore-forming region (Kramer et al., 1994). This experiment illustrates the strong structural homology that exists between the CNGCs and the voltage-gated K+ channels.

1.1.11 The Physiological Properties Of CNGCs

Under physiological conditions CNGCs carry both monovalent and divalent cations into the cell. Ion selectivity for monovalent cation is as follows; \( \text{NH}_4^+ > \text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Rb}^+ > \text{Cs}^+ \). As described in the above sections, divalent cations, such as \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \), present at mM concentrations also decrease single channel conductances by binding to sites within the channel pore and blocking the entry of monovalent cations. This divalent cation block is thought to be of physiological importance as through the gating of many low conductance channels, rather than a few high conductance channels, a relatively low noise membrane potential change can be achieved when intracellular cGMP concentrations change (Yau and Baylor, 1989). However, a major function of CNGCs may be to provide a pathway for \( \text{Ca}^{2+} \) entry into the cell. A recent study (Frings et al., 1995) has demonstrated that CNGCs differ significantly in their relative permeabilities to \( \text{Ca}^{2+} \) at physiological concentrations with the cone CNGC being most permeable followed by the olfactory CNGC and then the rod CNGC. The cone (CNG-3) and olfactory (CNG-2) type channels may be particularly important in the \( \text{Ca}^{2+} \) homeostasis of the non-sensory cells in which they are also expressed (Weyand et al., 1994; Frings et al., 1995). (Refer also to table 1.2)
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1.2 Mechanisms for Intracellular cGMP Elevation

1.2.1 Guanylate Cyclases

A major concern of this thesis is to investigate the possible signal transduction pathways which control the cGMP-gated channel isolated from cardiovascular tissue. Obviously, the mechanisms of interest are those which alter the intracellular concentrations of cGMP. There are two classes of guanylate cyclase, the soluble form and the particulate, membrane-bound form. The soluble guanylate cyclase elevates cGMP levels through a complex pathway whereby receptor stimulation causes nitric oxide (NO) production and NO in turn activates the guanylate cyclase. The signalling pathway of particulate guanylate cyclases simply involves the binding of a ligand to the protein extracellularly and this activates an intracellular guanylate cyclase domain which is an integral part of the protein. A type of particulate guanylate cyclase, the natriuretic peptide receptor, is expressed in cardiovascular tissue and is therefore a candidate for interaction with cardiovascular cGMP-gated cation channels.

1.2.2 Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is a hormone synthesised mainly in heart tissue and has multiple actions in kidney leading to increased glomerular filtration rate and excretion of fluid and electrolytes, decreased inner medullary hypertonicity and sodium reabsorption by tubular epithelial cells and modulation of renal vascular resistance in kidney. ANP also decreases arterial blood pressure, cardiac output and plasma volume. It is produced as a precursor of 152 amino acids which is cleaved to give a 126 residue molecule. ANP is stored in this state in the secretory granules of atrial myocytes. Upon the signal for hormone release this proANP molecule is cleaved into the amino terminal fragment, ANP1-99, and the biologically active protein, amino acid residues 99-126. The predominant signal for ANP release appears to be atrial stretch but the mechanical events involved in this release are unknown.

A further two types of natriuretic peptide have been isolated to date. These are the B and C type natriuretic peptide (BNP and CNP). BNP is also produced principally in the atrium whereas CNP is produced in central nervous system tissues. These peptides all produce similar pharmacological effects of natriuresis and muscle relaxation and they appear to mediate these effects through their receptors which have guanylate cyclase activity (Rosenzweig and Seidman, 1991).
1.2.3 Natriuretic Peptide Receptors

Two natriuretic peptide receptors with integral guanylate cyclase activity have been isolated from rat and human tissue. They are ANP receptor A (ANP-RA) and ANP receptor B (ANP-RB). The guanylate cyclase activity of ANP-RA is elevated equally by ANP and BNP whereas ANP-RB is responsive to CNP and shows low activity in the presence of physiological concentrations of ANP and BNP (Koller et al., 1991). The presence of ATP is also required to activate the guanylate cyclase. These membrane guanylate cyclases have a single transmembrane domain with an extracellular amino terminus and an intracellular carboxyl terminus. The extracellular domain consists of the ligand binding site while the intracellular regions, which represent about half of the protein, consist of a protein kinase-like domain and a carboxyl region with homology to the soluble guanylate cyclases and hence proposed to be the guanylate cyclase domain (Chinkers et al., 1989, Lowe et al., 1989, Chang et al., 1989, Schulz et al., 1989, Chinkers and Garbers, 1991).

The protein kinase-like domain of the ANP receptor has been found to function as a regulatory element as, when this kinase-like domain was removed by deletion mutagenesis, the ANP receptor retained guanylate cyclase activity, but independently of ANP activation. Stimulation of the guanylate cyclase by ATP was also reduced under these circumstances. A proposed model for signal transduction may therefore involve an ANP binding-initiated conformational change in the protein kinase domain which allows activation of guanylate cyclase activity (Chinkers and Garbers, 1989; Koller et al., 1992).
1.2.4 **Tissue Distribution of Particulate Guanylate Cyclase Expression**

Differential regional expression of the natriuretic peptide receptor genes has been studied within rhesus monkey tissues. ANP-RA was found to be abundantly expressed in kidney glomerulosa and endocardial endothelial cells of the right and left atrium and left ventricle. ANP-RB was found not to be expressed in heart (Wilcox et al., 1991). The expression of ANP-RA in cardiac tissue renders it the most suitable natriuretic peptide receptor to study in partnership with the cardiovascular cGMP-gated channel.

Another member of the particulate guanylate cyclase family which is activated by heat-stable enterotoxins, named STaR, has been cloned from small intestine. During bacterial infections these enterotoxins are released into the intestine where they bind to particulate guanylate cyclase receptors resulting in the elevation of cGMP levels which stimulates fluid secretion partly by the direct effects of cGMP on Cl⁻ channels (Schulz et al., 1990; de Sauvage et al., 1991). A natural ligand for STaR, called guanylin, is thought to regulate fluid and electrolyte homeostasis in the gut through activation of STaR (Currie et al., 1992). Sequences encoding particulate guanylate cyclases have also been cloned from retina, where it is possible that their protein products are involved in visual signal...
transduction. RetGC, cloned from a human retinal cDNA library and expressed in rod outer segments, shares 22% amino acid identity with ANP-RA in the extracellular domain and 44% in the intracellular domains but is not stimulated by any of the natriuretic peptides (Shyjan et al., 1992). It is possible that retGC and cGMP-gated cation channels exist in very close proximity to one another in rod outer segments and that retGC stimulation results in local cGMP elevations and direct activation of the cGMP-gated channel. ANP-RA has also been cloned from human retina where it too may be directly involved in the visual signal transduction pathway (Pardhasradhi et al., 1994). There is also some evidence for the expression of particulate guanylate cyclases in olfactory cilia which may be involved in the activation of olfactory cyclic nucleotide-gated channels (Steinlen et al., 1990).

1.2.5 NO as a Signalling Molecule

The free radical NO is recognised as an important signalling molecule in vascular tissue, where it controls smooth muscle tone. NO is produced by enzymes known as NO synthases in the endothelial cell layer lining the vessel. NO, a gas with a short half life, is then able to rapidly diffuse to the smooth muscle cells where it associates with soluble guanylate cyclases to elevate intracellular cGMP levels resulting in smooth muscle relaxation. As shown in Fig 1.6 binding of vasodilatory agonists circulating in the blood stream to their endothelial cell receptors results in an influx of Ca^{2+} into the cells. Ca^{2+} has the effect of activating NO synthase which uses arginine as its substrate. NO produced is then able to activate soluble guanylate cyclases in surrounding smooth muscle cells, resulting in smooth muscle relaxation through an unknown mechanism.

![Fig 1.6 Signal transduction pathway of endothelial-dependent smooth muscle relaxation. NOS=NO synthase, sGC=soluble guanylate cyclase, +ve=activation](image-url)
1.2.6 Endothelial NO Synthases

Endothelial NO synthases consist of a single subunit and it is thought that two of these subunits form homodimers in the native protein. The enzyme is also associated with FMN, FAD, one non-haem iron and one molecule of tetrahydrobiopterin per subunit, all of which are thought to be involved in the five electron-oxidation of arginine required to produce NO. The endothelial cell NO synthase is expressed constitutively and is also modulated by Ca\(^{2+}\)-calmodulin, possessing a consensus sequence for calmodulin binding, and is dependent on Ca\(^{2+}\) in the range 100-500nM for activation. Endothelial NO synthases are associated with membranes and this is possibly due to myristylation as a consensus sequence for myristylation is present at the amino terminus of the enzyme. Endothelial NO synthases can also be activated by shear luminal stress and association of the enzyme with the luminal plasma membrane may assist in such activation (Knowles and Moncada, 1992; Griffith and Stuehr, 1995; Umans and Levi, 1995).

1.2.7 Soluble Guanylate Cyclases

cDNA sequences encoding soluble guanylate cyclases have been cloned from rat lung, showing the protein to be a heterodimer of two subunits, an \(\alpha\) subunit of 82kDa and a \(\beta\) subunit of 70kDa. Northern blot analysis demonstrated that these proteins were expressed in lung, cerebellum, heart, kidney, liver and muscle (Nakane et al., 1990; Buechler et al., 1991). The carboxyl domains of both the \(\alpha\) and \(\beta\) subunits show strong homology to each other and also the carboxyl guanylate cyclase domain of the particulate guanylate cyclases, indicating that this is the catalytic domain of the soluble guanylate cyclase subunits. Soluble guanylate cyclases contain haem as a prosthetic group, although the haem-binding site remains unidentified, and activation of the enzyme is thought to be by NO binding to the haem prosthetic group. A further \(\beta\) subunit of soluble guanylate cyclase, named \(\beta_2\), has been isolated and shown to contain a consensus sequence for isoprenylation at the carboxy terminus, indicating that this subunit may be associated with the membrane (Yeun et al., 1990). The \(\alpha\) subunit with which \(\beta_2\) interacts has yet to be identified but the presence of the isoprenylation consensus sequence on \(\beta_2\) raises the possibility that at least one form of soluble guanylate cyclase is membrane-associated, favouring the possible interaction of this protein with other membrane-bound proteins such as ion channels (Chinkers and Garbers, 1991; Garbers and Lowe, 1994).
1.3 The Inward Rectifier Potassium Channel Family

1.3.1 The Gene Family

Recently, a new family of K⁺ channels, named the inward rectifier K⁺ channel family, has emerged. Members of this family are structurally and functionally different from those of the voltage-gated K⁺ channel family, and are characterised by their inclination to allow K⁺ to flow into the cell rather than out. Inward rectifier K⁺ channel cDNAs have been cloned from a number of tissues and species and five subfamilies of inward rectifier K⁺ channels, Kir 1.0-Kir 5.0, have been identified based on their degree of identity, with a level of identity between each subfamily of 50-60%. Homology alignment of these sequences has revealed a novel channel structure consisting of two transmembrane domains, M1 and M2, with both the amino and carboxyl termini on the intracellular side of the membrane (see fig. 1.7). Between transmembrane domains M1 and M2 there is a pore region, assigned as such due to its strong homology with the pore domain of voltage-gated K⁺ channels. It is expected that four of these subunits join to form functional channels, as is the case for the voltage-gated K⁺ channels (Ho et al., 1993; Kubo et al., 1993; Douplnik et al., 1995; Aldrich, 1993).
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The inwardly rectifying properties of these channels have been shown to be dependent on Mg$^{2+}$ blockage of the channel pore and areas of the carboxyl terminus appear to be involved in this blockade (Taglialatela et al., 1994). Site directed mutagenesis has also identified an aspartate residue in the M2 transmembrane domain which may form part of the Mg$^{2+}$ binding site and also appears to be partly responsible for the intrinsic voltage-dependent gating which occurs even in the absence of Mg$^{2+}$ in some members of the family (Stanfield et al., 1994). This negatively charged aspartic acid residue (or occasionally glutamic acid residue) occurs consistently in the M2 domain of strong inward rectifiers, such as members of the Kir2.0 subfamily, while for weakly rectifying family members this aspartate is replaced by a neutral asparagine residue. Polyamines, such as spermine, have also been shown to be involved in channel block in a similar manner to Mg$^{2+}$ (Ficker et al., 1994).

### Table 1.3 Features of the Five Subfamilies of Inward Rectifier K$^{\pm}$ Channels

<table>
<thead>
<tr>
<th>Standardised nomenclature</th>
<th>Clones</th>
<th>Subfamily Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir 1.0</td>
<td>ROMK-</td>
<td>Constitutively active, ATP regulated channels with weak inward rectification cloned from kidney. Possible function in K$^{+}$ export into the distal nephron collecting tubules.</td>
<td>Ho et al., 1993.</td>
</tr>
<tr>
<td>Kir 2.0</td>
<td>IRK-</td>
<td>Constitutively active and strongly inward rectifying channels which play a significant role in setting the resting membrane potential. Cloned from heart, brain, hippocampus and J744 cells.</td>
<td>Kubo et al., 1993a; Morishige et al., 1993.</td>
</tr>
<tr>
<td>Kir 3.0</td>
<td>GIRK- KATP</td>
<td>G-protein activated inward rectifiers cloned from heart and brain. Heart channel is responsible for I$<em>{K</em>{AC}}$; brain channels may act to suppress neuronal firing. Another subfamily member KATP, cloned from heart, has properties similar to native K$_{ATP}$ currents.</td>
<td>Kubo et al., 1993b; Lesage et al., 1994, Ashford et al., 1994.</td>
</tr>
<tr>
<td>Kir 4.0</td>
<td>BIR10</td>
<td>Cloned from rat brain but expressed a wide variety of tissues.</td>
<td>Bond et al., 1994.</td>
</tr>
<tr>
<td>Kir 5.0</td>
<td>BIR9</td>
<td>Cloned from rat brain but also expressed in a wide variety of tissues. Cannot form functional channels when expressed by itself in <em>Xenopus</em> oocytes.</td>
<td>Bond et al., 1994.</td>
</tr>
</tbody>
</table>

(The standardised nomenclature is according to Chandy and Gutman, 1993.)
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1.3.2 G-protein Gated Inward Rectifier Potassium Channels

One of the inward rectifier channel subfamilies, Kir3.0, includes K+ channels directly
gated by G-proteins. The most studied member of this subfamily is the channel responsible
for the acetylcholine activated potassium current (iKACH) present in pacemaker cells of the
sinoatrial node and in atrial myocytes which results in slowing of the heart beat during
cholinergic stimulation (Sakmann et al., 1983). The cDNA thought to encode the iKACH
channel has been cloned from rat atrium and it appears to have many of the properties of
the native channel such as activation by G-protein coupled receptors, slow endogenous
channel gating, strong inward rectification and expression principally in atrium as opposed
to ventricle (Kubo et al., 1993).

1.3.3 G-protein Activation of GIRK1

Binding of acetylcholine to its receptor in atrial tissue activates G-proteins which in turn activate K+ channels. This signalling pathway has been reproduced in Xenopus oocytes co-injected with M2-muscarinic receptor cRNA and GIRK1 cRNA. Extracellular application of carbachol to these co-expressing oocytes results in activation of iKACH-like currents, presumably through the involvement of endogenous G-proteins in the signalling pathway (Kubo et al., 1993b; Takao et al., 1994). On activation, heterotrimeric G-proteins dissociate to generate two possible transducing factors, an α-GTP subunit and βγ dimers. The α subunits of G-proteins are generally responsible for signal transmission but βγ subunits now also appear to have a role in the signalling pathway. The native muscarinic potassium channel has been shown to be activated by βγ dimers and not α subunits and this has also been shown to be the case for the cloned channel GIRK1 (Wickman et al., 1994; Reuveny et al., 1994). Regions of GIRK1 which interact with βγ dimers have been identified by forming chimaeric channel proteins between GIRK1 and IRK1, an inward rectifier potassium channel belonging to the Kir 2.0 subfamily which in its intact state is not affected by βγ dimers. Replacement of the carboxyl terminus of IRK1 with that of GIRK1 produced a chimaeric channel which was activated by βγ dimers (Takao et al., 1994). The carboxyl terminus region of GIRK1 also shares homology with the carboxyl terminus of the β-adrenergic receptor kinase 1, a protein which also interacts with G-protein βγ dimers, providing further evidence for the involvement of this region of GIRK1 in βγ dimer interactions (Reuveny et al., 1994).
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ACh channel closed

Binding of ACh to receptor results in G-protein activation and channel opening

![Diagram of ACh and GIRK1 interaction](image)

Fig 1.8 Activation of GIRK1 by βγ dimers on addition of ACh (acetylcholine)

1.3.4 **ATP-sensitive K⁺ Channels are also Members of the Kir 3.0 Subfamily**

Another member of the Kir 3.0 subfamily of inward rectifier K⁺ channels appears to be an ATP-sensitive K⁺ channel (K\textsubscript{ATP}). A cDNA, named rcK\textsubscript{ATP}, has been cloned from rat heart which expresses a protein with some of the characteristics of endogenous K\textsubscript{ATP} channels (Ashford et al., 1994). K\textsubscript{ATP} channels are expressed in many tissues such as smooth muscle, skeletal muscle, heart, neuronal tissue and also in pancreatic β cells. Channel function is best understood in pancreatic β cells, where increased blood glucose levels increase intracellular ATP levels, causing the closure of the K\textsubscript{ATP} channels which results in membrane depolarisation and insulin secretion. Native K\textsubscript{ATP} channels have several distinguishing features. They are inhibited by physiological concentrations of ATP (5-10mM), activated by G-proteins, opened by the K⁺ channel opener pinacidil and they are also specifically blocked by the sulphonylureas such as glibenclamide. The sulphonylureas, acting through their receptors, are of pharmacological importance in the treatment of noninsulin-dependent diabetes as they cause inhibition of K\textsubscript{ATP} channels resulting in insulin release from pancreatic β cells. K\textsubscript{ATP} channels link changes in the metabolic state of the cell to the membrane potential. In cardiovascular tissue reduced O₂ levels results in decreased concentrations of ATP which is thought to activate the K\textsubscript{ATP} channel resulting in membrane hyperpolarisation and relaxation (Nichols and Lederer, 1991). Although both GIRK1 and rcK\textsubscript{ATP} expressed heterologously share many characteristics with their respective native channels, the cloned channel proteins do not exhibit all the properties of native channels. It is possible that the native channels form...
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heteromultimers consisting of the cloned cDNA protein product and another inward rectifier $K^+$ channel subunit.
1.4 Heterologous Expression of Ion Channels

1.4.1 Expression in Xenopus Oocytes

Heterologous expression experiments are essential for confirmation of the physiological properties of cloned ion channels. To date one of the main techniques utilized for the expression of cloned ion channels has been the injection of cRNA into Xenopus oocytes (first reported by Timpe et al., 1988). There are several advantages to using Xenopus oocytes as an expression host. First, the oocyte is large (1mm diameter) and therefore fairly simple to microinject and record from using the two electrode voltage-clamp. Secondly, recordings are stable for long periods and extracellular solutions can be simply exchanged and thirdly, oocytes express very few endogenous channels. However, there are also several distinct disadvantages to using Xenopus oocytes for the expression of ion channel coding sequences. Oocytes tend to display seasonal variations in translational efficiency and expression and there can be variations in oocytes from different frogs and even from a single frog. The oocyte also has to be defollicated prior to voltage-clamping. This process involves removal of the outer layers of the oocyte by either enzymatic or mechanical methods, which often leads to damage of the inner vitelline membrane resulting in holes which can then allow the non-specific flow of ions across the cell membrane. As well as this, the few endogenous channels expressed in oocytes may interfere with recordings from certain heterologously expressed channels and finally, it is difficult to control accurately the intracellular solution of the oocyte using conventional two electrode voltage clamping. Due to the problems encountered in using Xenopus oocytes to heterologously express mammalian ion channels several expression systems involving heterologous expression in clonal mammalian cell lines have been developed.

1.4.2 Microinjection of Clonal Mammalian Cell lines

This technique was first reported for the functional expression of the voltage-gated potassium channel Kv2.1 in clonal mouse fibroblasts and also rat basophilic leukaemia cells (Ikeda et al., 1992). The technique simply involves injection of cRNA into the mammalian cell which, in the case of Kv2.1, results in protein expression within six hours with nearly 100% efficiency. There are several advantages of using mammalian cells as opposed to Xenopus oocytes. First a wide variety of different mammalian cell lines can be microinjected and therefore the properties displayed by the expression host can be selected according to each individual experiment. Post-translational processing in mammalian cells is probably also more suitable for expression of mammalian ion channels. Finally, whole cell patch-clamp techniques can be used on mammalian cells which allows for greater...
control of intracellular solutions compared to the methods that can be applied to *Xenopus* oocytes. The main disadvantages of microinjection of mammalian cell lines is that they are far smaller than *Xenopus* oocytes and microinjection equipment for mammalian cells can be expensive. Also, recordings from mammalian cell lines are fairly unstable when compared to those from *Xenopus* oocytes.

**METHODS OF HETEROLOGOUS EXPRESSION**

**INJECTION OF RNA**

- **Xenopus oocyte/mammalian cell**
- **RNA**
- **Inject**
- **Protein**

**TRANSIENT EXPRESSION**

- **DNA construct**
- **Mammalian cell**
- **RNA**
- **Protein**

**STABLE EXPRESSION**

- **Nucleus**
- **DNA inserted into genome**
- **RNA**
- **Protein**
- **Linearised DNA**
- **Mammalian cell**

Fig 1.9 Diagram illustrating different methods of heterologous expression.

1.4.3 **Viral Transient Heterologous Expression Systems**

Expression systems have been developed which do not require microinjection procedures. One such system involves the use of vaccinia virus, a lytic DNA virus. This system involves the cloning of the ion channel cDNA into a vaccinia virus recombination plasmid in a multiple cloning site just downstream of the efficient vaccinia virus 7.5kDa promoter. Mammalian cells infected with vaccinia virus are then transfected with the
Chapter 1 Introduction

recombination plasmid to produce recombinant viruses containing the ion channel cDNA of interest. Recombinant viruses can then be used to infect cell lines resulting in transient ion channel expression. Although this system bypasses the need to produce cRNA and perform microinjection the construction of the recombinant virus is lengthy and infected cells undergo cell death after several days in culture (Karschin et al., 1991; Karschin et al., 1992). Ion channels have also been expressed in cultured Spodoptera frugiperda (Sf9) insect cells using baculovirus. The viral vector used for expression is the Autographa californica nuclear polyhedrosis virus and recombinant viruses are produced in a time-consuming method similar to that of vaccinia virus vectors. This technique can also be unsuitable for the expression of mammalian proteins as post-translational protein modifications and endogenous protein expression will be different in insect cell lines (Kamb et al., 1992).

1.4.4 Mammalian Expression Vectors

A huge influx of information regarding cis and trans acting transcriptional elements over the last fifteen years has allowed the evolution of several gene-transfer systems which exploit this knowledge. The simian virus 40 (SV40) genome has been the most exploited in the development of mammalian expression vectors. SV40 late or early promoters are able to drive transcription of a cDNA inserted at a multiple cloning site downstream of the promoter. The cDNA can be followed by a SV40 polyadenylation sequence which will increase the stability and translatability of the transcript in the host cell. Inclusion of the SV40 origin of replication (ori) in the expression vector allows it to replicate in cells permissive for SV40 viral replication. Such permissive cells (COS-type cells) express the SV40 large T antigen which is a requirement for viral replication. Alternatively the gene encoding the large T antigen can be provided in cis on the expression vector or in trans on a cotransfected expression vector, thus allowing expression of the foreign protein in a larger variety of cell types. Enhancer/promoter elements have been identified which are more efficient in a wider variety of mammalian cell lines and these can also be used in conjunction with the SV40 ori. One particularly useful enhancer/promoter element is the human cytomegalovirus enhancer which has little cell type or species preference and is several-fold more active than the SV40 enhancer/promoter. The vector ori can also be changed to enable expression in specialist cell lines. One such example of this is use of the Epstein-Barr virus (EBV) ori (ori-P) which requires the trans acting factor EBV nuclear antigen-1 (EBNA-1) for replication. EBV DNA is maintained extrachromosomally as a double-stranded DNA molecule in primate cells and plasmids containing the ori-P sequence are also able to replicate stably in EBV-transformed host cells (expressing EBNA-1) as episomes. This makes the EBV-expression system ideal for the construction of
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expression cDNA libraries as plasmids of interest can easily be retrieved (Spickofsky et al., 1990; Kriegler, 1990).

1.4.5 Stable Heterologous Expression in Mammalian Cell Lines

The main problem with the techniques described above is that all of them, except the EBV episomal expression system, are transient systems. Cells die within a few days of transfection and therefore there is only a very limited time period over which the protein synthesised can be studied. To overcome this problem, methods of stably expressing heterologous proteins have emerged. Such methods rely on insertion of eukaryotic selectable markers into the expression construct. The DNA construct consisting of the cDNA of interest, an enhancer/promoter element and a selectable marker is linearised at a convenient enzyme site and transfected into a suitable host cell line. Production of stable cell-lines depends on the integration of the foreign DNA into the host genome. Stably transfected cells can then identified by utilisation of the selectable marker. One of the most efficient markers is the aminoglycoside phosphotransferase gene whose protein product confers G418-resistance to mammalian cells. The establishment of stably transfected cells usually occurs several orders of magnitude less frequently than that of transiently expressing cells and the process of selection may take up to two weeks. Constructs may also integrate in transcriptionally 'silent' regions of the host genome, resulting in low expression levels. However, once cell-lines are produced and screened for expression they can be frozen and stored under liquid nitrogen and the expressed protein in a stable cell line can be studied in depth over a long time period.

1.4.6 Heterologous Co-expression in Mammalian Cell Lines

Membrane proteins such as receptors and ion channels are important pharmacological targets. However, many drugs targeting these proteins exhibit serious side effects, largely because most receptor and ion channel proteins belong to multigene families. As well as interacting with their target molecule, drugs can also associate with homologous family members resulting in undesired effects. cDNAs are now available for many of the receptors and ion channels targeted by modern pharmacological agents and heterologous expression methods could enable the development of more specific drugs designed to interact with only the family member of interest (Lester, 1988). Receptors and ion channels often exist as heteromultimers and therefore a robust expression system is required to allow for co-expression. Production of stably expressing cell lines appears to be the ideal system for co-expression as once they are produced they can easily be screened for expression of heterologous proteins by Northern blot and Western techniques. Using such co-expression techniques it may be possible to reconstitute complex signalling pathways in suitable, well
characterised cell-lines, creating the perfect physiological model as a test system for new drugs.
Chapter 2

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2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals

General laboratory chemicals were supplied by Fisons (Loughborough), BDH (Poole) or Sigma (Poole) unless otherwise stated and were of analytical grade or equivalent. Bacterial culture media were obtained from Difco (East Molesley) and animal cell culture media from Gibco BRL (Paisley). Radiolabelled nucleotides were supplied by Amersham International (Amersham). Millipore Super-Q® system (Millipore Corporation, Bedford) treated water was used for all solutions.

2.1.2 Enzymes and Proteins

Restriction endonucleases were purchased from Gibco BRL or New England Biolabs (via CP Laboratories, Bishop's Stortford). DNA polymerase I (Klenow fragment) was supplied Gibco BRL. Taq DNA polymerase was obtained from Boehringer Mannheim (Lewes), Deep Vent DNA polymerase was obtained from New England Biolabs. T4 DNA ligase was purchased from Gibco BRL and New England Biolabs. Proteinase K, Pronase, RNase A (pancreatic RNase) and DNase I were obtained from Sigma. BSA (enzyme grade) was obtained from Gibco BRL.

2.1.3 Synthetic Oligonucleotides

Synthetic oligonucleotides used are shown in the following tables:

<table>
<thead>
<tr>
<th>Table of PCR Primers Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Primer</td>
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</tr>
<tr>
<td>M13 Rev (-21)</td>
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<tr>
<td>5'CNG1-seq</td>
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</tbody>
</table>
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### 2.1.4 Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>F' traD36 lacI4Δ(lacZ)M15proA+B+/Δ14- (McrA-) Δ(lac-proAB) thi gyrA96 (Nalr) endA1 hsdRI7 (rK-mK+) relA1 supE44 recA1</td>
<td>(Yanish-Perron, C. et al, 1985)</td>
</tr>
<tr>
<td>Epicurian®Coli</td>
<td>e14-(McrA-) Δ(mcrCB-hsdSMR-mrr)171 supE44 endA1 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC :: Tn5 (Kanr) uvrC [F' proAB lacI4ZΔM15 Tn10 (Tetr)]</td>
<td>(Greener, 1990)</td>
</tr>
</tbody>
</table>

### 2.1.5 Bacterial Culture Media

Bacterial strains were grown using the following solid and liquid culture media:

**Solid media:**
1. Luria agar (LA): tryptone, 1% (w/v); yeast extract, 0.5% (w/v); NaCl, 0.5% (w/v); agar, 1.5% (w/v).

**Liquid media:**
1. Luria broth (LB): tryptone, 1% (w/v); yeast extract, 0.5% (w/v); NaCl, 0.5% (w/v).
2. 2xYT broth (2xYT): tryptone, 1.6% (w/v); yeast extract, 1% (w/v); NaCl, 0.5% (w/v).

### 2.1.6 Cloning vectors

Routine subcloning was performed in the vector pBluescript KS®, which was purchased from Stratagene (San Diego). The eukaryotic expression vectors used were pcDNA3, which was purchased from Invitrogen (San Diego), and pCI-neo, which was purchased from Promega (Madison).
2.2 Methods of Sterilisation

**Autoclaving:** Autoclaves used to sterilise media and disposable plastic-ware were as follows: a Cabburn 8cu.ft. capacity autoclave (Cabburn Sterilisers, Shoeburyness) set to attain a temperature of 121°C for 30 minutes; a model ST19 portable electric autoclave (Dixon's surgical instruments, Wickford) set to attain a temperature of 121°C for 20 minutes.

**Dry sterilisation:** A B&T "Unitemp" sterilising cabinet (Laboratory thermal equipment, Oldham) was used to sterilise glass-ware. The cabinet was set to attain a temperature of 160°C for 6 hours.

**Filter sterilisation:** Small volumes (up to 50ml) were filter sterilised by passing through Acrodiscs (Gelman Sciences, Ann Arbor) with a pore size of 0.2μm. Larger volumes (50-500ml) were filter sterilised using Nalgene 0.2μm vacuum filter sterilising units (Nalgene, Rochester, New York).
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2.3 Nucleic Acid Methods

2.3.1 Solutions used During the Handling of Nucleic Acids

Ampicillin (25mg/ml): 2.5g of ampicillin (Sigma) was dissolved in 50% ethanol and stored at -20°C in a container wrapped in aluminium foil.

Chloroform/iso-amyl alcohol: 98% (v/v) chloroform (Fisons), 2% (v/v) iso-Amyl alcohol (Fisons) was prepared and stored at room temperature.

DEPC-treated Q water: 0.1% (v/v) DEPC was added to Q water. After shaking vigorously, the bottles of Q water were left overnight in a fume cupboard with loosened caps. The DEPC-treated Q water was then autoclaved and stored at room temperature.

dNTP solutions (2 mM): 20 μl of stock solutions of 100 mM dTTP, dCTP, dATP, dGTP (Pharmacia) were dissolved in 920 μl of Q water, and stored at -20°C.

EDTA (0.5 M, pH 8): 186.1 g of EDTA (Fisons) was dissolved in 1 L of Q water adjusted to pH to 8.0 with NaOH pellets and 10 M NaOH. Following sterilisation by autoclaving, the solution was stored at room temperature.

Ethidium bromide (10 mg/ml): Ethidium Bromide (Serva) was dissolved at the specified concentration in Q water and stored in opaque plastic bottles at room temperature.

Ethanol (100%, 80%, 70%): Ethanol (Fisons) was diluted as required with Q water and stored at -20°C.

Glycerol (50%): Glycerol (Fisons) was dissolved at a concentration of 50% (w/v) in Q water and filter sterilised. The solution was stored at room temperature.

HEPES (2 M, pH 6.6): 9.53 g of HEPES (Sigma) was dissolved in a final volume of 20 ml Q water after adjusting the pH to 6.6 with 5 M NaOH. After filter sterilisation, the solution was stored at 4°C.

HCl (0.2 M): 17.24ml of concentrated HCl (Fisons) was added to 982.76 ml of Q water. The solution was stored at room temperature.
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IPTG (200mg/ml): 200 mg of IPTG (Sigma) was dissolved in 1ml of Q water and stored at -20°C.

MOPS buffer (10x): 0.2 M MOPS, 50 mM sodium acetate.3H2O, 1 mM EDTA (pH 8). The pH was adjusted to 8 with NaOH prior to autoclaving. The solution was stored at room temperature.

PBS: 8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4 and 0.2g of KH2PO4 were dissolved in 800mls of H2O and the pH was adjusted to 7.4 with HCl. H2O was added to 11 and the solution was dispensed into aliquots and autoclaved.

PCI: 50% (v/v) phenol (Fisons), 48% (v/v) chloroform, 2% (v/v) iso-Amyl alcohol; equilibrated against 10 mM Tris-HCl (pH 8) and kept in the dark at 4°C.

Phenol (liquified): Liquified phenol containing 0.1% (w/v) 8-hydroxy-quinoline and equilibrated against 100 mM Tris (pH 7.6) was obtained from Fisons.

Pronase (20mg/ml): Pronase was dissolved in H2O to give a final stock solution of 20mg/ml and stored at -20°C

Proteinase K (20mg/ml): Proteinase K was dissolved in H2O to give a final stock solution of 20mg/ml and stored at -20°C

RNase A (10mg/ml): RNase A was dissolved at a concentration of 10mg/ml in 10mM Tris-HCl (pH 7.5), 15mM NaCl, heated to 100°C for 15mins and allowed to cool slowly to room temperature before dispensing into aliquots and storing at -20°C.

Sodium acetate, (3 M, pH 5.6): 40.8 g of sodium acetate.3H2O (Fisons) was dissolved in 100 ml of Q water, after adjusting the pH to 5.6 with glacial acetic acid. The solution was sterilised by autoclaving and stored at room temperature.

Sodium chloride (4 M): 23.4 g of sodium chloride (Fisons) was dissolved in 100 ml of Q water and sterilised by autoclaving. The solution was then stored at room temperature.

SDS (10%): 50 g of SDS (Fisons) was dissolved in 500 ml of Q water and stored at room temperature.
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Sodium hydroxide (10 M): 200 g of sodium hydroxide (Fisons) was added slowly to 400 ml of Q water. After adjusting the volume to 500 ml, the solution was stored at room temperature.

Southern denaturation solution: 0.5 M NaOH, 1.5 M NaCl; autoclaved and stored at room temperature.

Southern neutralisation solution: 0.5 M Tris-HCl (pH 7.4), 3 M NaCl; autoclaved and stored at room temperature.

SSC (20x): 3 M NaCl, 0.3 M tri-sodium citrate (Fisons); pH adjusted to 7 with NaOH, autoclaved and stored at room temperature.

TAE (10x): 48.4 g of Trizma base (Sigma) and 20 ml of 0.5 M EDTA (pH 8) were dissolved in 1 litre of Q water after the pH had been adjusted to 7.5 with glacial acetic acid (Fisons).

TE: 10 mM Tris-HCl (pH 7.2), 1 mM EDTA (pH 8); autoclaved and stored at room temperature.

Tris-HCl (1 M): 121.1 g of Trizma base was dissolved in 1 litre of Q water after adjusting the pH as required with concentrated HCl. Following sterilisation by autoclaving, the solution was stored at room temperature.

X-Gal: X-gal was dissolved in dimethylformamide to make a stock solution of 20mg/ml. This was stored in a glass tube wrapped in aluminium foil and stored at -20°C.

2.3.2 Large-Scale Preparation of Plasmid DNA

Alkaline lysis and purification of DNA by the use of Qiagen columns (Hybaid, Teddington) was performed according to the manufacturer's instructions. 500 mls of bacterial culture was harvested by centrifugation at 6,000g for 15 minutes at 4°C using a JA-14 rotor (Beckman) in a J2MC centrifuge (Beckman). The pellet was resuspended in 10mls of buffer P1 (100µg/ml RNase A, 50mM Tris-HCl, 10mM EDTA, pH 8) followed by addition of 10mls of buffer P2 (200mM NaOH, 1% SDS) and the mixture was incubated at room temperature for 5 minutes. 10mls of chilled buffer P3 (3M Potassium Acetate, pH5.5) were added and the mixture was incubated on ice for 20 minutes. The solution was then centrifuged for 30 minutes at 30,000g in
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a JA-20 rotor (Beckman) at 4°C and the supernatant was applied to a Qiagen-tip 500 previously equilibrated by applying 10mls of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH 7, 0.15% Triton X-100) and the column allowed to empty by gravity flow. The Qiagen tip was washed with 2x30mls of buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7) and then the plasmid DNA was eluted from the column with 15mls of buffer QF (1.25M NaCl, 50mM Tris-HCl, 15% ethanol, pH 8.5). The plasmid DNA was precipitated with 0.7 volumes of isopropanol and centrifuged immediately at 15,000g for 30 minutes at 4°C in a JS-13.1 rotor (Beckman). The DNA pellet was washed in 15mls of 70% ethanol and resuspended in a suitable volume of sterile water. Subsequent to purification the quantity and quality of the DNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

2.3.3 Small-Scale Preparation of Plasmid DNA

Two methods were used, the first for analysis of plasmid DNA and the second for producing template plasmid DNA suitable for sequencing.

**Method 1**

A 5ml overnight culture was grown from a single colony or a glycerol stock in 2xYT under appropriate antibiotic selection. A 1.6ml aliquot was spun down in a microcentrifuge tube at 13,000g, the pellet resuspended in 100μl sterile water and incubated on ice for 5-minutes. 200μl of alkaline SDS (1% SDS, 0.2M NaOH) were added, mixed and the cells incubated on ice a further 5minutes. Next, 150ml of potassium acetate solution (3M Potassium Acetate, pH5.5) was added, the solution gently mixed and incubated on ice for a final 5mins. Precipitated matter was collected by centrifugation and discarded; the supernatant was retained. RNase A was added to 100μg/ml and the solution incubated at 37°C for 1 hour. Precipitated protein was removed by phenol extraction and the plasmid DNA was recovered by ethanol precipitation.

**Method 2**

Plasmid DNA was purified using the Wizard™ Miniprep DNA Purification System (Promega). A 5ml overnight culture was grown from a single colony or a glycerol stock in 2xYT under appropriate antibiotic selection. 1.5 mls of cells were pelleted and resuspended in 200μls of cell resuspension solution (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100μg/ml RNase A). 200μls of cell lysis solution (0.2M NaOH, 1% SDS) were added and the solution mixed by inverting the tube several times. 200μls of neutralization solution (1.32M Potassium Acetate, pH 4.8) were then added and the mixture vortexed and then spun at top speed in a microcentrifuge for 5 minutes. The supernatant was transferred to a fresh tube and 1ml of Wizard
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Minipreps DNA Purification Resin was added. The resin/DNA mix was transferred into a 2ml syringe barrel placed in a Wizard Minicolumn and then pushed into the Minicolumn using the syringe plunger. 2mls of column wash solution (83mM NaCl, 8.3mM Tris-HCl, pH 7.5, 2mM EDTA and 55% ethanol) were then also pushed though the minicolumn. The minicolumn was then removed from the syringe, placed in a microfuge tube and spun at top speed for 20 seconds. The pure plasmid DNA was then eluted from the minicolumn with 50µls of sterile water.

2.3.4 Gel Electrophoresis

Nucleic acids were separated by electrophoresis through horizontal agarose slab gels which varied between 0.8 and 2% (w/v). Gels were made and run in 1 x TAE (DNA) buffer. Ethidium bromide was added to the gel at 100 ng/ml to allow visualisation of the DNA when illuminated by ultraviolet light (254nm wavelength). When a permanent record of a gel was required, the gel was photographed.

2.3.5 Restriction Enzyme Digestions

Restriction endonucleases were used according to the manufacturers’ instructions and incubated for sufficient time to allow complete digestion. 1/10 volume of loading buffer (20mM EDTA, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added and the sample loaded on an agarose gel together with a suitable size-marker.

2.3.6 Purification of DNA Fragments from Agarose Gels

The desired fragment was excised from an agarose gel, placed inside dialysis tubing containing a small amount of 1 x TAE and electrophoresed at 10 V/cm for 15 minutes. The eluted DNA was then ethanol precipitated, dried and resuspended in a suitable volume of Q water.

2.3.7 Ligation of DNA Molecules and Transformation of Competent E. coli Cells

Ligations were performed using T4 DNA ligase at 16°C. Standard reactions were performed in a 10µl volume with 10-100ng of vector and an appropriate amount of insert to give a 1:1 molar ratio of vector end to insert ends. 0.5µl of T4 DNA ligase from New England Biolabs at 400u/µl final concentration with the supplied 10 x buffer was used and incubations were for a minimum of 16hrs.
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Transformation of competent *E. coli* cells

**Method 1**

**Preparation of competent cells.** A single colony was picked from an agar plate and grown overnight in 10mls LB. A 1ml aliquot was diluted 1:100 in LB prewarmed to 37°C and the culture grown to an optical density at 600nm of 0.5. The culture was chilled on ice for 15 min and the cells collected as a pellet by centrifugation at 4°C, 3,500 rpm for 5min in a JA-14 rotor. The pellet was carefully drained of LB, the cells resuspended in 40ml of Tbef (30mM potassium acetate, 100mM RbCl, 10mM CaCl2, 50mM MnCl2 and 15%(v/v) glycerol at pH 5.8 and filter sterile) and incubated on ice for 5 min. The cells were collected by a second centrifugation as above and the pellet resuspended in 4mls TbfII (10mM MOPS pH 6.5, 75mM CaCl2, 10mM RbCl and 15%(v/v) glycerol, filter sterile). Following a 15 min incubation on ice the cells were flash-frozen in 200μl aliquots in microfuge tubes using liquid nitrogen. They could be stored in this state at -70°C until required.

**Introduction of DNA.** An aliquot of competent cells was thawed on ice for 15 min. 50-100ng of DNA from a ligation, or 5-10ng of closed circular DNA, was chilled on ice while the cells were thawing. The DNA was added to 100μls of cells, mixed by gentle agitation and incubated on ice for 30 minutes. The DNA-cell mix was incubated at 42°C for 90 seconds and finally returned to ice for 5 minutes.

**Selection of transformed cells.** 400μls of 2xYT was added and the cells incubated at 37°C with shaking for 1 hour. Following this cells were diluted as appropriate for each experiment and plated on LA, including appropriate antibiotics (usually 60μg/ml ampicillin) and chromogenic selective agents if required. Transformed cells could be seen as discrete colonies on one or more of the plates following an overnight incubation at 37°C.

**Method 2**

**Preparation of electrocompetent *E. coli* cells:** 1 liter of L-broth was inoculated with 1/100th volume of fresh overnight culture. The cells were grown at 37°C with vigorous shaking to an absorbance at 600nm of 0.4 to 0.6. The culture flask was then chilled on ice for 30 minutes and the cells then harvested by centrifugation at 4,000g for 5 minutes at 4°C in a pre-chilled JA-14 rotor. The supernatant was removed and the cell pellets resuspended by gentle pipetting in a total of 1 liter of ice-cold sterile water. The cells were harvested at 4,000g for 5 minutes at 4°C and the cell pellet resuspended in 500mls of ice-cold sterile water.
The cells were harvested at 4,000g for 5 minutes at 4°C and resuspended in 20mls of ice-cold, sterile 10% glycerol. The cells were harvested at 4,000g for 5 minutes at 4°C in a bench top centrifuge and the cell pellet resuspended in 2mls of ice-cold, sterile 10% glycerol. The cell suspension was frozen on dry ice in 40μl aliquots and stored at -70°C until needed.

**Electro-transformation and plating**: An aliquot of cells was gently thawed at room temperature and then placed immediately on ice. 1-2μl of DNA (containing 10-100ng DNA) was prepared in a low ionic strength buffer and then added to 40μl of the cell suspension. The mixture was then added to a chilled 0.2cm gap cuvette (BIO-RAD). The Gene Pulser apparatus (BIO-RAD) was set at 25mF and 2.50kV and the connected Pulse Controller (BIO-RAD) was set to 200 Ω. A pulse was delivered to the cells and 1ml of 2xYT, prewarmed at 37°C was added to the cells. The cells were then removed from the cuvette and placed in a polypropylene tube and incubated with shaking at 37°C for 1 hour. Following this cells were diluted as appropriate for each experiment and plated on LA, including appropriate antibiotics and chromogenic selective agents if required. Transformed cells could be seen as discrete colonies on one or more of the plates following an overnight incubation at 37°C.

2.3.8 **Testing Transformed Bacteria for α-Complementation**

40μl of X-Gal (20mg/ml) and 4μl of IPTG (200mg/ml) were spread on a pre-made LB agar plate containing the appropriate antibiotics. The plate was incubated at 37°C until all the liquid was absorbed. The plate was inoculated with the bacteria and the plate was incubated in an inverted position for 12-17hrs. The plate was stored at 4°C for several hours to allow the blue colour to develop.

2.3.9 **Purification of High Molecular Weight Genomic DNA from Tissues**

Bovine and porcine tissues were powdered under liquid nitrogen in a mortar and pestle. The powder was then resuspended in 5 volumes of 1xSE buffer (150mM NaCl; 100mM EDTA, pH 8) with 1/10 volume of 10% SDS in Corex tubes(DuPont Scientific Instruments, Delaware) and mixed gently. 1/2 volume of PCI was added and after mixing, the emulsion was spun at 10,000 rpm in a JA-20 rotor for 2 minutes. 2 volumes of 100% ethanol were added to the supernatant and this was mixed gently to produce a stringy precipitate which was spooled to a fresh tube and washed in 80% ethanol. The precipitate was redissolved in 0.5-1ml of 0.1xTNE pH7.5 buffer (5mM...
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Tris-HCl pH 7.5, 0.5mM EDTA, 10mM NaCl). 1/200 volume of pancreatic RNase (20mg/ml) was added and the solution incubated for 15min at 37°C. 1/10 volume of 10% SDS, 1/20 volume 20xTNE pH 8 (1M Tris-HCl pH 8, 100mM EDTA, 2M NaCl) and 1/200 volume of pronase (20mg/ml) was added, mixed, and incubated for 15min at 37°C. 1/2 volume of PCI was added and after mixing spun at 10,000 rpm for 2min. 1/10 volume of 3M sodium acetate pH5.6 and 2 volumes of 100% ethanol were added to the supernatant. A stringy precipitate formed which was spoiled into a fresh tube and rinsed with 80% ethanol. The precipitate was redissolved in 10mM Tris-HCl pH 7.5, reprecipitated at least 3 more times and finally redissolved in 50-200μl of 10mM Tris-HCl pH 7.5. The DNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm.

2.3.10  Purification of High Molecular Weight Genomic DNA from Cultured Mammalian Cells

Purification of genomic DNA from cultured cells was carried out using Qiagen genomic DNA columns according to the manufacturer's instructions. Cells growing in monolayer were removed from the culture dish by trypsin-treatment and placed in a chilled centrifuge tube. The cells were recovered by centrifugation at 1,000g for 10 minutes at 4°C in a bench-top centrifuge. The cells were washed once in PBS and again recovered by centrifugation. They were resuspended at a concentration of $10^7$ cells/ml. Cells grown in suspension were recovered by centrifugation at 1,000g for 10 minutes at 4°C. The cells were washed once in PBS and again recovered by centrifugation. They were resuspended at a concentration of $10^7$ cells/ml. To 2mls of washed cells 2mls of ice-cold buffer Cl (320mM Saccharose, 5mM MgCl2, 10mM Tris-HCl and 1% Triton X-100, pH7.5) and 6mls of ice-cold sterile water were added. The mixture was incubated on ice for 10 minutes. The lysed cells were then centrifuged at 1,300g for 15 minutes at 4°C and the supernatant was discarded. 1ml of ice-cold buffer Cl and 3mls of sterile water were added and the pelleted nuclei resuspended by vortexing. The nuclei were then centrifuged at 1,300g for 15 minutes at 4°C and the supernatant was discarded. The nuclei pellet was completely resuspended in 5mls of buffer G2 (800mM Guanidine HCl, 30mM EDTA, 30mM Tris-HCl, 5% Tween-20, 0.5% Triton X-100, pH 8) by vortexing for 15 seconds and 95μl of Proteinase K (20mg/ml) was added. The nuclei suspension was incubated at 50°C for 1 hour.

A Qiagen Genomic-tip 100 was equilibrated with 4mls of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH 7, 0.15% Triton X-100) and the column allowed to empty by gravity flow. The nuclei suspension was vortexed and then applied to the equilibrated column and allowed to enter the resin by gravity flow.
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The Qiagen Genomic-tip was washed with 15mls of buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7) and the genomic DNA was then eluted from the column with 5mls of buffer QF (1.25M NaCl, 50mM Tris-HCl, 15% ethanol, pH 8.5). The DNA was precipitated with 0.7 volumes of isopropanol and the DNA was spooled using a glass rod and placed in a fresh tube where it was washed with 70% ethanol and then air-dried. The DNA was resuspended in 200-300μls of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8) and allowed to dissolve over night at 4°C. The yield and purity of the DNA were measured by taking absorbance readings at 260nm and 280nm. The A260nm/A280nm ratio should be 1.7-1.9 for pure DNA. The quality of the DNA was checked by running a sample on a 0.3% Agarose gel.

2.3.11 Purification of Total RNA from Tissues

Total RNA was purified from whole tissues by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). All solutions were prepared using DEPC-treated Q water and all vessels containing RNA were soaked in DEPC-treated Q water prior to use.

Guanidinium thiocyanate extraction: Tissue was homogenised on ice in 1ml of extraction buffer (4M guanidium thiocyanate, 25mM sodium citrate, pH7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) then extracted with phenol. The aqueous phase was precipitated twice with isopropanol (Fisons), washed with 70% ethanol, dried and resuspended in 0.5% SDS or DEPC-treated Q water. The quantity and quality of the RNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

2.3.12 Purification of Poly (A)+ mRNA from Tissues

Poly (A)+ mRNA was isolated using a fast lysis oligo (dT) selection method (FastTrack™, Invitrogen) according to the manufacturer’s instructions. Briefly 1g of tissue was homogenized in 15ml of Lysis buffer using a Polytron (Status, Lucern) for 15-30 seconds. The lysate was incubated at 45°C for 30 minutes, and the NaCl concentration was adjusted to 0.5 M with 5 M NaCl, before the addition of one oligo (dT) cellulose tablet. After mixing at room temperature for 15 minutes, the oligo (dT) cellulose was pelleted, and washed in low salt buffer, before being resuspended in a final volume of 0.8 ml. The sample was pipetted into a spin-column and the poly (A)+ mRNA was eluted in 400μl of elution buffer, ethanol precipitated, resuspended in DEPC-treated Q water and stored at -70°C.
2.3.13 Purification of Total RNA from Cultured Mammalian Cells

Total RNA was isolated from cultured cells using TRIzol™ Reagent (GIBCO BRL), a mono-phasic solution of phenol and guanidine isothiocynate. Cells grown in monolayer were lysed directly in 1ml of TRIzol per 10cm² area of culture dish. Cells grown in suspension were pelleted by centrifugation at 4000g at room temperature for 3 minutes and then lysed in 1ml of TRIzol reagent per 5x10⁶ cells by repetitive pipetting. The homogenized samples were were incubated at room temperature for 5 minutes followed by addition of 0.2mls of chloroform per 1ml of TRIzol reagent. Samples were shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. The samples were then centrifuged at 12,000g for 15 minutes at 4°C and the resulting aqueous phase removed to a fresh tube. 0.5mls of isopropanol per 1ml of TRIzol used for the initial homogenization were added to the aqueous layer to precipitate the RNA. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000g for 10 minutes at 4°C. The RNA precipitate was washed with 75% ethanol and then resuspended in RNase-free water. The quantity and quality of the RNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

2.3.14 First Strand cDNA Synthesis from Total RNA

First strand cDNA synthesis was carried out using the SUPERSCRIPT™ Premplification System for First Strand cDNA Synthesis (GIBCO BRL). 1μl of oligo(dT) was added to 5μg of total RNA in a final volume of 14μl. The mixture was heated to 70°C for 10 minutes and then incubated on ice for 1 minute. The contents of the tube were collected by brief centrifugation and then 2μl of 10x synthesis buffer, 1μl of 10mM dNTP mix, 2μl of 0.1mM DTT and 1μl of SuperScript II Reverse Transcriptase were added in a final volume of 20μl. This was incubated at room temperature for 10 minutes and then placed at 42°C for 50 minutes. The reaction was terminated by incubating the tube at 70°C for 15 minutes and then placing on ice. 1μl of RNase H was then added and the mixture was incubated at 37°C for 20 minutes. The cDNA was then ready for use in PCR amplifications.

2.3.15 PCR Methods

PCR was used for amplifying sequences from DNA/cDNA in several protocols. Amplifications were performed using a Perkin Elmer Cetus DNA thermal cycler. The 10 x buffer used was supplied with the Taq DNA polymerase (Boehringer
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Mannheim). The reaction volume was 100µl containing 2.5u of Taq polymerase, 250µM dNTPs and overlayed with 50µl of mineral oil (Fisons). Primers were present at 0.2pmoles and template DNA at 10-300ng for genomic DNA amplifications and at up to 3µg for amplification of cDNA where the cDNA of interest was believed to be present at very low levels. When necessary extra MgSO4 was added to the reaction at concentrations of 4mM or 6mM.

For amplification of genomic DNA sequences using two known primers, the reactions were set up according to the protocol supplied with the DNA thermal cycler and amplification performed using 30 cycles of: 94°C melt for 1 minute, anneal for 1 minute and extension at 72°C for 3 minutes. The final extension was for 10 minutes, followed by a 4°C soak. Annealing temperatures were usually 5°C below the calculated melting temperature of the oligonucleotide primers. For amplification of cDNAs a 40 cycle reaction was used.

2.3.16 Purification of PCR Products for Cloning

PCR products of 200-400bp were purified using Wizard™ PCR Preps DNA Purification System for Rapid Purification of DNA Fragments (Promega) according to the manufacturer's instructions. The purified DNA was then used in cloning steps as described below.

2.3.17 Cloning of PCR Products for Sequencing

PCR products were cloned into pUC18 SmaI dephosphorylated vector using the SureClone™ kit (Pharmacia) following the manufacturer's instructions. Cloned PCR products were then sequenced as described below.

2.3.18 Southern (DNA) Blotting

Genomic DNA was prepared from cultured cells by the method described. The appropriate restriction endonuclease was used to digest the genomic DNA as previously described and the digested DNA was then run out on a 0.8% agarose gel. Following electrophoresis and photography, vacuum transfer of the DNA to a nylon membrane (Hybond-N, Amersham) was performed using a VacuGene XL blotting unit (Pharmacia). The apparatus was set up as instructed by the manufacturer. Depurination was carried out for 20 minutes in 0.2M HCl followed by denaturation for 20 minutes in Southern denaturing solution and then neutralisation for 20 minutes in Southern neutralising solution. Transfer was then carried out for 90 minutes in 20 x SSC. The filter was dried and the DNA cross-linked to the membrane by irradiation.
with ultraviolet light using a UV Stratalinker™ 2400 on the autocrosslink setting (Stratagene).

2.3.19 RNA Dot Blots
Total RNA was prepared from cell lines using TRIzol™ Reagent (Gibco BRL) as described. 5μg of each RNA sample to be analysed was dotted onto a nylon filter, allowed to dry and the RNA was cross-linked to the membrane by irradiation with ultraviolet light using a UV Stratalinker™ 2400 on the autocrosslink setting (Stratagene).

2.3.20 Preparation of 32P-Radiolabelled DNA Probes by Random Priming

The method used was that of Feinberg and Vogelstein (1983). Briefly, DNA fragments were radiolabelled using random primers, α32P dCTP and dTTP, dGTP and dATP. 10ng of the fragment to be labelled in 17μl of H2O was boiled for 10 min with 5μl of oligo-labelling buffer (250mM Tris-HCl pH 8, 25mM MgCl₂, 0.3% β-mercaptoethanol, 100μM each dATP, dCTP, dGTP, dTTP, 1M HEPES pH 6.6 and 0.5μM hexanucleotides) and cooled to allow the random hexanucleotides to anneal to the fragment template. 1μl α32P dCTP(0.37MBq/μl) was added with 6.5μl of Klenow fragment and 1μl of BSA(10mg/ml) to a final volume of 25μl and the reaction allowed to incorporate the radiolabel by incubating at room temperature for 1 hour. The reaction mixture was added directly to the hybridisation chamber.

2.3.21 Hybridisation of Membrane-Immobilised Nucleic Acids

Prehybridisation was performed for a minimum of 10 minutes at 60°C in Church and Gilbert buffer (Church and Gilbert, 1984), 7% (w/v) SDS, 1% (w/v) BSA, 0.5M NaPO₄ pH7 and 1mM EDTA. Hybridisation was performed in this solution with 0.5ng/ml of 32P-radiolabelled probe for 16hours at 60°C. Prehybridisation and hybridisation were performed in a volume of 10ml in sealed Hybaid oven hybridisation bottles.

After hybridisation the filters were washed initially in 2 x SSC, 0.1% (w/v) SDS at 60°C for 2 x 20 minutes and then in 1 x SSC, 0.1% (w/v) SDS at 60°C for 1 x 20 minutes and then in 0.5 x SSC, 0.1% SDS at 60°C for 1 x 20 minutes. Filters were blotted dry and autoradiographed, using Kodak X-Omat™ (Eastman Kodak Company, New York), at -70°C with intensifying screens.
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2.3.22 **Screening Bacterial Colonies using DNA Probes**

A gridded nylon filter (orientated) was placed on an agar plate and individual colonies picked onto the membrane using sterile tooth-picks (the same colonies were also replicated onto a master plate). The plate was then inverted and grown at 37°C until the colonies became visible. At this time the filter was peeled off the plate and transferred to 3MM Whatman filter paper soaked in 10% SDS for 3 minutes and then transferred to 3MM paper soaked in Southern denaturing solution. After 5 minutes, the filter was transferred to 3MM paper soaked in Southern neutralising solution for 5 minutes. The filter was then washed briefly in 1 x SSC to remove bacterial debris before drying and cross-linking the DNA to the filter by irradiation with ultraviolet light using a UV Stratalinker™ 2400 on the autocrosslink setting (Stratagene). The filter was then probed as described for Southern blots.

2.3.23 **Automated Sequencing of DNA**

Double stranded DNA templates were prepared by alkaline-lysis plasmid DNA preparation as previously described and single stranded DNA templates were prepared as described below. Double-stranded DNA samples were adjusted in concentration to 250ng/ml based on the absorbance at 260nm. Single-stranded templates were prepared at a concentration of 100ng/ml. Both DyeDeoxy™ Terminator and Dye Primer sequencing were performed using the cycle sequencing procedure and the Cycle Sequencing Kits essentially as described by the supplier (ABI). The reactions were carried out using the PCR System 480 Thermal Cycler (Perkin Elmer-Cetus). The samples were electrophoresed on an ABI 373 automated DNA Sequencer. The primary sequence data was edited using the program package SeqEd™ 675 DNA Sequence Editor (ABI) on a Macintosh Ilci.

2.3.24 **Recovery of Single-Stranded Phagemid DNA.**

5mls of 2xYT containing ampicillin at 60mg/ml was inoculated with a single *E.coli*(F') colony containing pBluescript. The culture was incubated overnight at 37°C with strong agitation. 50μl of the overnight culture was used to inoculate a further 5mls of 2xYT and these grown to give A600nm of 0.5. At this stage M13K07 helper 'phage was added to the culture to give a concentration of 1x10^{11} pfu/ml. The subculture was allowed to incubate overnight. Single stranded DNA was isolated by spinning down 1.5 ml of the infected culture and recovering the supernatant. The phage particles were precipitated using 20% Polyethylene glycol (PEG 8000) in 2.5M
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NaCl and recovered by centrifugation at 13000g for 5 minutes. The pellet was resuspended in 100µl of sterile water and the protein coat from the particles was removed by phenol extraction. The DNA released was precipitated using 10µl of 3M Sodium acetate (pH 5.2) and 250 mls 100% ethanol and washed using 70% ethanol before resuspending in 50ml sterile water. The concentration was then determined by electrophoresis and by spectrophotometric techniques.

2.4 In Vitro Cell Culture Methods

2.4.1 Solutions used During the Culture and Transformation of Animal Cells

DMEM:MEL cells: DMEM (without sodium pyruvate, with 500mg/l glucose) was purchased from Gibco BRL and stored at 4°C.
HEK 293 cells: DMEM Nut Mix F-12 (without L-glutamine) from Gibco BRL and stored at 4°C.

The medium for both cell types was supplemented with 10% (v/v) FCS (Gibco BRL) and antibiotic (100µ/ml penicillin and 100µ/ml streptomycin, Gibco BRL).

PBS (tissue culture): Dulbecco’s modified PBS (without Mg^2+ or Ca^2+) was prepared by dissolving ten tablets (ICN Flow) in 1000ml of Q water. The PBS was dispensed (250ml aliquots) into 500ml tissue culture bottles (Gibco BRL). Following sterilisation by autoclaving, the solution was stored at room temperature.

Electroshock buffer for MEL-cells: Electroshock buffer for the electroporation of MEL-cells contained 140mM NaCl, 25mM HEPES pH 7.5 (correct pH obtained using 4M NaOH) and 0.75mM Na2HPO4. 500ml aliquots of the buffer were filter sterilised. The final pH of the buffer was approximately pH 7.

Gene pulser cuvettes: Gene pulser cuvettes with a 0.4cm electrode gap, for the electroporation of MEL cells, were obtained from Biorad.

Geneticin (G418): Geneticin (G418) powder was dissolved in Q water to a final concentration of 200mg/ml (w/v) and stored as 1ml aliquots at -20°C.

Trypsin/EDTA: A solution containing 0.5g Trypsin and 0.2g EDTA in 1l H2O was used.
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Cell lines cultured In Vitro: MEL-C88 murine erythroleukaemia cells (Deisseroth, 1978) and HEK 293 human embryonal kidney cells (Graham et al., 1977) were employed. (Both cell lines were tested for the absence of mycoplasma.)

2.4.2 General Cell Culture

Cell manipulations were performed in a class II microbiological safety cabinet (Medical Air Technology Ltd., Manchester). Cells were routinely cultured in 15ml of medium in 260ml Nunclon® (Gibco BRL) flasks. All mammalian cells were incubated at 37°C (5% CO2 atmosphere) in a humidified incubator.

2.4.3 Subculturing and Handling of Cells

MEL cells
To concentrate MEL-cell lines (non-adherent cells), the medium containing cells was centrifuged in a sterile 15ml tube (Sarstedt) for 5 minutes at 1000rpm in a bench top centrifuge. The medium was aspirated and the cells resuspended in a suitable volume of fresh medium. To subculture these cell-types, log-phase cells were diluted as required (up to one hundred-fold) into fresh medium in 260ml tissue culture flasks.

HEK293 cells
To concentrate HEK293 cells (adherent cells) the medium was removed from the cells in a 260 ml flask which were then incubated for 5mins at room temperature in 2mls trypsin/EDTA. The trypsin/EDTA was then inactivated by the addition of 10mls of complete medium and this medium containing the cells was centrifuged in a sterile 15ml tube for 5 minutes at 1000rpm in a bench top centrifuge. The medium was aspirated and the cells were washed in PBS. The pellet was then resuspended in a suitable volume of medium and the cells seeded into fresh flasks at a density of 5×10⁴ cells/ml.

2.4.5 Cryogenic Storage of Cells

Cells from one 50ml flask (Nunclon®) were pelleted as above and resuspended at 1×10⁶ cells/ml in medium containing 10% DMSO (Sigma, cell culture grade) and 50% FCS. 1ml aliquots were dispensed into 1.5ml ampules and cooled slowly to -196°C before storing in liquid nitrogen in a model LR40 cryostorage refrigerator (Jencons [Scientific] Ltd., Leighton Buzzard).

Growth of cells from frozen samples was performed as follows: a vial of frozen cells was placed in a water bath at the normal culture temperature of the cells until thawed. The cells were then transferred to 15ml of culture medium in a 260ml
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flask and incubated overnight. The following day, the medium was changed for fresh medium and incubation continued.

2.4.6 Stable Transfection of MEL-cells

The DNAs of expression constructs were linearized with a suitable restriction enzyme prior to electroporation. 1 x 10^7 MEL cells in logarithmic growth phase were washed twice with PBS and resuspended in 0.9 ml of electroshock buffer before being mixed with 25 μg quantities of expression constructs. The cell-DNA mixture was placed in a gene pulser cuvette and electroporated in a Bio-Rad Gene Pulsar™ at 960 mF and 250 V in sterile electroshock buffer. Following electroporation, cells were diluted in fresh culture medium to 10^4 - 10^5 cells/ml; 24-hours later, G418 was added (final concentration 1 mg/ml) to select for transfectants. Semi-adherent colonies (at a size visible to the naked eye) appeared 10-14 days post-transfection, and were either pooled or grown in separate wells under G418 selection. Independently-propagated cell-lines were numbered and aliquots taken for storage in liquid nitrogen.

2.4.7 Induction of MEL-Cells for Expression Studies

For expression studies cells were thawed at 37°C, and 50 μl diluted into 10 ml complete DMEM medium. Exponential growth was maintained for a minimum of four days prior to addition of DMSO (2 % v/v) to induce differentiation and expression. Cells were suitable for electrophysiological analysis and RNA expression assay between 0.5 to 5 days post-induction.

2.4.8 Stable Transfection of HEK 293 cells

HEK 293 cells were transfected using LipofectAmine™ Reagent (Gibco BRL) according to the manufacturer's instructions. Briefly, cells were seeded in a 6 well tissue culture dish (Nunclon®, Gibco BRL) at a density of 2x10^5 cells per well and incubated until the cells were 50% confluent. For each transfection 1-2 μg of linearised DNA was added to 100 μl of serum-free medium and 10 μl of LipofectAmine Reagent was diluted into 100 μl of serum-free medium. These two solutions were combined, mixed and incubated at room temperature for 30 mins. The liposome/DNA complexes were diluted by addition of 800 μl of serum-free medium and then added to the cells which had previously been washed in 2mls of serum-free medium. The cells were incubated for 6-7hrs at 37°C in a CO2 incubator and following incubation 1ml of growth medium containing 20% FCS was added. 18-24 hours following the start of transfection the medium was replaced with fresh complete
medium. At 72hrs after transfection the cells were passaged 1:10 into fresh medium containing 1mg/ml G418 and after 10 days-2 weeks G418 resistant colonies appeared which were transferred to separate 50ml tissue culture flasks (Nunclon®).

2.5 Protein Techniques

2.5.1 Preparation of Total Protein from Mammalian Cell Lines

Cells in suspension were spun down for 5 minutes at 2000rpm in a bench-top centrifuge and the cell pellet washed once in PBS. The cells were then resuspended at a concentration of 2x10^6 cells/50μl in lysis buffer (2% SDS, 100mM dithiothreitol, 60mM Tris-HCl pH 6.8, 5% β-mercapto-ethanol and 0.01% bromophenol blue). The lysed cells were placed in a boiling water bath for 5mins and then sonicated for 20secs at 5 microns in a Soniprep 150 Ultrasonic Disintegrator (Sanyo). The protein was then spun in a microfuge for 5mins and the supernatant removed to a fresh tube. The sample was now ready for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein solutions were stored at -20°C.

2.5.2 Preparation of Total Protein from Tissues

Approximately 0.5g of tissue was frozen in liquid N₂ and then ground, using a pestle and mortar, into a powder. Lysis buffer (2% SDS, 100mM dithiothreitol, 60mM Tris-HCl pH 6.8) was then added and the mixture vortexed and incubated on ice for 20mins. The protein solution was then placed in boiling water bath for 10mins and then sonicated for 20secs at 5 microns in a Soniprep 150 Ultrasonic Disintegrator (Sanyo). The protein was then spun in a microfuge for 5mins and the supernatant removed to a fresh tube. The protein concentration was estimated by measuring A_{280nm}. Before analysing by SDS-PAGE the protein solution was adjusted to contain 5% β-mercapto-ethanol and 0.01% bromophenol blue.

2.5.3 Gel Electrophoresis of Proteins

Proteins were separated by electrophoresis through SDS-containing discontinuous polyacrylamide gels (SDS/PAGE) according to the method of Laemmli (1970). The separating gel percentage was optimum for the molecular weight of the particular protein.
2.5.4 Coomassie Blue Staining of Protein Gels

After electrophoresis the protein gel was transferred to a clean plastic container and 5 gel volumes of Coomassie blue stain (0.25% Coomassie brilliant blue G-250, 50% methanol and 10% acetic acid) were added and incubated overnight with shaking at room temperature. The gel was then destained by successive incubations in 10% acetic acid, 30% methanol at room temperature with shaking.

2.5.6 Western (protein) Blotting

Proteins were transferred from SDS/PAGE gels to nitrocellulose membranes (Hybond-C, Amersham) in transfer buffer (160mM glycine, 12mM Tris base, 10% methanol) essentially by the method of Towbin et al. (1985). Transfer was performed using the Trans-blot® cell (from Bio-rad) at 0-4°C at 60V overnight. Rainbow markers (Amersham) were used to indicate the transfer of protein onto the nitrocellulose.

2.5.7 Antigen Detection

The nitrocellulose membrane containing the proteins was incubated in 5% Marvel diluted in TBST (10mM Tris-HCl, pH 8, 0.9% NaCl, 0.05% Tween 20) for 1hr at room temperature or overnight at 4°C to block protein binding sites on the membrane. The membrane was then transferred to 5% Marvel in TBST containing the primary antibody at a suitable dilution and incubated with gentle agitation for 1hr at room temperature. After 3 x 5 minute washes in TBST the filter was transferred to 5% Marvel in TBST containing the secondary antibody (horseradish peroxidase conjugated) at 1/1000 dilution and incubated with gentle agitation for 1hr at room temperature. The membrane was then washed in TBST for 1 x 15 minutes and then 4 x 5 minutes prior to development. The blot was developed using the ECL™ Western Blotting Kit (Amersham) according to the manufacturer's instructions. Detection solution (containing luminol) was applied to the protein surface of the membrane and incubated for 1 minute at room temperature. The membrane was then wrapped in SaranWrap and placed in a film cassette. In the dark a sheet of autoradiography film (Hyperfilm-ECL, Amersham) was placed on top of the membrane and the cassette closed for 15 seconds. The film was then developed and further film was exposed to the membrane for various times depending on the signal strength.
2.6 **cGMP Assays**

HEK 293 cells were plated out in a 6 well plate (Nunclon®) at a density of 1x10⁶ cells/well and incubated at 37°C, 5% CO₂ overnight. The cells were washed with fresh serum-free medium and then incubated in 1ml of Lockes (154mM NaCl, 5.6mM KCl, 1mM CaCl₂, 1mM MgCl₂, 3.6mM NaHCO₃, 5.6mM Glucose 10mM HEPES and 0.1mM IBMX) for 10mins at 37°C. The media was removed and agonist (50nM ANP or 5µM SNP) was added in 1ml of Lockes and incubated for 3mins at 37°C. The reaction was terminated by the addition of 50µl of 10M HCl. The lysed cells were removed to an Eppendorf and incubated on ice for 20mins. The cells were then neutralised to pH 7 by addition of 10M NaOH. The lysed cells were centrifuged in a microfuge at 13,000g for 20mins and the cell extract was aspirated and used for assays. For MEL cells 10⁶ cells were aliquoted into an Eppendorf and pelleted by spinning for five minutes at 6,500g in a microfuge. The cells were washed in serum-free medium and pelleted again. The cells were then resuspended in 1ml of Lockes for 10 minutes at 37°C. 50nM ANP or 5µM SNP were added directly to the cell suspension and incubated for a further 3 minutes at 37°C. The remainder of the procedure was as for HEK293 cells. The cGMP concentration was measured using the BIOTRAK cGMP enzymeimmunoassay (EIA) system (Amersham Life Science) according to the manufacturer's instructions.
Chapter 3

The Tissue Distribution and Cloning of a Cardiovascular cGMP-Gated Cation Channel
Chapter 3 The Tissue Distribution and Cloning of a Cardiovascular CNGC

3.0 The Tissue Distribution and Cloning of a Cardiovascular cGMP-Gated Cation Channel

3.1 The Distribution of the cGMP-Gated Cation Channel (CNG-1) in Cardiovascular Tissue

3.1.1 Introduction

An isoform of the photo-receptor cGMP-gated channel (CNG-1) has been located in a rabbit sino-atrial node cDNA library (Hundal et al., 1993) and there are also reports of its location in an unspecified area of heart tissue (Ahmad et al., 1992). cGMP is an established second messenger in cardiovascular tissue and plays an important role in cardiac chronotropy (Balligand et al., 1993). However, the events occurring downstream of cGMP elevation in these tissues which result in relaxation are unknown. A cGMP-gated channel may have an important role to play in such events. To investigate the possible functions of the channel in cardiac tissue it is necessary to know whether expression is limited to particular regions of the heart, such as the sino-atrial node, or whether expression occurs universally in this tissue. Therefore, initial studies were carried out to examine the expression of this channel in different regions of cardiovascular tissue.

The species chosen for this distribution study was bovine, as this tissue is freely available and, as the organ is large, it is easier to dissect specific regions of heart tissue. The sequence of the bovine CNG-1 cDNA is also available (Kaupp et al., 1989). A reverse-transcription PCR-based assay (RT-PCR) was chosen for the distribution study because this method is much more sensitive than the Northern method also used to detect mRNA.

3.1.2 A Panel of Heart Tissue Samples

Tissue samples were prepared by isolating cubes of tissue, approximately 0.5mm², from ten regions of the heart. These were: aorta, right atrial wall, left atrial wall, right ventricle inner wall, left ventricle inner wall, right ventricle outer wall, left ventricle outer wall, moderator band, upper left ventricle near atrio-ventricular node and left atrial free wall. Total RNA was prepared from these samples for use in RT-PCR experiments.
Chapter 3 The Tissue Distribution and Cloning of a Cardiovascular CNGC

Fig 3.1 The gene structure of the human cGMP-gated cation channel (Dhallan et al. 1992).

Pre-splicing (chromosomal) structure

It is estimated that the transcription unit of the cGMP-gated channel is >40 kb in length.

Note that in the original mapping of the genomic DNA, clones encompassing exon 2 (including the Alu element present as an insert between nucleotides -217 and -15) were not isolated.

Exon numbering is tentative, based on exon 1 containing the most 5' proximal sequences found in the cDNA.

Seven small exons encode the amino-terminal one-third (229 aa) of the protein.

Alternative splicing removes one of the small exons (exon 8, encoding part of the H1 domain) in a subset of transcripts within the human retina, producing an internal in-frame deletion of 36 codons.

One large exon encodes the C-terminal two-thirds (457 aa) of the protein.
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3.1.3 PCR Primer Design

The gene encoding the human rod photo-receptor CNGC has been shown to consist of at least ten exons with one large exon, exon ten, encoding the carboxy-terminal two-thirds of the protein and seven smaller exons encoding the amino terminus one third (Dhallan et al., 1992; see fig 3.1). It was assumed that the structure of the bovine gene would be very similar. The bovine cDNA sequence was aligned to the human genomic sequence exons, allowing a 5' forward primer, cG-A, to be designed in exon 8 and a 3' reverse primer, cG-B, in exon 10, corresponding to bases 469-494 and 794-819 of the published cDNA (Kaupp et al., 1989). These primers were designed to amplify a 345bp band from cDNA. The design of primers to give products spanning intron/exon junctions allows discrimination between products arising from cDNA and any that may result from contaminating genomic DNA in the template preparation. Forty-cycle PCR reactions with these primers gave indistinct bands of the correct size for several samples and clear bands for right atrial tissue.

Design of PCR primers to CNG-1

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
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<tr>
<td>8</td>
<td>cG-A</td>
</tr>
<tr>
<td>9</td>
<td>cG-C</td>
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<td>10</td>
<td>cG-D</td>
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<tr>
<td>10</td>
<td>cG-B</td>
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Fig 3.2 Schematic diagram of CNG-1 cDNA showing the positions of the forward primers cG-A and cG-C and reverse primers cG-B and cG-D with respect to the position of intron-exon junction splice sites predicted from the gene sequence.

Analysis of cGMP-gated channel expression in kidney cell lines (Ahmad et al., 1992) showed that after a single RT-PCR reaction no positive DNA bands could be visualised on an ethidium bromide stained gel. However strong bands of the correct size were observed when aliquots of the PCR reaction were further amplified with a set of nested primers. Such a protocol was therefore applied to this distribution study and a further pair of nested primers was designed which would result in a product which also spanned an intron/exon junction. The 5' primer cG-C was designed in exon 8 and the 3' primer cG-D in exon 10, corresponding to bases 505-522 and 740-757 of the published cDNA, to give a 251 bp band from cDNA. When the PCR products obtained from
amplification with cG-A and cG-B were re-amplified with primers cG-C and cG-D strong bands, of the expected size, were produced for all samples in the panel (see fig 3.3). Extra PCR products just below 400bps in size, are present in lanes h and l (fig. 3.3). This is due to mis-priming where the PCR primers are annealing to and amplifying sequence highly related to the sequence of interest. In this case primers cGC and cGD may be recognising a related channel also expressed in these regions of heart as well as the channel sequence to which they are specifically designed.

3.1.4 CNG-1 is also expressed in bovine and porcine vascular tissue

The results of the distribution study show that an isoform of CNG-1 is expressed throughout cardiac tissue. A sample of bovine aorta was also included in the panel of tissues screened and, as shown in fig 3.3, a PCR product of the correct size was also amplified from this tissue. Electrophysiological studies carried out at Leicester have shown the possible presence of a cGMP-activated current in porcine coronary artery smooth muscle, although the recordings observed were equivocal (Boyle, J.P. and Dart, C., unpublished observations). In an attempt to validate this data total RNA was prepared from porcine coronary artery and included in the RT-PCR screen of the cardiovascular bovine tissues. As with many of the bovine samples a forty cycle amplification reaction with primers cG-A and cG-B gave a smear when viewed on an agarose gel but when the PCR product was re-amplified using the nested primers, cG-C and cG-D, an intense band of the correct size was observed (see fig 3.3).

3.1.5 Analysis of the Vascular PCR Products by DNA Sequencing

These PCR results indicate the presence of a cGMP-gated channel in vascular tissue, a previously unreported tissue type for CNGC expression. The bovine aorta and coronary artery PCR products were sequenced to verify their relationship to the photoreceptor sequence and also to check that they are not derived from a cloned human cGMP-gated channel contaminant. The PCR products were purified and cloned into pUC 18 for automated, double-stranded sequencing using the DyeDeoxy™ Primer kit with the M13 Rev primer (-21). Sequence data are shown in fig 3.4 and fig. 3.5. The sequence for the bovine aorta PCR product has three base pair changes from the bovine photo-receptor cGMP-gated channel sequence and thirty two base pair changes from the human sequence confirming that the sequence is derived from bovine and not human tissue. The sequence for the porcine coronary artery PCR product has two base pair differences from the human sequence (not including primer-determined sequence) and twenty nine base pair differences from the bovine sequence. The porcine
sequence for the CNG-1 channel is unknown however porcine sequences tend to be very similar to human sequences and it is therefore probable that the porcine CNG-1 sequence is very closely related to the human CNG-1 sequence. There are two amino acid differences between the porcine and human sequence (see fig 3.4b), both of which are conservative (S to L) and (K to Q). These amino acid changes may be a result of errors produced by mismatching of base pairs during PCR amplification. This is probably the case for the two base pair differences between the aorta PCR product sequence and the published bovine CNG-1 sequence. This results in one conservative (I to V) and one non conservative (N to D) amino acid change although allelic differences in sequence may also be responsible.
Fig 3.3 Re-amplification of cGA and cGB PCR products using primers cGC and cGD. A 250bp band is observed in all samples in the panel showing that CNG-1 is expressed throughout cardiac tissue. A band is also observed in lanes h and m indicating the expression of CNG-1 in arteries also. Lanes are as follows (lanes a-l from bovine): a) left atrial free wall b) upper left ventricle wall near a.v. node c) inner left ventricle wall d) left atrial wall e) right ventricle inner wall f) moderator band g) inner left ventricle wall h) aorta i) right ventricle inner wall j) right ventricle outer wall k) upper left ventricle wall near a.v. node l) left ventricle outer wall m) porcine coronary artery smooth muscle n) positive control (bovine genomic DNA) o) negative control p) molecular weight markers: 1000, 700, 500, 400, 300, 200, 100 bps. The positive control appears as a smear as the product from bovine genomic DNA using primers cGC and cGD includes an intron and is too large to be amplified efficiently under the conditions for this experiment.
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Fig 3.4 a) DNA sequence alignment of the cGC-cGD PCR product from porcine coronary artery smooth muscle (PCASM) with human CNG-1. The underlined sequence represents the primers cGC and cGD which were designed to the bovine CNG-1 cDNA sequence.

PCASM TGCATCACCTTACTCTGTAATGTACAACTGGACAATGGTTA  [40]
Hum CNG-1 ........a........t........................

PCASM TGGCACAGCATGnTnTnATGACTTTAATCTnATTACCT  [80]
Hum CNG-1 ............t...g..........c....g......

PCASM AGAAATTGGGTCATTTTGATTACGTATCAnACATAGTC  [120]
Hum CNG-1 ..................................g........

PCASM TATTTAATCGATATGTnTTGTACGAACAAGACAGGTTACC  [150]
Hum CNG-1 ............................................

PCASM TAGAACAAGGACTGCTGGTAAAGGAAGAACTTAAACTCAT  [200]
Hum CNG-1 ............................................

PCASM AAATAAATATAAATCCAACTCGCAATTTAACTTGATGTT  [240]
Hum CNG-1 .....................t.....................

PCASM CTATCAGTGATCCG  [254]
Hum CNG-1 ..g...c....a.c

Fig 3.4 b) Amino acid sequence alignment of the cGC-cGD PCR product from porcine coronary artery smooth muscle with the corresponding amino acid sequence for human CNG-1. An X represents a codon which could not be allocated due to unreadable DNA sequence. The underlined sequence represents primer-determined sequence.

PCASM CITLEPVNYWTVARACXXELKSXYWILDYVSXIV
Hum CNG-1 ..........fd..q.................d..

PCASM YLIDMPVTRRTGYLEQLLVKEELKLINKYSNSQFKLQV
Hum CNG-1 ...............................l....

PCASM LSVIP
Hum CNG-1

67
Figure 3.5 a) DNA sequence alignment of the bovine aorta cGC-cGD PCR product with the bovine CNG-1 sequence. Two nucleotides could not be identified by the Sequence Editor programme and are denoted 'n'. There are three further base pair differences between the cloned PCR sequence and the published bovine CNG-1 sequence. This is probably due to mismatch mutations introduced into the PCR reaction by Taq DNA polymerase during the 80 PCR cycles required to amplify this product. Underlined sequence represents the primers cGC and cGD which were designed to the published bovine CNG-1 sequence.

Figure 3.5 b) Amino acid sequence alignment of the bovine aorta cGC-cGD PCR product with the bovine CNG-1 sequence. The underlined sequence represents primer-determined sequence.
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3.2 Cloning of a cGMP-Gated Cation Channel from Porcine Coronary Artery

3.2.1 Introduction

The PCR-based screen described above showed the presence of a CNGC, very closely related to the retinal channel in porcine coronary artery. To date there have been no reports of expression of this CNGC family member (CNG-1) in vascular tissue, although a member of the family with a CNG-2-related sequence has been cloned from rabbit aorta (Biel et al., 1993). To establish whether the CNGC sequence identified in vascular tissue is an entirely new member of the channel family or simply an isoform of the CNG-1 channel expressed in rod retina a PCR-based approach was designed to attempt to retrieve a full length cDNA for this CNG-1 type channel from porcine coronary artery.

3.2.2 Amplification of Overlapping PCR Products Spanning the Coding Region of the Porcine Coronary Artery cGMP-Gated Cation Channel

As porcine sequences tend to be highly related to human sequences, PCR primers were designed to the human photoreceptor CNGC sequence (Dhallan et al., 1992). Four primer pairs were designed using the PCR Primer Design facility on the MacVector™ programme with the very 5' and 3' primer designed to span the full coding sequence of the channel. These were named cG1-cG8, as shown in fig 3.7, and their predicted products should produce overlapping fragments encompassing the entire CNG-1 coding sequence. Primers cG1, cG4, cG5 and cG8 had a Not I site incorporated into their 5' ends to allow easy cloning of PCR products into the Not I site of a cloning vector. The 5' restriction site on the primer was designed with care to allow sufficient binding of the restriction enzyme close to the end of the DNA molecule. The protocol used was recommended by New England Biolabs and required the addition of ten bases upstream of the restriction site. This procedure allowed >90% enzyme efficiency when the reaction was incubated at 37°C for 20 hours. The most 5' primer, cG1, also included a 'Kozak' consensus sequence (Kozak, 1986) just prior to the initiating ATG to allow for optimal expression of any new cloned CNGC from porcine coronary artery.

Poly A+ RNA was prepared from porcine coronary artery tissue and used in reverse transcription experiments to produce cDNA which could then be used as a PCR template. Use of primers cG1 and cG8 in PCR reactions amplified a band of approximately 2.05Kb from this reverse transcribed PCASM mRNA after thirty five cycles using Taq DNA polymerase (Fig 3.6). No products were produced if a proof
Fig 3.6 Amplification of porcine coronary artery smooth muscle cDNA using primers cG1 and cG8. Lane a) λ Hind III molecular weight markers (23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564bps) b) 10μg cDNA amplified using Deep Vent DNA polymerase, c) negative control using Deep Vent polymerase, d) 10μg cDNA amplified using Taq DNA polymerase, e) negative control using Taq DNA polymerase, f) 123bp ladder molecular weight markers. A band of approximately 2,100bp, the expected size for CNG-1, is observed in lane d. but Deep Vent DNA polymerase was unable to amplify any distinct bands producing only a smear in lane b.
Fig 3.7 Re-amplification of cG1-cG8 porcine coronary artery PCR product (see fig 3.6) using nested primers cG1,cG2,cG3,cG4,cG5,cG6,cG7 and cG8. The above diagram shows the positions to which each primer anneals to CNG-1 and the expected length of amplification products.

The photograph shows the results from the actual PCR experiment with bands of the size predicted from the human CNG-1 sequence confirming the expression of a CNG-1 sequence in porcine coronary artery. Samples in lanes as follows a) cG1-cG2 product b) negative control c) cG3-cG4 product d) negative control e) cG1-cG4 product f) negative control g) cG5-cG6 product h) negative control i) cG7-cG8 product j) negative control k) cG5-cG8 product l) negative control m) cG3-cG6 product n) negative control o) λ.Bst E2 molecular weight markers 8454,7242,6369,5686,4822, 4324,3675,2323,1929,1371,1264,702bps.
reading heat stable polymerase such as Deep Vent DNA polymerase was used instead of \textit{Taq} DNA polymerase. This band was very faint but when the nested primers cG2-cG7 were used to re-amplify the gel-purified PCR product produced by cG1 and cG8 in a thirty five cycle reaction bands of the expected sizes for a CNGC sequence similar to CNG-1 were observed on an agarose gel (see fig 3.7). Negative control experiments were carried out for each primer pair using only water as a template and as can be seen from fig 3.7 no products were observed in these lanes. Some mis-priming is occurring during amplification probably due to the presence of related channel sequences in porcine coronary artery although by far the clearest products are those of the expected size for a CNG-1 channel. These results confirm that a mRNA encoding a cGMP-gated channel with very strong homology to the human photoreceptor channel, CNG-1, is present in porcine coronary artery.

3.2.3 Cloning and Sequencing of Overlapping PCR Products Spanning the Coding Region of the Porcine Coronary Artery Smooth Muscle cGMP-Gated Cation Channel

Unfortunately, the full length product produced by the first round of PCR was present at levels too low to allow it to be cloned, although this was attempted several times. Therefore, the second round PCR products produced by primers cG1 and cG4 and cG5 and cG8 were purified, cut for twenty hours with \textit{Not I} at 37°C, as recommended by NewEngland Biolabs, and ligated into the \textit{Not I} site of pBSK-.

Recombinants containing the correct inserts were selected by colony hybridisation (see fig 3.9). Four recombinant clones were selected for each PCR product and single stranded sequencing was carried out on each clone. This number of clones were analysed to check for any mutations introduced into the DNA by mismatches made by \textit{Taq} DNA polymerase. For the first sequencing reaction the Dye Deoxy\textsuperscript{TM} Primer kit (M13 Rev -21) was used. Following this sequential sequencing primers, 3'cGseq1 and 2 and 5'cGseq1-4, designed within the cDNA coding region were used with the DyeDeoxy\textsuperscript{TM} Terminator kit.

Following sequencing the porcine CNGC sequence was found to be highly related to the human retina CNG-1 sequence. At the nucleotide level there were fourteen differences (fig 3.10) between the porcine and human sequences, resulting in only eight amino acid differences, with six of these eight amino acid differences between the human and porcine sequences conserved among the porcine, bovine and rabbit CNG-1 sequences (see fig 3.11). There is an overall homology between the human rod photoreceptor amino acid CNG-1 sequence and the porcine coronary artery CNG-1 amino acid sequence of 98.8%, reflecting the very close relationship between human and porcine sequences in general. There are two base pair differences between
the sequence from primers cGC and cGD (fig 3.4) and the sequence from primers cG1 and cG4. As only one clone for this cGC-cGD product was sequenced this is probably due to errors introduced into the sequence during PCR.

**Cloning of the Porcine Coronary Artery Smooth Muscle CNG-1**

![Diagram of cloning process](image)

Fig 3.8 This diagram displays the method used to clone PCR fragments spanning the coding region of the porcine coronary artery CNG-1. Shaded areas represent regions of sequence determined by the primer sequence (not to scale).
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Fig 3.9 Colony Hybridisation of cG1-cG5 PCR products amplified from porcine coronary artery smooth muscle cloned into pBKS-.

Following ligation of the Not I digested PCR product into Not I digested pBKS- the ligation mixture was used to transform competent JM109 E.coli. Blue/white selection was used to identify bacterial colonies containing the recombinant plasmid. However white colonies, which should contain recombinant plasmid were found to contain wild type pBKS- vector which had recircularised. This may be due to contaminating DNA exonucleases in the commercial Not I enzyme preparation 'nibbling' the ends of the linearised vector during digestion. Some of these degraded ends were still able to recircularise during ligation resulting in the observation of 'false' positives following blue/white selection. Therefore colony hybridisation was employed to select for true positives using the PCR product itself as a probe. The filter shown here contains fifty 'white' colonies from the original transformation, three of which light up (see arrows) following colony hybridisation. These three colonies were grown up and all contained recombinant plasmids with the cG1-cG5 PCR product inserted at the Not I site.
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Fig 3.10 A MacVector™ DNA sequence alignment of the porcine coronary artery smooth muscle CNG-1 sequence to the human CNG-1 sequence. There are a total of fourteen base pair differences between the human and porcine sequences, resulting in eight amino acid differences. The top line (Porcine cG) displays the porcine coronary artery sequence and the bottom line (@ Human) the human CNG-1 sequence. Conserved base pairs in the human sequence are represented by a . and changes are represented by a lower case letter.
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Porcine CNG-1
Human CNG-1
Bovine CNG-1
Rabbit CNG-1
Rabbit CNG-2

Porcine CNG-1
Human CNG-1
Bovine CNG-1
Rabbit CNG-1
Rabbit CNG-2

Porcine CNG-1
Human CNG-1
Bovine CNG-1
Rabbit CNG-1
Rabbit CNG-2

Porcine CNG-1
Human CNG-1
Bovine CNG-1
Rabbit CNG-1
Rabbit CNG-2

Porcine CNG-1
Human CNG-1
Bovine CNG-1
Rabbit CNG-1
Rabbit CNG-2

MKNNIINTQQSFVTMPNVIVPDIEKEIRRMENGACSSFS-E [40]

DDDSASTSEESESNHPARGSFYKSRLKGDPSQREQTLT [80]

GAIALFNVNSNENKQEPEEKKKKEEKSKGDKNKNKKN [120]

DEKKKKK-KDEKKKKEEKSKDEKKKKEEKKVUVIPDSGNT [160]

YYNNLPCITLPNVYNTMTVAPFQPDDQYLVWYML [200]

YPDVLVSIIPTDLFFKILGWSFYREIRNLRLRFSRHF [280]

FGQIERTYGPNIIFRSLNYMIVVIIIHNACVFYISKA [320]

IGGQDMTHYPOINDPHEGLARKVYLWVSLZLTMPTIT [360]

KTPPVRGEGYVYVFVFDLGFLVPARIYVGKSHMSNHNN [400]

77
Fig 3.11 Amino acid alignment of the porcine coronary artery smooth muscle CNG-1 sequence with the human photoreceptor CNG-1 sequence, the bovine photoreceptor CNG-1 sequence, the rabbit SAN CNG-1 sequence and the rabbit aorta CNG-2 sequence. Lower case letters represent amino acid changes, dots represent conserved amino acids and dashes represent deleted amino acids. Bold letters represent regions of primer-determined sequence.
Chapter 3 The Tissue Distribution and Cloning of a Cardiovascular CNGC

3.3 Is the Porcine Coronary Artery cGMP-Gated Cation Channel Expressed in Vascular Endothelial Cells?

3.3.1 Introduction

Vascular tissue consists of two distinct tissue-types; the layer of smooth muscle cells which control the vessel diameter and a flat monolayer of endothelial cells lining the inside of the vessel wall and in direct contact with the blood stream. As these two different cell layers are very difficult to separate, the tissue sample used to prepare the cDNA template for the above PCR reactions contained both smooth muscle and endothelial cells. However, smooth muscle cells and endothelial cells carry out different functions. Endothelial cells detect agonists in the blood stream and convey the information from these primary messengers to the surrounding layer of smooth muscle cells which respond by contracting or dilating. In smooth muscle cells a rise in cGMP is thought to result in membrane hyperpolarisation which leads to vessel relaxation. In vascular endothelial cells rises in cGMP appear to occur in a response to agonists such as histamine (Groschner et al., 1994) during processes that result in Ca^{2+} uptake into intracellular stores. Expression of CNGCs in one of these cell types and not the other could give clues towards the function of such channels in the vasculature. The most convenient way to study expression in vascular endothelial cells is by looking at cultured cell lines. During this study two cell lines were examined; bovine aorta endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs). Unfortunately smooth muscle cell lines, completely free of contaminating endothelial and fibroblast cells, are not available and therefore CNGC expression distribution could only be studied in endothelial cells.

3.3.2 Expression of CNG-1 in BAECs

Primers cG-A and cG-B, designed for the PCR-based screen of the bovine heart tissue panel, amplified a faint 345bp product from cDNA prepared from cultured BAECs during a thirty five cycle PCR reaction using Taq DNA polymerase. Re-amplification of this product with nested primers cG-C and cG-D, once again employing a thirty five cycle PCR reaction, gave an intense band of 251bp when the reaction product was viewed on an agarose gel. This 251bp PCR product was purified and cloned into the Sma I site of the cloning vector pUC18. The cloned PCR product was then sequenced using the M13 Reverse (-21) primer. Sequencing data from four recombinant clones revealed that these 251 nucleotides, cloned from BAECs, was
identical to the corresponding segment of sequence from bovine CNG-1 (Kaupp et al., 1989). Therefore, these results indicate that CNG-1 is expressed in BAECs.

3.3.3 Expression of CNG-1 in HUVECs

Primers were designed to the human CNG-1 sequence at positions corresponding to cG-A and cG-B and they were named cG-E and cG-F respectively. Amplification of cDNA prepared from cultured HUVECs using these primers gave products of the expected 345bps. Nested primers, cGH and cGI were designed to the human CNG-1 sequence, corresponding to primers cGC and cGD, and they also amplified bands of the expected size (251bp) from the cGE and cGF PCR product (Fig 3.12). This product was purified and cloned into the Sma I site of pUC18. The cloned product was sequenced using the M13 reverse (-21) primer. Sequence data obtained from four recombinant clones showed that the fragment cloned from HUVECs was identical to the corresponding sequence of the human photoreceptor CNG-1. CNG-1 is therefore also expressed in HUVECs.

A similar strategy to that used to clone the porcine coronary artery CNG-1 was employed in an attempt to clone a full length CNG-1 sequence from HUVECs using primers cG1-cG8. Primers cG1 and cG8 were unable to amplify any full length channel products from HUVEC cDNA. Neither were primer pairs cG1 and cG2 and cG3 and cG4. However, products of the expected size for CNG-1 were obtained using primers cG5 and cG6 and cG7 and cG8. An 878bp product was obtained by amplifying HUVEC cDNA using primer cG5 in conjunction with cG8. Both cG5 and cG8 amplify regions within the large exon ten of the genomic sequence (Dhallan et al., 1992) but the possibility that the 878bp PCR product resulted from contamination of the HUVEC cDNA preparation with genomic DNA was eliminated by treating HUVEC total RNA with 5units of RNase free DNase (for fifteen minutes at 37°C followed by ten minutes at 65°C) prior to reverse transcription. This 878bp product was purified and cut with Not I for twenty hours at 37°C and following this it was cloned into the Not I site of pBKS-. Four recombinant clones were sequenced and sequence data revealed that the 3' end of the CNGC cloned from HUVECs was identical to the corresponding segment of CNG-1 cloned from human rod photoreceptors (Dhallan et al., 1992). These results confirm that CNG-1 is expressed in HUVECs.
Fig 3.12 PCR experiment to demonstrate the expression of a CNG-1 type channel in HUVECs. Lanes as follows: a) 123bp ladder molecular weight marker, b) Re-amplification of 10μl of PCR product in lane e. using nested primers cGH and cGI., c) negative control using nested primers cGH and cGI, d) positive control using primers cGH and cGI using pcD/CNG-1(human) as a template, e) Amplification of 10μl of HUVEC cDNA using primers cGE and cGF, f) negative control using primers cGH and cGI.
3.4 Electrophysiological Evidence for the Expression of a cGMP-Gated Cation Channel in HUVECs

In collaboration with Dr. Klaus Groschner electrophysiological experiments have been carried out to investigate whether a cGMP-activated current is present in HUVECs. Under physiological conditions using the whole-cell perforated patch method the cell does not appear to respond significantly to addition of DEA-NO, a particularly pure NO donor, when voltage-clamped at negative or positive membrane potentials. The cell does respond when as a control experiment ionomycin, a Ca^{2+} ionophore, is added to the cell (see fig 3.13) indicating that the cells under analysis are viable. Addition of La^{3+}, a Ca^{2+} channel blocker, quenched the response of the cell to ionomycin, as would be expected. Pretreatment of cells with cGMP phosphodiesterase inhibitors such as IBMX did not change this situation and cells did not respond to the addition of ANP. However, when internal Ca^{2+} was strongly buffered with high concentrations of the Ca^{2+} chelating agent BAPTA approximately 50% of the cells studied responded to addition of DEA-NO (see fig 3.14). Under these buffered Ca^{2+} conditions the response of the cell to ionomycin was less marked, as would be expected. These data indicate that there may be some form of Ca^{2+}-dependent modulation of the channel. The current-voltage relationship of the channel (fig 3.14b) is similar to that for the cloned CNG-1 from bovine (Kaupp et al., 1989). These results give a preliminary indication that a functional CNGC exists in HUVECs.
Fig 3.13 Whole cell current recordings from HUVECs. Under physiological conditions including 2mM external Ca\(^{2+}\), as shown here, small currents are observed only very occasionally in the presence of 0.2mM of the NO donor DEA-NO. In this figure these small currents are most obvious at a holding potential of -80mV. On the addition of 0.1\(\mu\)m of the Ca\(^{2+}\) ionophore, ionomycin, there is a large response confirming the viability of the cell. The response to ionomycin is blocked by addition of 20\(\mu\)m of the Ca\(^{2+}\) channel blocker La\(^{3+}\) as would be expected.
Fig 3.14 a) This shows whole cell patch clamp current recordings from HUVECs as in fig 3.13 but this time with internal Ca^{2+} strongly buffered by 5mM BAPTA. Open circle indicate readings taken at a holding potential of 0mV and closed circles indicate readings taken at a holding potential of -80mV. The response to 0.2mM DEA-NO is more clear resulting in inward current at a holding potential of -80mV and outward current at a holding potential of 0mV. These results suggest that a cGMP-activated channel which is modulated by internal Ca^{2+} is present in HUVECs. As would be expected the response of the cell to ionomycin is blunted due to the presence of BAPTA in the pipette solution.

b) The current-voltage relationship of the cGMP-activated channel in HUVECs. The current-voltage relationship was measured at two points taken from fig a: A, without 0.2mM DEA-NO and B with 0.2mM DEA-NO.
3.5 Is CNG-2 or CNG-3 Expressed in Vascular Endothelial Cells?

3.5.1 PCR Primer Design

It is possible that the other members of the CNGC family, CNG-2 and CNG-3, are also expressed in vascular endothelial cells. To investigate this possibility a further PCR-based experiment was designed. Only the bovine sequences for both CNG-2 and CNG-3 are known (Ludwig et al., 1990; Weyand et al., 1994) and these were aligned with the bovine CNG-1 sequence to look for regions of strong homology between CNG-2 and CNG-3 which were not homologous to CNG-1. Such regions were identified within the predicted H4 domain and at the end of the predicted H5 domain and a forward and reverse primer, cG13 and cG14 respectively, were designed to these regions. Within primer cG13 (22 nucleotides long) there are no base pair differences between CNG-2 and CNG-3 and within primer cG14 (23 nucleotides long) there is only one base pair difference. However, both primers differ significantly from the CNG-1 sequence (see fig 3.15).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence Alignment</th>
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<tbody>
<tr>
<td>cG13</td>
<td>5' CACTGGAATGCTCCATCTACT 3' TM 66</td>
</tr>
<tr>
<td>CNG-1</td>
<td>5' .................G..TG.G.... 3' TM 52</td>
</tr>
<tr>
<td>CNG-2</td>
<td>5' .................... 3' TM 66</td>
</tr>
<tr>
<td>CNG-3</td>
<td>5' .................... 3' TM 66</td>
</tr>
<tr>
<td>cG14</td>
<td>5' ATGTTTGGAGATCATGAGCCAC 3' TM 68</td>
</tr>
<tr>
<td>CNG-1</td>
<td>5' ....G..A.....A..A..T.T 3' TM 46</td>
</tr>
<tr>
<td>CNG-2</td>
<td>5' .................... 3' TM 66</td>
</tr>
<tr>
<td>CNG-3</td>
<td>5' .................... 3' TM 68</td>
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Fig 3.15 Sequence alignments of PCR primers cG13 and cG14 with bovine CNG-1, CNG-2 and CNG-3 sequences. The TM values (in °C) show the optimal temperature for each primer binding to sequence 100% homologous and also the values for the primer binding to CNG-1, CNG-2 or CNG-3.

3.5.2 Is CNG-2 or CNG-3 Expressed in HUVECs?

As the main interest in this area of the project was focused on CNGC expression in HUVECs primers cG13 and cG14, designed from bovine sequence, were tested for their ability to amplify products from human genomic DNA. Amplification products of the correct size (275bp) were observed on an agarose gel following a thirty five cycle PCR reaction employing primers cG13 and cG14 and human genomic DNA.
as a template at an annealing temperature of 58°C. However, at this annealing temperature, primers cG13 and cG14 could also amplify a 275bp product from human CNG-1 cloned cDNA (provided by Jeremy Nathans). Therefore the annealing temperature of the reaction was increased in steps of 2°C until no product was observed in the reaction containing human CNG-1 cDNA but a reasonably intense product was observed in the reaction containing human genomic DNA. This balance was achieved at an annealing temperature of 64°C. As primers cG13 and cG14 amplified a band of 275bp from genomic DNA or cDNA it can be presumed that the product does not span an intron/exon junction. Therefore HUVEC total RNA was treated with RNase free DNase prior to reverse transcription. Following a PCR reaction using primers cG13 and cG14 at an annealing temperature of 64°C with HUVEC cDNA as template no product was observed. A control experiment was carried out simultaneously. In this experiment primers designed to the 3’ end of the human ANP-RA receptor, ANP1 and ANP4, were used to amplify a product from HUVEC cDNA also. These primers are designed to amplify products of 229bp from cDNA or 707bp from genomic DNA. In this experiment they amplified only a product of 229bp indicating that no genomic DNA was present in the reaction and that the cDNA resulting from reverse transcription was a viable template. Taking this control experiment into account it can be presumed that neither CNG-2 or CNG-3 sequences are expressed in HUVECs at detectable levels and that CNG-1 is the only CNGC expressed in these vascular endothelial cells. (see fig 3.16)

Fig 3.16 Amplification of HUVEC cDNA with cG13 and cG14. Lanes as follows: a) 123bp DNA ladder b) Amplification of HUVEC cDNA with primers cG13 and cG14 c) Amplification of HUVEC RNase-free DNase treated-total RNA with primers cG13 and cG14. d) Negative control with primers cG13 and cG14. e) Amplification of HUVEC cDNA with primers ANP1 and ANP4. f) Amplification of HUVEC RNase-free DNase treated-total RNA with primers ANP1 and ANP4. g) Negative control with primers ANP1 and ANP4. h) 123bp DNA ladder
3.6 Discussion

PCR results from the panel of bovine cardiac tissues show that the cGMP-gated channel is expressed throughout heart tissue and is not isolated to the sino-atrial node. Therefore, it is probably not directly involved in pacemaking and carries out a more general function in cardiac cells, possibly in relaxation mechanisms. It is fair to assume that the channel is expressed at very low levels in cardiac tissue as two rounds of PCR amplification were necessary to obtain products clearly visible on ethidium bromide-stained gels. This low abundance of expression makes functional studies of the cardiac CNG-1 channel extremely difficult.

This PCR-based screen also revealed the presence of a CNG-1 channel in vascular tissue and a full length sequence for the porcine coronary artery CNG-1 channel was subsequently obtained (Ratcliffe et al., 1995). Once again the level of expression in porcine coronary artery was extremely low and two rounds of PCR were required to obtain products which could be cloned. This low abundance of expression indicated that the channel may be expressed only in the vascular endothelium and not in the surrounding layers of smooth muscle. Following experiments demonstrated that CNG-1 was expressed in vascular endothelial cell lines, once again at fairly low expression levels, requiring several micrograms of template cDNA to obtain products which were visible on an ethidium bromide-stained agarose gel. Unfortunately no homogeneous cultured vascular smooth muscle cell lines are available, so the expression of CNG-1 in smooth muscle alone could not be investigated.

The low level of channel expression presented many difficulties when attempting to clone full length cDNAs for CNG-1 from cardiovascular tissue. As described in this chapter the full length PCR product obtained for the porcine coronary artery CNG-1 could not be cloned due to its low abundance. There were also problems in attempting to clone a full length cDNA from HUVEC cDNA. In this case the 5' end of the coding region could not be amplified probably due to the very low copy number of the mRNA for this channel in vascular endothelial cells. As the channel is large, especially when compared to other members of the superfamily (2.1 Kb as opposed to 1.2Kb for an inward rectifier K+ channel), reverse transcription of the mRNA results in an abundance of cDNA fragments towards the 3' end of the message but relatively few encompassing the entire channel coding region. The low abundance of the message and also the cDNA size provided an inefficient PCR template from which detectable products could not be amplified.

Electrophysiological studies carried out on HUVECs provide evidence for a cGMP-activated current in HUVECs. Addition of DEA-NO, a NO donor, onto the cells produces an increase of intracellular cGMP through activation of NO synthase. The
requirement of intracellular Ca\(^{2+}\) to be buffered for a cGMP-activated current to be observed indicates that some form of Ca\(^{2+}\) dependent modulation of the channel is occurring. This is consistent with observations that Ca\(^{2+}\)-calmodulin complexes bind to the photoreceptor channel with the effect of increasing the apparent Michaelis constant of the channel for cGMP and therefore decreasing the rate of cation flux through the channel (Hsu and Molday, 1993 and 1994). Divalent cation block of the channel may also contribute to the low cation flux observed at high levels of intracellular Ca\(^{2+}\) (Frings et al., 1995).

Although no expressible clones were obtained, due to low abundance of channel mRNA, the data presented here show that a CNG-1 channel is expressed throughout cardiac tissue and also in vascular endothelial cells. The relatively low abundance of channel expression makes future functional studies \textit{in situ} difficult. However, as cGMP is an important second messenger in this tissue type it will be important to investigate further the role of the cardiovascular CNG-1.
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4.0 Heterologous Co-expression of Inward Rectifier Potassium Channels using the MEL/LCR Expression System

4.1 The MEL/LCR Expression System

To produce a model signalling pathway by heterologous co-expression of a receptor and ion channel in a mammalian cell-line a highly efficient expression system is required. Both components must be expressed and this is not guaranteed in most systems which require integration of the foreign DNA into an 'active' area of the genome. Integration of the foreign DNA is a random procedure and often the DNA will be inserted into a 'silent' region of the genome and not be expressed. When two components are to be co-expressed it is unlikely that they will both be expressed efficiently in the same cell and extensive screening procedures need to be carried out to identify cell-lines expressing both transfected cDNA products at sufficient levels.

The MEL/LCR system overcomes this problem of position effects because expression occurs independently of the position of integration (Nandi et al., 1988). Murine erythroleukaemia (MEL) cells are erythroid progenitor cells arrested at the proerythroblast stage of development. MEL cells can be induced to undergo erythroid differentiation by addition of chemicals such as dimethyl sulfoxide (DMSO) (Friend et al., 1971).

This expression system utilises features of the β-globin gene, which is naturally expressed in the MEL cell-line. The β-globin cluster contains five genes (ε, Gγ, Aγ, δ and β) which are successively expressed during development. The expression of individual genes in the cluster depends on a regulatory element named the locus control region (LCR), which lies at the extreme 5' end of the complex, meaning that the LCR is able to activate genes, still within the β-globin locus, but 11-60Kb downstream. The array of transcription factors present at each developmental stage and acting on the LCR is thought to influence the expression of specific genes in the cluster. Four DNase1-hypersensitive sites have been identified on the β-globin LCR and it is thought that some of the effects of the LCR involve the unfolding of the chromatin, allowing the access of regulating factors, followed by gene transcription (Orkin, 1990; Crossley and Orkin, 1993). When MEL cells are induced with DMSO, these transcription factors appear to be activated thus influencing the LCR and causing transcription of the β-globin gene. In fact, three to four days following induction the colour of pelleted MEL cells has changed visibly from a white to red indicating the expression of β globin and the formation of haemoglobin.

To take advantage of the globin gene locus control system expression vectors have been developed which include an LCR region linked to a β-globin promoter.
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The cDNA of interest is inserted into a multiple cloning site (MCS) directly after the promoter. The second intron, untranslated region and polyadenylation signal sequence of the β-globin gene are present just 3' of the MCS to enable the production of more stable RNA transcripts from the construct (see fig 4.1) (Needham et al., 1992). Following transfection of MEL cells with this vector some of the foreign DNA is inserted into the MEL cell genome and, when the cells are induced, the LCR is activated and the foreign cDNA is expressed at high levels. Gene expression occurs independently of the position of integration into the host genome. The human growth hormone cDNA and gene were the first sequences expressed in this system and expression levels were found to be similar to those of murine β-globin in induced MEL cells (Needham et al., 1992). Two LCR expression vectors were used in the experiments described in this thesis. They are identical, except for the sizes of their LCR regions. The vector pEV3 contains the LCR 'microlocus' and the vector pNV1 contains the LCR 'nanolocus' (fig 4.2). The LCR 6.5Kb 'microlocus' incorporates all the regions of the native LCR required to give optimal gene activation (Talbot et al., 1989). The 'nanolocus' is only 0.95Kb in size and contains only the LCR regions absolutely essential for gene activation. Although the 'nanolocus' is not quite as active as the 'microlocus', its small size makes it much more useful in the construction of DNA expression vectors.

The MEL/LCR system has since been used successfully for the stable electrophysiological expression of K⁺ channels. The voltage-dependent K⁺ channel hPCN1 was expressed using its own promoter to drive expression in conjunction with the β-globin LCR. The same K⁺ channel was also expressed successfully with expression driven from the β-globin promoter (Shelton et al., 1993). MEL cells are particularly suitable for the analysis of heterologously expressed K⁺ channel proteins as differentiated native cells do not appear to produce any interfering voltage-dependent currents (Shelton et al., 1993). In fact, only three types of ion channel have been reported to exist in these cells, a Ca²⁺-activated K⁺ channel, a stretch-activated channel and a Cl⁻ channel, the latter two of which are fairly labile (Arcangeli et al., 1987), and these endogenous currents did not appear to interfere with measurements of heterologous channel currents when low free [Ca²⁺] was present in the pipette solution during patch-clamping protocols (Shelton et al., 1993). Further studies have resulted in expression of a member of the inward rectifier Kir 2.0 family using this system (Stanfield et al., 1994) confirming the suitability of this expression system for members of this family as well as the family of voltage-activated K⁺ channels.
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Fig 4.1 Diagram to show the coupling of a channel cDNA to the human β-globin promoter and LCR. The positions of the β-globin gene second exon, second intron, polyadenylation signal sequence (poly A) and 3' untranslated region (3'UTR) are also shown.
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Fig 4.2 Restriction maps of the LCR expression vectors pNV1 and pEV3. Amp=ampicillin resistance selectable marker; tk-neo=G418 resistance selectable marker
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4.2 Heterologous Co-expression of the Muscarinic M2 Receptor with GIRK1 in MEL Cells

4.2.1 Introduction

GIRK1, cloned from rat heart (Kubo et al., 1993b), appears to be one of the main molecular components underlying the muscarinic K⁺ channel. Activation of M2 muscarinic acetylcholine receptors in the right atrium result in activation of the muscarinic K⁺ channel causing a slowing of the heart beat (Sakmann et al., 1983). This signalling pathway, which couples with endogenous oocyte G-proteins, has been reconstituted in *Xenopus* oocytes co-injected with GIRK1 and M2 receptor cRNA (Kubo et al., 1993b). GIRK1 has also been activated by heterologous co-expression with the serotonin 1A receptor in *Xenopus* oocytes (Doupnik et al., 1995) and also with the neuronal cannabinoid receptor, again in *Xenopus* oocytes (Henry and Chavkin, 1995). However, such signalling pathways have yet to be reconstituted in a mammalian cell-line. As the components involved in the opening of GIRK1 through activation of a M2 receptor are well established, it is a useful system to attempt to reconstitute in MEL cells to test for the versatility of the MEL/LCR system when compared to other expression systems, especially within the context of co-expression.

The suitability of the MEL/LCR system for the expression of GIRK1 has already been shown in experiments carried out within the group (Jacobsen, 1994). The GIRK1 cDNA (kindly provided by Y.N. Jan and L.Y. Jan) was cloned into pNV1 and expressed in MEL cells to produce a strong inwardly rectifying K⁺ current when the cells were stimulated with GTP-γ-S, a non-hydrolysable analogue of GTP. These results indicated that GTP-γ-S is interacting with endogenous MEL cell G-proteins to activate GIRK1 and are very similar to those observed in *Xenopus* oocytes by Kubo et al., (1993b). Activation in MEL cells of heterologously expressed GIRK1 by a heterologously co-expressed M2 receptor employing endogenous MEL cell G-proteins would demonstrate the usefulness of the MEL/LCR system for co-expression experiments. The advantages of the MEL/LCR system over other expression systems is that stable, well-analysed cell-lines can be produced which are then easily available and also the LCR ensures that expression of all constructs is unhindered by position effects.
4.2.2 Co-transfection of MEL Cells with the M2 Muscarinic Receptor and GIRK1

The GIRK1 ORF had previously been subcloned into the Eco R1 site of pNV1 (Jacobsen, 1993) and this construct is named pNV1[GIRK1]. An M2 muscarinic receptor cDNA was kindly provided by Noel Buckley, already cloned into a mammalian expression vector, downstream of an SV40 promoter, and named pcDM2 (Bonner et al., 1987). To allow efficient integration of the constructs into the MEL cell genomic DNA, in order to produce stably transfected cell-lines, the DNA constructs were first linearised. pNV1[GIRK1] and pcDM2 were linearised at the Pvu I restriction site within the ampicillin resistance genes of each expression vector and the digested samples were purified by gel-isolation followed by electroelution. 15µg of each expression construct was used to co-transfect MEL cells by electroporation and in a control experiment 15µg of pNV1[GIRK1] alone was used to transfect a second aliquot of MEL cells, also by electroporation. Twenty four hours following transfection G418 was added to the cells at a final concentration of 1mg/ml, and 10 days following transfection G418-resistant colonies were picked and stably transfected cell-lines were established. The pcDM2 expression construct did not contain a G418 resistance selectable marker gene and therefore cells transfected with pcDM2 alone would die following addition of G418. However, during co-transfection approximately 80% of cells appear to take up both constructs for reasons as yet unknown (Angelotti et al., 1993), and therefore most cells taking up pNV1[GIRK1] would also be transfected with the pcDM2 construct.

4.2.3 Analysis of Co-transfected Cell-lines by Southern Blotting

Southern blots were carried out to check that both constructs had integrated into the genomic DNA of all selected stable cell-lines. No complete restriction maps were available for either pcDM2 or pNV1 and therefore several restriction digests were carried out to determine which enzyme would be most suitable for use in this experiment (see fig 4.3). It was calculated that digestion of the transfected cell-line genomic DNA with Pst I would result in a fragment of approximately 4.8Kb, containing the M2 receptor cDNA, being released from the integrated pcDM2 construct and a fragment of approximately 3Kb, containing the GIRK1 cDNA, being released from the integrated pNV1[GIRK1] construct. Genomic DNA was prepared from eight cell-lines and also non-transfected MEL cells as a negative control and 20µg of each sample was digested overnight at 37°C with 20 units of Pst I. The digested DNA was then run on a 0.8% agarose gel with λ Hind III-digested DNA.
molecular weight markers. This procedure was carried out twice to produce replicated agarose gels for each set of samples. The DNA on these agarose gels was then transferred onto nylon membranes and hybridised to $^{32}$P-radiolabelled DNA probes. DNA probes were prepared by a method of random priming using the coding regions of the M2 receptor and GIRK1 as templates. Briefly, fragments were excised from pcDM2 by digesting the construct with Bam HI (giving a 710bp fragment from the M2 coding region) and from pNV1[GIRK1] by digesting with Hind III (giving a 1.4Kb fragment from the GIRK1 coding region). These fragments were purified by gel-isolation followed by electrophoresis and 10ng of each were then used as templates to prepare $^{32}$P-radiolabelled DNA probes. Following overnight hybridisation to these probes at 58°C the nylon filters were washed and then autoradiographed with intensifying screens at -70°C for five days before developing the films. The results of this Southern blot showed that both pNV1[GIRK1] and pcDM2 had integrated into the genomic DNA of all eight cell-lines analysed (fig 4.4).

4.2.4 Analysis of Co-transfected Cell-lines using RNA Dot-blots

Although the constructs, pcDM2 and pNV1[GIRK1] had stably integrated into the genomic DNA of all cell-lines analysed by Southern blotting this was no indication that the GIRK1 and M2 protein products were co-expressed in every cell-line. No antibodies were available to study protein expression in the cell membrane so the next best method was to look for the presence of GIRK1 and M2 mRNA in each cell-line.

Four cell lines, M2/GIRK1 5-8, and non-transfected MEL cells were induced and the cells were harvested four days post-induction as it has been observed that the accumulation of heterologous mRNAs peaks at this time (Needham et al., 1992). Of course, the expression of the M2 receptor is driven from an SV40 promoter which should be operative whether the cells are induced or not. Total RNA was prepared from each cell-line and the non-transfected MEL cells and then run out on a 1% agarose gel to check for the integrity and estimate the concentration of the RNA. Approximately 5μg of each sample was dotted onto nylon membranes and hybridised to $^{32}$P-radiolabelled DNA probes prepared from M2 and GIRK1 cDNA fragments as described above for the Southern blotting procedure. Following overnight hybridisation at 58°C, the nylon membranes were washed and then autoradiographed with intensifying screens at -70°C for ten days before developing the films. The results from this experiment (fig 4.5) show that mRNAs for the M2 receptor and GIRK1 are present in all four cell-lines analysed. M2 receptor mRNA does not appear to be present at such high levels as GIRK1 mRNA and this is probably due to more efficient transcription of GIRK1 cDNA from the LCR-activated β-globin promoter.
Similar dot-blot experiments were used to check that there was adequate levels of GIRK1 mRNA present in the control MEL cells stably transfected with GIRK1 alone.

4.2.5 Electrophysiological Analysis of MEL Cells Heterologously Co-expressing GIRK1 and the Muscarinic M2 Receptor

Control MEL cell-lines, stably expressing GIRK1 alone, were analysed first using the whole-cell patch-clamp technique with GTP-γ-S present in the pipette solution. No K+ currents were evoked by voltage step experiments from -80mV to +80mV with a holding potential of -20mV (fig 4.12). MEL cells, stably transfected with GIRK1 and M2 receptors, also failed to produce K+ currents under similar experimental conditions with GTP-γ-S in the pipette solution and carbachol (to stimulate the heterologously co-expressed muscarinic M2 receptors) in the bath solution (fig 4.6). Therefore, although RNA dot-blot experiments showed that GIRK1 mRNA was present in these cell-lines, the GIRK1 subunit does not appear to be able to produce functional K+ channels on its own.
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Fig 4.3 Diagram illustrating the digestions, run out on an agarose gel, carried out to obtain suitable DNA fragments to analyse genomic DNA from cell-lines MEL/[M2/GIRK1] 1-8 for the integration of pNV1[GIRK1] and pcDM2.a) For pNV1[GIRK1] digestion with Pst I gives three distinct fragments of 4.8Kb, 3.4Kb and 2.7Kb and some small fragments which could not be accurately sized. Digestion with Bgl I, which cuts twice within the GIRK1 cDNA sequence, and Pst I completely removes the 3.4Kb fragment indicating that the GIRK1 cDNA sequence is present within this fragment. The pNV1[GIRK1] construct was linearised with Pvu I prior to transfection and digestion with Pst I and Pvu I reduces the 3.4Kb fragment to 3Kb. Therefore, following digestion of cell-line genomic DNA with Pst I, a band of 3Kb should light up following southern blotting and hybridisation with a ^32P-radiolabelled DNA probe prepared from GIRK1 cDNA. b) For pcDM2 digestion with Pst I gives a 4.8Kb and a 1.3Kb fragment. Digestion with Bam HI, which cuts twice within M2 cDNA, and Pst I removes the 4.8Kb fragment to give multiple fragments indicating that the M2 cDNA sequence is present within this 4.8Kb band. pcDM2 was also linearised with Pvu I prior to transfection but digestion with Pst I and Pvu I does not affect the 4.8Kb fragment. Therefore, following digestion of cell-line genomic DNA with Pst I, a band of 4.8Kb should light up after southern blotting and hybridisation with a 32P-radiolabelled DNA probe prepared from M2 cDNA. All fragment sizes are rounded up to the nearest 0.1Kb and solid lines represent accurately sized fragments while dashed lines represent fragments too small to accurately size. All molecular weight size fragments are in Kb.

[N.B. λB=λBst EII DNA Markers]
Fig 4.4 Southern hybridisations of $^{32}$P-radiolabelled GIRK1 and M2 DNA probes to Pst I-digested genomic DNA prepared from the cell-lines MEL[M2/GIRK1] 5-9. A) A band lights up at 3Kb, as expected for the integrated pNV1[GIRK1] construct, in all lanes but the negative control lane which contains the non-transfected MEL cell genomic DNA sample. The smaller sized band appearing in all lanes is probably the fragment released from the genomic copy of GIRK1. B) A band lights up at 4.8Kb, as expected for the integrated pcDM2 construct, in all lanes but the negative control lane. The multiple bands observed for samples M2/GIRK1 5, 6 and 7 probably results from partial digestion of the genomic DNA. For fig A) and B) lane DNA sample run was a) M2/GIRK1 5, b) M2/GIRK1 6 c) M2/GIRK1 7 d) M2/GIRK1 8 e) M2/GIRK1 9, f) Non-transfected MEL cell.
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Fig 4.5 Analysis of MEL[M2/GIRK1] 5-8 co-transfected cell-lines using RNA dot-blot.

a) Hybridisation of a $^{32}$P-radiolabelled GIRK1 probe to total RNA prepared from induced cell-lines. No signal is obtained from the non-transfected induced MEL cell total RNA sample indicating that there is no endogenous expression of GIRK1 in MEL cells. The positive control was provided by spotting 10ng of the DNA template for the GIRK1 probe onto the membrane.

b) Hybridisation of a $^{32}$P-radiolabelled M2 probe to total RNA prepared from induced cell-lines. No signal is obtained from the non-transfected induced MEL cell total RNA sample indicating that there is no endogenous expression of M2 in MEL cells. The positive control was provided by spotting 10ng of the DNA template for the M2 probe onto the membrane.
Fig 4.6 Recordings showing the effect of voltage jumps from -80mV to +80mV at a holding potential of -20mV during whole-cell patch-clamp (see below) experiments on MEL cells stably transfected with muscarinic M2 receptors and GIRK1. The pipette solution contained 140mM KCl, 10mM EGTA, 10mM HEPES, 1mM MgCl$_2$, and 200μM GTP-γ-S (pH 7.2) and the bathing solution contained 70mM KCl, 70mM NaCl, 2mM CaCl$_2$, 2mM MgCl$_2$, 10mM HEPES and 100μM carbachol (pH 7.4).

Whole-Cell Patch-Clamp: Clean glass pipettes can fuse to clean cell membranes to form a seal with high resistance and mechanical stability (known as a gigaseal). When the pipette is sealed to the cell membrane the on-cell patch may be deliberately ruptured by suction to allow recording in the whole-cell configuration (Hille, 1992).
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4.3 Heterologous Expression of re\textsubscript{c}K\textsubscript{ATP} in HEK293 cells and MEL cells

4.3.1 Introduction

\( \text{K}_{\text{ATP}} \) channel currents have been identified in a wide variety of tissues including heart, skeletal muscle, neuronal tissue, non-arterial smooth muscle, arterial smooth muscle and pancreatic \( \beta \) cells. \( \text{K}_{\text{ATP}} \) channels have several distinguishing features. They are inhibited by physiological concentrations of ATP (5-10mM), activated by G-proteins and nucleoside diphosphates, opened by the K\(^+\) channel openers such as diazoxide and pinacidil and they are also specifically blocked by sulphonylurea compounds, such as glibenclamide. A cDNA, named \( \text{reK}_{\text{ATP}} \), with many of the characteristics of a \( \text{K}_{\text{ATP}} \) channel, and belonging to the Kir 3.0 subfamily of inward rectifier K\(^+\) channels (and hence also named Kir 3.4 using the standardised nomenclature according to Chandy and Gutman, 1993), has been cloned from rat heart muscle (Ashford et al., 1994). When heterologously expressed in HEK 293 cells, \( \text{reK}_{\text{ATP}} \) displayed many of the characteristics of the native \( \text{K}_{\text{ATP}} \) channel. Channel activity was abolished by ATP concentration greater than 0.2mM, reactivated by addition of nucleoside diphosphates to the intracellular side of the patch and activated by the K\(^+\) channel opener pinacidil. As may be expected, due to the absence of an endogenous sulphonylurea receptor in HEK 293 cells, \( \text{reK}_{\text{ATP}} \) was not inhibited by sulphonylureas. Northern blot analysis revealed the presence of \( \text{reK}_{\text{ATP}} \) mRNA in heart ventricle and atrium and also in a variety of other tissues (Ashford et al., 1994).

Notably, \( \text{reK}_{\text{ATP}} \) is not expressed in skeletal muscle, a tissue from which \( \text{K}_{\text{ATP}} \) channel currents have been recorded (Spruce et al., 1985).

Following publication of these data (Ashford et al., 1994) many unsuccessful attempts have been made to repeat the observations reported. In collaboration with John Adelman, a co-author of the aforementioned paper, it was decided to independently express the \( \text{reK}_{\text{ATP}} \) clone in HEK293 cells in order to verify the published results. It was also decided to express the cloned channel using the MEL/LCR expression system as this system had already proved to be appropriate for the expression of inward rectifier K\(^+\) channels.

While these experiments were underway a further paper was published in which it was suggested that \( \text{reK}_{\text{ATP}} \) was not, in fact, a component of the \( \text{K}_{\text{ATP}} \) channel but instead formed a heteromultimeric complex with GIRK1 to create a channel showing all the characteristics of the native muscarinic K\(^+\) channel (Krapivinsky et al., 1995). In this paper the cloning of a cDNA, named cardiac inward rectifier or CIR, is described and alignment of the CIR protein sequence with the...
The rcKATP protein sequence reveals only two amino acid differences, a valine for an isoleucine at position 188 and a glutamine for a glutamate at position 375. Co-immunoprecipitation experiments using an antibody designed to the GIRK1 protein revealed that CIR and GIRK1 are associated in atrial tissue. Heterologous co-expression of CIR and GIRK1 with the M2 receptor in *Xenopus* oocytes produced an inwardly rectifying K⁺ channel on activation with acetylcholine. Expression of GIRK1 alone with an M2 receptor produced currents eight times smaller than in co-expressing cells on agonist addition, with higher basal channel activity in the absence of agonists. Only very small currents were produced on addition of agonist when CIR was expressed alone (but with M2). All the above observations were of whole-cell currents. Single channel currents observed in cells expressing GIRK1 alone or co-expressing GIRK1 and CIR were not particularly different. This suggested that *Xenopus* oocytes expressed low levels of an endogenous channel similar to CIR with which, in previous reports (Kubo et al., 1992b), GIRK1 was forming heteromultimeric functional channels. The effect of co-expression of GIRK1 with CIR appeared to increase the number of functional iKACh-like channels in *Xenopus* oocytes. In fact, Krapivinsky et al. report that preliminary northern analysis indicates that oocytes possess a CIR homologue. Krapivinsky et al. also present the results from experiments in which CIR and GIRK1 are heterologously co-expressed in CHO and HEK 293 cells. Co-expression of CIR and GIRK1 in these mammalian cell-lines produced iKACh-like currents on activation with GTP-γ-S, although expression of GIRK1 alone produced no detectable currents, possibly due to the lack of expression of an endogenous CIR homologue in these mammalian cell-lines. It is possible that MEL cells do express such a homologue as heterologous expression of GIRK1 in MEL cells did produce GTP-γ-S-activated currents (Jacobsen, 1994). Expression of CIR alone in CHO cells produced small currents which were activated by GTP-γ-S. Krapivinsky et al. also reported that heterologously expressed CIR in a number of different cell-lines did not produce channels which were inhibited by ATP or activated by pinacidil.

Due to these opposing reports on the function of rcKATP (Ashford et al., 1994) or CIR (Krapivinsky et al., 1995) further investigations into the true nature of this channel, and its possible association with GIRK1, were necessary.

### 4.3.2 Heterologous Expression of rcKATP in HEK293 Cells

John Adelman kindly provided his rcKATP cDNA already cloned into the *EcoRV* site of the mammalian expression vector pcDNA3 (Invitrogen) (fig 4.7) and it is named pcDNA3[rcKATP]. The pcDNA3[rcKATP] construct was linearised at the *Pvu* I site within the ampicillin resistance gene during a three hour digestion at 37°C.
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The linearised construct was purified by agarose gel-isolation and electrophoresis. 1μg of this purified DNA was then used to transfect each well of a six well plate containing approximately 2x10^5 HEK 293 cells/well by lipofection using LipofectAmine™ Reagent (Gibco BRL). Three days after transfection the cells were passaged into fresh media containing 1mg/ml G418 to select for stably transfected cells. Two weeks following this G418-resistant colonies were picked and cell lines stably transfected with the pcDNA3[rcKATP] construct were established.

To check that the construct had indeed inserted into the HEK 293 cell genome, a PCR-based assay was devised to screen for the presence of the pcDNA3[rcKATP] construct in the genomic DNA of each established cell line. A forward PCR primer was designed to the 3’ end of the rcKATP cDNA coding region (bases 1061-1081) and used in conjunction with the primer pcDNA3(rev) which was designed to the SP6 site on pcDNA3 at the 3’ of the MCS. Used together these primers amplified a band of 270bp from genomic DNA prepared from stably transfected cell-lines containing the pcDNA3[rcKATP] construct. As the PCR product spanned both cloned cDNA and vector sequence there could be no question of false positive results obtained from the HEK 293 genomic copy of the KATP gene. The results of this amplification were run on a 1% agarose gel (fig 4.8) and they show clearly that the pcDNA3[rcKATP] construct has integrated into the genomes of all five cell-lines (HEK293[rcKATP] 1-5) analysed.

An antibody designed to the extreme N-terminus of the rcKATP channel protein (a gift from John Adelman) was then used to study the expression of rcKATP in the HEK 293 cell lines. Total cell protein was prepared from the five cell-lines analysed in the PCR screen, HEK293[rcKATP] 1-5, and run out on a 10% SDS polyacrylamide gel. The protein from 10^6 cells was loaded for each sample and non-transfected HEK 293 cells were used as a negative control. Western blotting using the rcKATP antibody revealed that the rcKATP protein product is expressed in only three of the five cell-lines analysed even though the PCR screen revealed that the pcDNA3[rcKATP] construct is present in all five cell lines. This is probably due to the 'position effects' often observed during heterologous expression protocols of this kind. The negative control lane containing non-transfected HEK293 cell protein, contains no visible bands at the size expected for rcKATP revealing that there is no endogenous expression of the channel (fig 4.9).
Fig 4.7 Vector map of pcDNA3 (Invitrogen). The main features of this expression vector are:
Enhancer promoters sequences from the immediate early gene from the human cytomegalovirus (pCMV) for high levels of transcription.
Polyadenylation signal and transcription termination sequences from the bovine growth hormone gene (BGH pA) which increases the stability of RNA.
An ampicillin resistance gene (Amp) and CoLE1 origin for selection and maintenance in E.Coli.
A neomycin resistance gene to allow for selection in mammalian cell-lines.
The vector also contains an SV40 origin for episomal replication in cell-lines expressing the SV40 large T antigen, T7 and Sp6 promoters flanking the MCS for in vitro transcription of RNA and an f1 origin for the production of single stranded DNA.
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Fig 4.8A) Diagram illustrating the design of PCR primers to pcDNA3[rcKATP] to check for the stable integration of the construct into the genomic DNA of HEK 293 cell-lines following transfection.

Fig 4.8B) PCR results using primers rcKATP1 and pcDNA3 rev to check for the stable integration of the construct pcDNA3[KATP] into the genomic DNA of cell-lines HEK293[rcKATP]1-5 following transfection and selection of G418 cell-lines. Lanes are as follows a)HEK293[rcKATP]1 b)HEK293[rcKATP]2 c)HEK293[rcKATP]3 d)HEK293[rcKATP]4 e) HEK293[rcKATP]5 f) non-transfected HEK293 cells g) negative control h) positive control-pcDNA3[rcKATP]. All cell-lines give a PCR product of 270bp as expected from the integrated construct. The DNA marker is a 123bp ladder. The larger bands appearing in lanes b-f are probably due to mis-primed amplification reactions.
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COMPARISON OF THE RELIABILITY OF EXPRESSION WITH THE pcDNA3 EXPRESSION VECTOR AND THE MEL/LCR SYSTEM

Fig 4.9 The position-independent properties of MEL/LCR expression system mean that 100% of cell lines transfected with an LCR construct express the linked cDNA’s protein product. Other expression vectors employing a promoter such as the CMV promoter (as in these experiments) are susceptible to position effects. This figure demonstrates the greater expression efficiency obtained using an LCR vector as opposed to pcDNA3. Five HEK293[rcKATP] cell lines and five MEL[rcKATP] cell lines were analysed in western blot experiments using the rcKATP antibody. Only three of the five HEK293 cell lines which had the pcDNA3[rcKATP] construct inserted into their genomic DNA (see fig 4.6) actually express the rcKATP protein product, as shown above, while all the MEL cell lines express the rcKATP protein product. HEK293 cell lines 1-5 were run in lanes a-e with the negative control of non-transfected HEK 293 cells in lane f and MEL cell lines 1-5 were run in lanes g-k with the negative control of non-transfected MEL cells in lane l. High molecular weight rainbow markers (Amersham) of mass 97kDA,66kDa,46kDa and 30kDa were used. The 46kDa size marker is shown by an arrow. Protein bands observed at higher molecular weight are due to non-specific antibody binding.
4.3.3 Electrophysiological Analysis of rcK\textsubscript{ATP} Heterologously Expressed in HEK 293 Cells

Before transfected cell-lines were examined native HEK293 cells were analysed by whole-cell patch-clamp recording techniques. However, distinct K\textsuperscript{+} channel currents were observed in these non-transfected cells, including a voltage-activated K\textsuperscript{+} current and also a barium-sensitive inward rectifying K\textsuperscript{+} current (fig 4.10). The presence of these background K\textsuperscript{+} currents in native HEK293 cells renders them unsuitable for the recording of heterologously expressed inward rectifier K\textsuperscript{+} channel currents.

4.3.4 Heterologous Expression of rcK\textsubscript{ATP} in MEL Cells

As MEL cells do not appear to possess any background inward rectifier K\textsuperscript{+} currents (Arcangeli et al., 1987) they are suitable for use in the electrophysiological analysis of heterologously expressed rcK\textsubscript{ATP}. The rcK\textsubscript{ATP} cDNA was provided cloned into the Sal I and Not I sites in the MCS of pEV3 (a gift from John Adelman) and this expression construct was named pEV3[rcK\textsubscript{ATP}]. The construct was linearised at the Pvu I site within the ampicillin resistance gene and, following purification by agarose gel-isolation and electroelution, 25\mu g of the construct was used to transfect MEL cells by a method of electroporation. Twenty four hours after transfection G418 was added to the cells at a final concentration of 1mg/ml and ten days post-transfection G418-resistant colonies were picked and cell-lines stably transfected with the pEV3[rcK\textsubscript{ATP}] were established.

To check that these cell-lines were actually expressing the rcK\textsubscript{ATP} protein western blotting techniques, using the K\textsubscript{ATP} antibody, were carried out on total cell protein preparations from five transfected cell-lines, MEL[rcK\textsubscript{ATP}]1-5. At four days post-induction protein samples were prepared from these five cell-lines and non-transfected MEL cells and for each sample protein from 10\textsuperscript{6} cells was loaded onto a 10% SDS polyacrylamide gel. This western blotting experiment showed that the rcK\textsubscript{ATP} protein was present in all five cell-lines analysed and was not expressed in non-transfected MEL cells (fig 4.9). When an rcK\textsubscript{ATP} expression construct, in which the channel cDNA is under the control of a CMV promoter, was used to transfect HEK293 cells only three of five cell-lines analysed actually expressed the channel protein (see section 4.3.2). However, using the MEL/LCR system, as described here, all cell-lines analysed express the rcK\textsubscript{ATP} protein. This demonstrates the advantage of the position-independent properties of the MEL/LCR expression system.
4.3.5 Electrophysiological Analysis of $hcK_{ATP}$ Heterologously Expressed in MEL Cells

The human cDNA clone of the cardiac $K_{ATP}$ channel was also provided by John Adelman. The cDNA was provided cloned into the LCR expression vector $pEV3$. MEL cells were transfected with the $pEV3[hcK_{ATP}]$ construct using exactly the same procedure as described above in section 4.3.4. Stably transfected cell-lines were induced by addition of 2% DMSO and at three to four days post-induction they were analysed using whole-cell patch-clamp techniques.

Under conditions which should activate a $K_{ATP}$ current, with a solution of $140\text{mM}[K^+]$, $1\text{mM}[Mg^{2+}]$, $0.1\text{mM}[ATP]$ and $0.1\text{mM}[ADP]$ in the pipette solution and $60\text{mM}[K^+]$ in the bath solution, no currents could be recorded when the cell was clamped at a holding potential of -70mV. Therefore, it can be concluded that the $hcK_{ATP}$ protein product did not produce channels with the characteristics of native $K_{ATP}$ channels.

Following the reports of Krapivinsky et al., where CIR was shown to be activated by G-proteins, it was also important to investigate whether $hcK_{ATP}$ could be activated in a similar manner. 160$\mu$M GTP-$\gamma$-S was included in the pipette solution and preliminary experiments showed that this resulted in the activation of inwardly rectifying currents which were inhibited by barium, an inward rectifier $K^+$ channel inhibitor (fig 4.11).

4.3.6 Electrophysiological Analysis of $rcK_{ATP}$ Heterologously Expressed in MEL Cells

MEL cells, stably transfected with $pEV3[rcK_{ATP}]$ were analysed using the whole-cell patch-clamp technique. Once again preliminary data shows the presence of a small inwardly rectifying $K^+$ current in these cells when GTP-$\gamma$-S was present in the pipette solution (fig 4.12).
Fig 4.10 Whole-cell patch-clamp recordings from non-transfected HEK293 cells.  
A) Currents produced as a result of voltage steps from -60mV to +50mV from a holding potential of -70mV with 6mM K⁺ in the pipette solution and 140mM K⁺ in the bath solution. A voltage-dependent K⁺ current is clearly observed.  
B) Recording of a barium-sensitive inwardly rectifying K⁺ current evoked through a voltage ramp from -140mV to +50mV with 140mM K⁺ in the pipette solution and 60mM K⁺ in the bath solution. Addition of 50μM Ba²⁺ to the bath solution inhibited this current.
Fig 4.11 Whole-cell patch-clamp recordings from MEL cells stably transfected with hcKATP. A small barium-sensitive inward rectifying K⁺ current was produced by a voltage change from -140mV to +50mV. No outward current was observed at voltages positive to $E_K$ which is -21mV. The pipette solution contained 107mM KCl, 33mM KOH, 10mM HEPES, 10mM EGTA, 1mM CaCl₂, 1mM MgCl₂, 0.1mM ADP, 0.1mM ATP and 150μM GTP-γ-S. The bathing solution contained 60mM KCl, 80mM NaCl, 10mM HEPES, 1mM MgCl₂, and 0.1mM CaCl₂.
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Fig 4.12A) Whole-cell patch-clamp recordings from MEL cells stably transfected with rcKATP. Voltage step experiments from -80mV to +40mV, with a holding potential of -20mV, produced small K+ currents which appeared to inwardly rectify.

Fig 4.12B) Whole-cell patch-clamp recordings from MEL cells stably transfected with GIRK1. Voltage step experiments from -80mV to +80mV, with a holding potential of -20mV, failed to produce any current. For both rcKATP-transfected cells and GIRK1-transfected cells the pipette solution contained 140mM KCl, 10mM EGTA, 10mM HEPES, 1mM MgCl₂ and 200μM GTP-γ-S (pH 7.2) and the bathing solution contained 70mM KCl, 70mM NaCl, 2mM CaCl₂, 2mM MgCl₂ and 10mM HEPES (pH 7.4)
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4.4  The Distribution of the rcK\textsubscript{ATP} Channel Protein in Cardiovascular Tissue

4.4.1  Introduction

Ashford et al., (1994) used Northern blotting techniques to show that tc\textsubscript{K}\textsuperscript{ATP} mRNA is present primarily in rat ventricle but also in rat atrium. This is the tissue distribution which would be expected for a K\textsubscript{ATP} channel. Conversely, Krapivinsky et al., (1995) report that CIR is expressed predominantly in rat atria and at much lower levels in rat ventricles. The muscarinic K\textsuperscript{+} channel, responsible for I\textsubscript{KACb}, functions to regulate atria and the result of Krapivinsky et al., when considered together with all the other data presented in their paper, indicates that CIR, whose protein sequence differs from the rc\textsubscript{K}\textsuperscript{ATP} protein sequence by only two amino acids, is more likely to be one of the subunits forming this muscarinic K\textsuperscript{+} channel than a component of the K\textsubscript{ATP} channel. In an attempt to resolve the cardiac distribution of CIR or rc\textsubscript{K}\textsubscript{ATP} the rc\textsubscript{K}\textsubscript{ATP} antibody was used in Western blot experiments on protein prepared from rat atria, ventricles and also aorta, as the true K\textsubscript{ATP} channel is also expressed in vascular smooth muscle tissue.

4.4.2  Investigation into the Distribution of rc\textsubscript{K}\textsubscript{ATP} in Cardiovascular Tissue using Western Blotting Techniques

Total cell protein was prepared from rat atrium, ventricle and aorta and the protein concentration was estimated by measuring the absorbance of the sample at 280nm. Approximately 0.5mgs of total cell protein was run out for each sample on a 10% SDS polyacrylamide gel. A positive control of MEL cell-line MEL[rc\textsubscript{K}\textsubscript{ATP}] 1 total cell protein and a negative control of non-transfected MEL cell protein were also run on the same gel. The gel was transferred onto a nitrocellulose membrane and antigens were detected using the rc\textsubscript{K}\textsubscript{ATP} antibody. The results from this western blot experiment (fig4.13) show a band of 45kDa, the expected size for the rc\textsubscript{K}\textsubscript{ATP} protein, in the rat atrial sample lane but not in the rat ventricle or aorta sample lanes. A 45kDa band was also detected in the positive control lane, as would be expected.
Fig 4.13 Western blot of cardiac protein samples using the N-terminal rcK\textsubscript{ATP} antibody. A) Photograph of a coomassie blue stained 10% SDS-PAGE gel on which cardiac protein samples were run as follows; a) rat aorta, b) rat ventricle, c) rat atrial, d) non-transfected MEL cell, e) MEL[rcK\textsubscript{ATP}]\textsubscript{1} and f) High molecular weight Rainbow markers (Amersham) sized 97.4kDa, 66kDa, 46kDa, 30kDa and 21.5kDa.

B) Western blot of gel identical to that shown in fig 4.13A. A protein of approximately 45kDa (the correct size for the rcK\textsubscript{ATP} protein) has lit up in the positive control lane and also in lane c) on which the rat atrial protein sample has been run. No rcK\textsubscript{ATP} protein band is detected in lanes a) or b).
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4.5 Heterologous Co-expression of rcKATP and GIRK1 in MEL Cells

4.5.1 Introduction

The rcKATP protein sequence and the CIR protein sequence are identical apart from two amino acid differences one of which is conservative. It is reasonable to assume that rcKATP and CIR are, in fact, the same channel due to their very strong homology and the very small sequence differences may be due to the use of different rat strains, small sequencing errors or even possibly allelic differences. The experiments carried out by Krapivinsky et al., (1995) strongly suggest that rcKATP (or CIR) does not encode a KATP channel but is a subunit of the muscarinic K+ channel forming a heteromultimer with GIRK1. To confirm the reports of Krapivinsky et al., their experiments were repeated co-expressing rcKATP and GIRK1 using the MEL/LCR expression system. These experiments were designed to test whether rcKATP would display all the properties of CIR when co-expressed with GIRK1, despite the slight sequence disparity, and also to investigate the usefulness of the MEL/LCR system for co-expression experiments.

4.5.2 Preparation of Stable MEL Cell-lines Co-expressing rcKATP and GIRK1

The constructs pNV1[GIRK1] and pEV3[rcKATP] were linearised at the Pvu I site within the ampicillin resistance gene. The linearised constructs were purified by gel-isolation followed by electroelution and then 15µg of each were mixed together and used to co-transfect MEL cells using an electroporation method. Twenty four hours after electroporation G418 was added to the cells to a final concentration of 1mg/ml and after ten days G418 resistant colonies were picked and stable cell-lines were established.

A PCR-based screen was devised to look for cell-lines which had been transfected with both constructs. A forward primer, β-globin 1, was designed to sequence within the β-globin promoter region of the LCR vectors just 5' of the MCS. Reverse primers were designed within the extreme 5' ends of the rcKATP cDNA sequence and the GIRK1 cDNA sequence. Primer rcKATP 2 was designed to nucleotides 180-201 of the rcKATP cDNA and primer GIRK1 1 was designed to nucleotides 233-256 of the GIRK1 cDNA (fig 4.14). Amplification of genomic DNA prepared from the transfected cell-lines produced a band of 300bp using primers β-globin 1 and rcKATP 2 in cell-lines stably transfected with pEV3[rcKATP] and a band
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of 370bp using primers β-globin 1 and GIRK1 1 in cell-lines stably transfected with pNV1[GIRK1]. Of fourteen cell-lines analysed in this PCR screen only five cell-lines, G/K14, G/KA, G/KE, G/KM and G/KO, were found to be co-transfected with both pEV3[rcKATP] and pNV1[GIRK1].

![Diagram illustrating the design of PCR primers β-globin 1 and GIRK1 1 to pNV1[GIRK1] for the analysis of cell-lines transfected with pNV1[GIRK1]](image)

Fig 4.14 Diagram illustrating the design of PCR primers β-globin 1 and GIRK1 1 to pNV1[GIRK1] for the analysis of cell-lines transfected with pNV1[GIRK1]

To check for the expression of rcKATP protein in these five cell-lines western blots were carried out using the rcKATP antibody. Cells were harvested at four days post-induction and total cell protein was prepared. The protein from 10^6 cells was run out for each sample on a 10% SDS polyacrylamide gel. Western blotting procedures revealed that the rcKATP protein was present in all but one of the co-transfected cell-lines. One cell-line G/KA appeared to express the channel protein at far lower levels than the other three (fig 4.15).

No antibody was available to check for expression of the GIRK1 protein product and so the levels of mRNA transcribed from the integrated pNV1[GIRK1] construct was measured instead. Total RNA was prepared from cells harvested at four days post-induction and the RNA was run out on a 1% agarose gel to check the yield and integrity of the RNA. Following this 5μg of each sample was dotted onto nylon membranes and hybridised to 32P-radiolabelled DNA probes prepared from GIRK1 cDNA fragments as described in section 4.2.3. The results of this RNA dot-blot showed that GIRK1 mRNA is present in all five co-transfected cell-lines. No GIRK1 mRNA was detected in the control sample of induced, non-transfected MEL cells (fig 4.16).

4.5.3 Electrophysiological Analysis of MEL Cell-lines Co-expressing GIRK1 and rcKATP

MEL cell-line G/K14 was analysed using whole-cell patch-clamp techniques with GTP-γ-S present in the pipette solution. Voltage-step experiments clearly showed the presence of an inwardly rectifying current in these cells (fig 4.17a). The distinct inwardly rectifying properties of this channel are illustrated in fig 4.17c.
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Fig 4.15 Western blot, using the N-terminal rcKATP antibody to show the levels of rcKATP expression in co-transfected cell-lines G/K14, G/KA, G/KE, G/KM and G/KO. Protein samples were run in lanes as follows a) G/K14, b) G/KA, c) G/KE, d) G/KM, e) G/KO, f) non-transfected MEL cell and g) High molecular weight Rainbow Markers (Amersham). The rcKATP protein was found to be expressed at high levels in G/K14, G/KE and G/KM, at low levels in G/KA and not at all in G/KO. Lack of expression in G/KO may have been because the cell-line was not transfected with pEV3[rcKATP] but had been selected due to a contaminated PCR reaction.

Fig 4.16 RNA dot-blot of RNA prepared from cell-lines G/K14, G/KA, G/KE, G/KM and G/KO showing the presence of GIRK1 mRNA in all five cell-lines. Positive and negative controls were carried out as in fig 4.5.
Fig 4.17 Whole-cell patch-clamp analysis of MEL cell stably co-transfected with rεK\textsubscript{ATP} and GIRK1. The pipette solution contained 140mM KCl, 10mM EGTA, 10mM HEPES, 1mM MgCl\textsubscript{2} and 200\muM GTP-γ-S (pH 7.2) and the bathing solution contained 70mM KCl, 70mM NaCl, 2mM CaCl\textsubscript{2}, 2mM MgCl\textsubscript{2} and 10mM HEPES (pH 7.4). A) Currents produced by voltage-steps from -80mV to +80mV, in 10mV steps, at a holding potential of -20mV. An inwardly rectifying K\textsuperscript{+} current is observed. B) Control experiment carried out on a non-transfected MEL cell. No currents were evoked by voltage commands from -80mV to +80mV at a holding potential of -20mV. C) The I/V relationship of this K\textsuperscript{+} current, clearly illustrating its inwardly rectifying properties.
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A.

B.

C.

command potential (mV)

peak current (pA)
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4.6 Discussion

The rcKATP clone was originally heterologously expressed in HEK293 cells (Ashford et al., 1994). However, control experiments reported in this chapter demonstrate that non-transfected HEK293 cells contain both voltage-activated and inward rectifier K+ currents making them unsuitable for the heterologous expression of members of either of these families of K+ channels. When rcKATP was heterologously expressed in MEL cells, which do not endogenously express either inward rectifier or voltage-activated K+ channels, preliminary electrophysiological investigations did not detect any KATP-type currents. However, when the experiments of Krapivinsky et al. (1995) were repeated and GTP-γ-S was included in the pipette solution small inwardly rectifying currents were observed. Similarly, MEL cells heterologously expressing hcKATP did not display any K+ currents with the characteristics of I_KATP but they did produce inwardly rectifying, barium-sensitive K+ currents on addition of GTP-γ-S to the pipette solution. Therefore, in our hands, the rcKATP and the hcKATP channel proteins are functioning as described in the report made by Krapivinsky et al. (1995). This was also shown to be the case when rcKATP was heterologously co-expressed in MEL cells with rat atrial GIRK1. Co-expression of these two components resulted in the production of inwardly rectifying K+ channel currents on the addition of GTP-γ-S to the pipette solution and no background current was observed in the same cells without the addition of GTP-γ-S. Co-expression of GIRK1 with rcKATP significantly increased the size of currents produced when compared to those observed when rcKATP was expressed on its own. This result is similar to that obtained by Krapivinsky et al. (1995). When GIRK1 is expressed on its own in Xenopus oocytes, the basal current without G-protein activation is fairly significant (Kubo et al., 1993b). This leakage is uncharacteristic of the native I_KACb but addition of a second subunit, rcKATP, to the heterologously expressed channel complex results in a non-leaky current, only activated in the presence of GTP-γ-S and very similar to the native channel. Western blotting experiments also described in this chapter show that the rcKATP channel protein is expressed only in the rat atrium and not in the ventricle. The findings reported here strongly support the results of Krapivinsky et al. (1995), who proposed that rcKATP (or CIR) is not responsible for I_KATP but instead forms a heteromultimeric channel complex with GIRK1 which is responsible for I_KACb.

Previous experiments carried out within the group showed that GIRK1 could be successfully, heterologously co-expressed in MEL cells (Jacobsen, 1994) producing recognisable inward rectifying K+ currents. However, attempts to repeat these experiments by heterologous co-expression of GIRK1 with an M2 receptor
failed. With GTP-γ-S present in the pipette solution, no inward rectifying K⁺ channel currents were observed in these cell-lines. This result also supports the findings of Krapivinsky et al. (1995), who were unable to functionally express GIRK1 in Sf9, CHO or HEK293 cells. As mentioned in section 4.3.1., this may be due to a lack of endogenous CIR-type channels in these mammalian cell-lines. RNA dot-blots, using the rcKATP cDNA as a probe, were unable to detect the expression of any endogenous channel in native MEL cells under medium stringency washing conditions. Therefore, the current observed in GIRK1-transfected cells by Jacobsen (1994), was possibly an artefact.
Chapter 5

Heterologous Co-expression of the Cyclic Nucleotide-Gated Cation Channel with an ANP Receptor
Chapter 5 Heterologous Co-expression of the Cyclic Nucleotide-Gated Cation Channel with an ANP Receptor

5.0 Heterologous Co-expression of the Cyclic Nucleotide-Gated Cation Channel with an ANP Receptor

5.1 Introduction

There are two forms of guanylate cyclase, a soluble haem-associated form and a particulate membrane-spanning form such as the ANP receptor (section 1.2). A major aim of this thesis was to heterologously co-express the cGMP-gated cation channel expressed in cardiovascular tissue, CNG-1, with a cardiovascular cGMP-generating component. This type of experiment would allow the study of channel gating through activation of a second messenger pathway and provide a model signalling pathway which could help to elucidate the function of the CNG-1 in cardiovascular tissue. The particulate guanylate cyclase, ANP receptor type A (ANP-RA) and soluble guanylate cyclases are both expressed in cardiovascular tissue (section 1.2) and it is possible that either or even both of these cGMP-generating molecules activate the cGMP-gated cation channel expressed in cardiovascular tissue (fig 5.1).

Fig 5.1 Diagram illustrating the gating of the cGMP-gated cation channel (CNGC) by either a soluble or particulate guanylate cyclase (G.C.). The particulate guanylate cyclase is ANP-RA and is activated by ANP and the soluble guanylate cyclase by NO. cGMP molecules are represented by the shaded circles.
It was decided to concentrate on the co-expression of ANP-RA, and not the soluble guanylate cyclase, with CNG-1 for three reasons. First, members of the particulate guanylate cyclase family have previously been suggested to be closely coupled to cGMP-gated cation channels expressed in the retina (Shyjan et al., 1992; Pardhasradhi et al., 1994) and therefore this may be an established signalling pathways adapted through evolution to carry out other functions in different tissues. Secondly, an abstract published by Seiss-Geuder et al., 1993, demonstrated that the application of ANP to cultured guinea-pig coronary endothelial cells resulted in a rapid depolarisation of 12mV which was repeated by addition of a membrane-permeable cGMP derivative, 8-bromo-cGMP. These observations provided clear evidence that ANP activation of its receptor resulted in an elevation in intracellular cGMP which was directly affecting a cGMP-regulated ion channel. Thirdly, ANP-RA is a single polypeptide encoded by a 3,171bp cDNA (rat ANP-RA), while the soluble guanylate cyclase consists of an α and β subunit and requires a haem group. Therefore it was simpler to heterologously co-express CNG-1 with ANP-RA, a single peptide, than with the soluble guanylate cyclase which required the expression of two subunits and the presence of a haem group.

The advantages of the MEL/LCR expression system for heterologous co-expression procedures were described in detail in section 4.1. The MEL/LCR system is also ideal for heterologous co-expression of CNG-1 and ANP-RA as high levels of position-independent co-expression of both heterologous proteins is insured. However, to date only voltage-activated K⁺ channels and inward rectifier K⁺ channels have been heterologously expressed in MEL cells and the suitability of MEL cells for the functional expression of cGMP-gated cation channels is not known. Human erythrocytes have been shown to possess a muscarinic cholinergic receptor which, when activated, stimulates transient increases in Ca²⁺ uptake and subsequently results in the production of cGMP (Tang et al., 1984; Bennekou, 1993). MEL cells, which are derived from mouse erythrocytes, may also possess this cGMP-generating signalling pathway which may interfere with a heterologously expressed ANP receptor and CNG-1. The MEL cell differentiation process, which occurs following induction, is also thought to involve an initial period of Ca²⁺ influx (Gillo et al., 1993) which may also interfere with patch-clamp recordings of a heterologously expressed cGMP-gated cation channel. Therefore, to test the whether the heterologous expression of cGMP-gated cation channels in MEL cells was compatible it was decided to first express CNG-1 in MEL cells without co-expression of ANP-RA. However, the presence of endogenous cGMP signalling pathways in MEL cells could be beneficial in coupling experiments between cGMP-generating pathways and a heterologously expressed cGMP-gated cation channel. The report of Bennekou (1993)
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suggests that the carbachol-induced Ca^{2+} elevation is activating NO synthase and NO in turn is activating a soluble guanylate cyclase causing cGMP elevations. This signalling pathway may be present in MEL cells in which case it would be possible to activate this endogenous soluble guanylate cyclase with a NO-donor, such as sodium nitroprusside (SNP), causing elevations in cGMP in vivo and opening of heterologously expressed CNG-1 channels. It has also been reported that human erythrocytes express an endogenous ANP receptor (Petrov et al., 1994). MEL cells may also express this receptor and it may be possible to couple this endogenous particulate guanylate cyclase with heterologously expressed CNG-1, thus by-passing the need to co-express ANP-RA. Experiments to investigate the presence of these cGMP-signalling pathways in MEL cells needed to be carried out to enable detailed design of coupling experiments. The existence of both the particulate and soluble guanylate cyclases in MEL cells this would create a model system for cGMP-gated channel activation which requires the heterologous expression of only the channel protein.

Functional heterologous expression of cGMP-gated cation channels in HEK293 cells has been reported in several recent papers (Dhallan et al., 1992; Weyand et al., 1994). As the suitability of MEL cells for the functional study of cGMP-gated cation channels is unknown, it was also decided that heterologous co-expression experiments would be carried out in HEK293 cells. ANP-RA has been reported to be expressed at negligible levels, if at all, in HEK293 cells (Chrisman et al., 1993) and therefore this makes HEK293 cells an ideal cell-line in which to study the interactions of heterologously co-expressed CNG-1 and ANP-RA. However, the selection of co-expressing HEK293 cell-lines may be more complicated than in the MEL/LCR system as this approach is susceptible to 'position effects'.
5.2 Direct Evidence for the Co-expression of ANP-RA and the cGMP-Gated Cation Channel in the Same Cell

In section 3.3.3. PCR experiments were used to demonstrate the expression of CNG-1 in HUVECs. Primers cG-E and cG-F amplified a product of 345bp from 1μg of HUVEC cDNA. At the same time another PCR amplification was carried out on the same sample of HUVEC cDNA using primers ANP1 and ANP4, designed to the human ANP-RA cDNA sequence, which have been described previously in section 3.5.2. These primers were able to amplify a product of 229bp from 1μg of HUVEC cDNA (fig 5.3). HUVECs are highly unlikely to be contaminated with any other cell type and therefore this result strongly indicates that ANP-RA and CNG-1 are expressed in the same cell and therefore as ANP-RA is a cGMP-generating protein and CNG-1 is opened by cGMP these two membrane proteins could well be interacting.

![Agarose gel](image)

Fig 5.2 Photograph of an agarose gel which shows the co-expression of CNG-1 and ANP-RA in HUVECs. Lane a) Amplification product from HUVEC cDNA template with primers cG-E and cG-F, b) negative control PCR using primers cG-E and cG-F, c) 123bp DNA ladder, d) Amplification product from HUVEC cDNA template with primers ANP1 and ANP4, e) negative control PCR using primers ANP1 and ANP4
5.3 Heterologous Expression of CNG-1 in MEL Cells

5.3.1 Subcloning of CNG-1 into pNV1 and pEV3 and Transfection into MEL Cells

Human CNG-1 cDNA was kindly donated by Jeremy Nathans cloned into the Sal I and Hind III sites of pUC 119. Study of the 5' untranslated sequence of this cDNA clone detected an inframe translation initiating codon (ATG) 12 nucleotides upstream of the true initiating ATG. However, as this clone had previously been functionally expressed using a transient expression system in HEK293 cells (Dhallan et al., 1992) and the inframe, upstream ATG does not seem to effect the levels of functional protein expression it was not removed before cloning of the cDNA into the LCR vectors. The CNG-1 cDNA was removed from pUC 119 on the Eco RI and Hind III fragment and cloned into the Eco RI and Hind III sites of pBKSII-. The CNG-1 cDNA fragment was then removed from pBKSII- on the Eco RI and Sal I sites and cloned into the Eco RI and Sal I sites of pNV1 and pEV3. These expression constructs were named pNV1[CNG-1] and pEV3[CNG-1] respectively. Both pNV1[CNG-1] and pEV3[CNG-1] were linearised by overnight digestion with Pvu I which cuts both constructs within the ampicillin resistance gene. The linearised constructs were purified by isolation on a 0.8% agarose gel followed by electroelution and ethanol precipitation and 20μg of each construct was used to transfect MEL cells. G418 was added to the cells to a concentration of 1mg/ml at twenty four hours post-transfection and at ten days post-transfection positive G418-resistant cell-lines were selected.

5.3.2 Southern Analysis of MEL Cell-lines Stably Transfected with pNV1[CNG-1] and pEV3[CNG-1]

Detailed restriction maps are not available for pNV1 or pEV3. Therefore several restriction digests of pNV1[CNG-1] and pEV3[CNG-1] were carried out to determine which enzyme would digest the constructs to give suitably-sized fragments for probing in a Southern blot. Digestion of pNV1[CNG-1] with Pst I gives a band of approximately 5.5Kb and three smaller fragments. Bgl II cuts once approximately 1.9Kb into the CNG-1 cDNA and the Bgl II site present in pNV1 has been removed from the construct during subcloning. Digestion of pNV1[CNG-1] with Bgl II and Pst I removes the 5.5Kb band observed when digesting with Pst I alone and instead a 3Kb and 2.5Kb band are observed when the digestion is run out on a 0.8% agarose gel. This digestion shows that the complete CNG-1 cDNA is present on the 5.5Kb
fragment produced when pNV1[CNG-1] is digested with Pst I. Similar experiments were carried out for pEV3[CNG-1] and in this case digestion with Pst I results in a 9.5Kb fragment (and several much smaller fragments) which is cleaved into fragments of 7Kb and 2.5Kb when pEV3[CNG-1] is digested with both Pst I and Bgl II. Digestion of both pNV1[CNG-1] and pEV3[CNG-1] with Pst I, Bgl II and Pvu I does not affect the sizes of the 5.5Kb and 9.5Kb bands respectively.

Genomic DNA was prepared from two MEL cell-lines transfected with pNV1[CNG-1] and two MEL cell-lines transfected with pEV3[CNG-1] and also from non-transfected MEL cells. 15|g of each genomic DNA sample was digested overnight with 20 units of Pst I and each digested sample was run on a 0.8% agarose gel with λ Hind III cut DNA markers. The gel was blotted onto a nylon filter and then hybridised overnight to a 32P-radiolabelled DNA probe prepared by random priming. The template for random priming was prepared by digesting pBKSII[CNG-1] with Eco RI and Sal I and purifying the 2.3Kb fragment obtained by gel isolation and electroelution. 10ng of this fragment was then used in the random priming reaction. Following hybridisation and washing the filter was autoradiographed against X-ray film with an intensifying screen at -70°C for twenty four hours. The results from this Southern blot showed that the LCR constructs have stably integrated into the genomic DNA of each transfected cell-line analysed.

5.3.3 Analysis of Stably Transfected MEL Cell-lines for the Expression of CNG-1 by Western Blotting

An antibody to the C-terminus of the cGMP-gated cation channel was kindly provided by Robert Molday. The monoclonal antibody, PMc ID 1, was raised against the bovine CNG-1 protein (Cook et al., 1989) but was shown to cross-react with the human, pig, mouse, rat and guinea-pig CNG-1 proteins also (Molday et al., 1991). The four cell-lines in which the stable integration of the pNV1[CNG-1] or pEV3[CNG-1] constructs had previously been shown (see 5.3.2) were induced and the cells were harvested at four days post-induction. Total cell protein was prepared from each of these cell-lines and the protein from 10^6 cells was run for each sample in duplicate on an 8% SDS polyacrylamide gel. Non-transfected-induced MEL cell protein was loaded as a negative control and 50μg of bovine ROS (rod outer segment) membrane protein (kindly provided by Dr. I. Weyand) was run as a positive control. Following electrophoresis half of the gel was stained with Coomassie blue stain to check the integrity of the protein and the duplicated half was transferred onto a nitrocellulose filter overnight. Western blotting using PMc ID 1 detected a band of 63kDa consistent with the expected size of the native channel protein in the positive
control lane in which ROS membrane protein was run but no bands were detected for any of the transfected cell-lines.

5.3.4 Are Soluble and Particulate Guanylate Cyclases Already Expressed in MEL Cells?

Human erythrocytes have been shown to express an ANP receptor which elevates intracellular cGMP concentrations and increases the rate of Na⁺/H⁺ exchange (Petrov et al., 1994). It is possible that MEL cells, which are derived from erythrocytes, may also express this receptor. If so, heterologously expressed CNG-1 could be gated through activation of an endogenous ANP receptor. Human erythrocytes may also possess a soluble guanylate cyclase (Bennekou, 1993). Primers ANP1 and ANP4, designed to the human ANP-RA cDNA sequence, and described previously in section 3.5.2 were able to amplify a product of 229bp from 100ng of MEL cell cDNA confirming that ANP-RA is expressed in MEL cells. To investigate whether this receptor is functional and also to see whether soluble guanylate cyclases are also expressed in MEL cells, experiments were carried out to measure intracellular cGMP concentrations following addition of the agonist ANP or the NO-donor SNP. MEL cells were induced and experiments were carried out at four days post-induction. 10⁶ MEL cells were used for each experiment in which cells were incubated with 50nM ANP or 5μM SNP or saline solution. Following cGMP assays only negligible levels of cGMP (under 1pmol of cGMP per 10⁶ cells) could be detected in the control experiment and the cells which had been stimulated with either ANP or SNP. Therefore, neither soluble nor particulate guanylate cyclases appear to be functional in differentiating MEL cells.
5.4 Heterologous Co-expression of CNG-1 and ANP-RA in HEK293 Cells

5.4.1 Subcloning of CNG-1 and ANP-RA into pcDNA3 and Transfection into HEK293 Cells

The human CNG-1 cDNA was cloned into the Eco RI and Hind III restriction sites of pBKS- (see section 5.3.1). Following this the CNG-1 cDNA fragment was excised from pBKSII- on the Eco RI and Xho I sites and cloned into the Eco RI and Xho I sites of the mammalian expression vector pcDNA3 (see section 4.3.2) and this construct was named pcDNA3[CNG-1]. A rat ANP-RA cDNA was kindly provided by David Garbers cloned into the Eco RI site of pBKSII-. The ANP-RA cDNA fragment was excised from pBKSII- at the Kpn I and Not I sites and cloned into the Kpn I and Not I sites of pcDNA3 and this construct was named pcDNA3[ANP-RA].

Both pcDNA3[CNG-1] and pcDNA3[ANP-RA] were linearised by overnight digestion with Pvu I which cuts within the ampicillin resistance gene of pcDNA3. The linearised constructs were purified by isolation on a 0.8% agarose gel followed by electroelution and ethanol precipitation. 1μg of each construct was mixed together and used to transfect HEK293 cells by a method of lipofection. 1μg of each construct was also used to separately transfect HEK293 cells again by a method of lipofection. At seventy two hours post-transfection cells were passaged 1:10 into fresh media containing 1mg/ml G418 and after two weeks G418-resistant colonies were picked and stably transfected cell-lines were established.

5.4.2 Analysis of Stably Transfected Cell-lines by PCR

To check that the constructs had inserted into the HEK293 cell genomic DNA a PCR-based experiment was used to screen for the integration of pcDNA3[CNG-1] and pcDNA3[ANP-RA] in the genomic DNA of each established cell-line. The PCR primer pcDNA3(rev) (see section 4.3.2) was used in conjunction with PCR primers cG7 (see section 3.2.2) and ANP-RA2 which is a forward primer designed to bases of the rat ANP-RA cDNA. Genomic DNA was prepared from five cell-lines transfected with pcDNA3[CNG-1] alone, six cell-lines transfected with pcDNA3[ANP-RA] alone and ten cell-lines which were co-transfected with both constructs. This genomic DNA was then used as the template in a series of PCR reactions using primers pcDNA3(rev), cG7 and ANP-RA2. The product size expected following amplification with cG7 and pcDNA3(rev) is approximately 800bp and the product size expected following amplification with ANP-RA2 and pcDNA3(rev) is...
approximately 1Kb. Following amplification four of the five cell-lines transfected with pcDNA3[CNG-I] were found to definitely have the construct integrated into the genomic DNA and all of the cell-lines transfected with pcDNA3[ANP-RA] had the construct integrated into their genomic DNA. For the co-transfected cell-lines six of the ten lines analysed clearly had both constructs integrated into their genomic DNA.

5.4.3 Is the Heterologously Expressed ANP-RA Active?

cGMP assays were carried out to check that the ANP-RA receptor expression construct integrated into the genomic DNA of the cell-lines analysed as described above (section 5.4.2) was being functionally expressed. 10^6 HEK293 cells were used for each assay in which cells were incubated with 50nM ANP or saline solution (as a control). Non-transfected HEK293 cells were used as a negative control to check that the cells had no endogenous ANP receptor and 5μM SNP, a NO donor, was also applied to non-transfected HEK293 cells to check that the cells had no endogenous soluble guanylate cyclase. Five cell-lines transfected with pcDNA3[ANP-RA] alone and five cell-lines which had been co-transfected with both pcDNA3[ANP-RA] and pcDNA3[CNG-I] were analysed in the assays. The results of cGMP assays showed cGMP elevations on addition of ANP in all ten cell-lines of between 210pmoles cGMP/10^6 cells and 3600pmoles cGMP/10^6 cells (fig 5.3). Addition of ANP to non-transfected HEK293 cells did not result in a rise in cGMP indicating that HEK293 cells do not have an endogenous ANP receptor as has previously been indicated in the report of Chrisman et al. (1993). However, the addition of SNP to non-transfected HEK293 cells consistently resulted in a very small increase in cGMP from negligible levels of under 1pmoles cGMP/10^6 cells to which saline had been applied to approximately 5pmoles cGMP/10^6 cells to which 5μM SNP had been applied. This indicates that an endogenous soluble guanylate cyclase is expressed in HEK293 cells at very low levels (see fig 5.4).

5.4.4 Analysis of Stably Transfected HEK293 Cell-lines for the Expression of CNG-I

Total cell protein was prepared from all six co-transfected cell-lines in which both pcDNA3[ANP-RA] and pcDNA3[CNG-I] had been shown to be stably integrated and also from non-transfected HEK293 cells. The protein prepared from 10^6 cells was run in each lane of duplicated 8% SDS polyacrylamide gel. A positive control of 50μg of bovine ROS protein was also run. One gel was stained in Coomassie blue stain to check the integrity of the protein and the other gel was transferred overnight onto nitrocellulose. The protein bound to the nitrocellulose was
Fig 5.3a) Histogram displaying the elevation of intracellular cGMP/10^6 cells without addition of ANP (-) and following addition of 50nM ANP (+) in HEK293 cells which have been stably co-transfected with ANP-RA and CNG-1. Cell-lines analysed were lines 1,10,11,15 and 16.

Fig 5.3b) Histogram displaying the elevation of intracellular cGMP/10^6 cells without addition of ANP (-) and following addition of 50nM ANP (+) in HEK293 cells which have been stably transfected with ANP-RA. Cell-lines analysed were lines 2,3,6,7 and 8.
Fig 5.4 Histogram showing the intracellular cGMP concentration/10^6 cells of non-transfected HEK293 cells following the addition of saline (control), 200nM ANP (+ANP) or 20mM of SNP (+NO).
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detected using the antibody PMc 1D1. However, the antibody was only able to detect channel protein of molecular weight 63kDa in the positive control lane.

5.4.5 Analysis of the 5' Upstream Region of the Human CNG-1 cDNA

Western blot experiments could detect CNG-1 channel protein in neither transfected MEL cells nor transfected HEK293 cells. This indicated that there may be a problem with either transcription from the integrated construct or with the initiation of translation. It seems unlikely the problem lies with transcription as both the LCR/β-globin promoter and the CMV promoter have been shown to drive transcription of downstream cDNAs very effectively (as described in chapter 4 and as shown for ANP-RA expression in this chapter). Therefore these expression problems are probably derived from the cDNA sequence itself and such problems are more likely arise at a translational level. As described in section 5.3.1, the CNG-1 cDNA contains an in frame upstream ATG. In transient expression experiments carried out by Dhallan et al. (1992) this in frame ATG was not removed and functional expression of CNG-1 was achieved in HEK293 cells. Therefore this upstream, inframe ATG was not removed prior to sub-cloning into the LCR expression vectors and pcDNA3. However, the Kozak consensus sequences for both the inframe, upstream ATG and also the true initiating ATG are not particularly strong and this may be affecting the initiation of translation. Studies carried out by Kozak (1986) reveal that the optimal sequence for initiation by eukaryotic ribosomes is A(or G)CCATGG. The upstream inframe ATG in the CNG-1 cDNA has an initiating sequence of GATATGA and the true initiating ATG of the CNG-1 cDNA had a sequence of TCCATGA. It was thought that the weak Kozak consensus sequences together with the presence of two competing initiating ATGs may be affecting levels of translation. Therefore a PCR primer, cG12, was designed which would eliminate the upstream, inframe ATG and introduce an optimal Kozak consensus sequence around the true ATG, although, of course, the nucleotide at position +4 could not be changed. An Eco RI restriction site was included at the 5' of cG12 to allow easy cloning with two extra bases at the very 5' end of the primer to allow efficient digestion. cG12 was used with primer cG8, which has a Not I site included at its 5' end (section 3.2.2), to amplify the channel cDNA using a proof reading DNA polymerase (Deep Vent) and 10ng of the original CNG-1 cDNA construct pUC119[CNG-1] as the template. Following amplification the 2.1Kb product was purified and digested for twenty hours at 37°C with Eco RI and Not I. The digested CNG-1 PCR product was purified by running on a 0.8% agarose gel followed by electroelution and ethanol precipitation. Following this the purified PCR product was cloned into the Eco RI and Not I sites of the expression vector pCIneo (Promega).
Fig 5.5. Vector map of the construct pCI-neo.

CMV I.E. Enhancer/Promoter: Cytomegalovirus immediate early enhancer promoter

Intron: A chimeric intron consisting of the 5'-donor site from the first intron of the human β-globin gene and the 3'-acceptor site from the intron of an immunoglobulin gene heavy chain variable region. Both the donor and acceptor sites have been changed to match the consensus sequences for splicing. Transfection studies have shown that an intron flanking the cDNA insert often increases the level of gene expression.

SV40 late poly A+: The SV40 polyadenylation signal terminates transcription and adds adenosine residues to the 3' end of the RNA increasing its stability.

Neo: Neomycin phosphotransferase gene

fl: fl origin of replication for generation of single strand DNA.

Ampf: Ampicillin selectable marker for selection in E.coli.
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This vector is identical to pcDNA3 except that an intron has been inserted upstream of the MCS which has been shown to increase the levels of gene expression from the construct (fig 5.5). This construct was named pCIneo[CNG-1].

The initiating sequence of CNG-1 in pCIneo[CNG-1] was sequenced using sequencing primer 5’CNG-1, which is an antisense primer designed to the human CNG-1 sequence at bases 85-104. Sequencing showed that the upstream, inframe ATG had been removed and that the initiating sequence was GCCATGA, which has been shown by Kozak (1986) to be near optimal for the initiation of translation. This sequencing reaction also revealed an amino acid difference from the published sequence (Dhallan et al., 1992) in the cDNA used in these experiments. The amino acid change (Lys-Asn), observed following several different sequencing reactions, occurred immediately after the initiating methionine and resulted from a single base pair change at the third codon position (AAG-AAT).

5.4.6  Transfection and Heterologous Expression of pCIneo[CNG-1] in HEK293 Cells

pCIneo[CNG-1] was linearised by overnight digestion with Bam HI which cuts at a unique site. The linearised construct was purified by gel isolation followed by ethanol precipitation and 1μg of the purified construct was used to transfect each well of a six well plate of HEK293 cells by a method of lipofection. At seventy two hours post-transfection cells were passaged 1:10 into fresh media containing 1 mg/ml G418 and after two weeks G418-resistant colonies were picked and stably transfected cell-lines were established.

To establish whether CNG-1 had stably integrated into the HEK293 cell genomic DNA a fast PCR screen was employed using PCR primers cG-E and cG-F with an extension period at 72°C of thirty seconds. These primers are designed to amplify a 345bp band from the human CNG-1 cDNA sequence but, as the product of this amplification spans an intron/exon junction, no products are observed from a genomic DNA template under the experimental conditions described here. Genomic DNA was prepared from seven G418-resistant colonies and used as templates in this rapid PCR screen. All seven amplifications produced a 345bp band. No band was observed when non-transfected HEK293 cell genomic DNA was used as a template. This screen gives a good indication that the pCIneo[CNG-1] construct has stably integrated into the genomic DNA of all seven cell-lines analysed.

Total cell protein was prepared from all seven co-transfected cell-lines in which pcDNA3[CNG-1] had been shown to be stably integrated and also from non-transfected HEK293 cells. The protein prepared from 10⁶ cells was run in each lane of duplicated 8% SDS polyacrylamide gel. A positive control of 50μg of bovine ROS
protein was also run. One gel was stained in Coomassie blue stain to check the integrity of the protein and the other gel was transferred overnight onto nitrocellulose. The protein bound to the nitrocellulose was detected using the antibody PMc 1D1. However, once again the antibody was only able to detect channel protein of molecular weight 63kDa in the positive control lane.
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Fig 5.6a) Results from rapid PCR screen using PCR primers cG-E and cG-F. Lanes are as follows: 1) 123bp DNA ladder, 2)-8) Amplification product from genomic DNA prepared from cell-lines HEK293/pCIneo[CNG-1] 1-7 respectively, 9) Amplification product from non-transfected HEK293 cells, 10) negative control PCR 11) positive control 12) 123bp DNA ladder. A 345bp product is observed following amplification of genomic DNA prepared from all seven cell-lines indicating that the pCIneo[CNG-1] construct has integrated stably into each line.

Fig 5.6b) Photograph of a coomassie-stained SDS-PAGE gel on which protein from samples HEK293/pCIneo[CNG-1] 1-7 was run in lanes 1-7, non-transfected HEK293 cell protein was run in lane 8 and 50μg of ROS membrane protein in lane 9. Lane 10 contains high molecular weight Rainbow Markers (Amersham). (For HEK293 cell samples total cell protein prepared from $10^6$ cells was run in each lane.)

Fig 5.6c) Western blot of an identical gel to the Coomassie-stained gel shown in fig 5.6b using the monoclonal antibody PMc 1D1. Lane samples are identical to those in fig 5.6b. As can be seen PMc 1D1 only detects a band of 63kDa, corresponding to the native CNG-1 channel, in the positive control lane containing ROS membranes.
5.5 Discussion

Initial PCR experiments showed that CNG-1 and ANP-RA are co-expressed in HUVECs which strongly suggests that there is an interaction between these two components in vivo. This result confirmed the importance of studying the coupling of these molecules within a model system. As described in the introduction the MEL cell system may be ideal for this particular experiment as human erythrocytes, and therefore possibly MEL cells, have been shown to express ANP receptors and there is strong evidence for the presence of soluble guanylate cyclases in erythrocytes also. An RT-PCR experiment was used to detect the expression of ANP-RA in MEL cells but this receptor was subsequently shown to be inactive. This may be due to substantial decreases in the expression of ANP receptors in cultured MEL cells. The experiments demonstrating the functional expression of ANP receptors in human erythrocytes were carried out on freshly isolated cells (Petrov et al., 1994) while MEL cells have been transformed and passaged many times. As PCR is a highly sensitive technique, minute quantities of the ANP receptor cDNA template can still result in the amplification of a detectable product. The loss of muscarinic receptor expression has been demonstrated in BAECs where receptor types M1, M2 and M3 are shown to be present in fresh cells, while only M2 was still expressed (Tracey and Peach, 1992). A similar phenomenon could be occurring in passaged MEL cells. This process may also occur with soluble guanylate cyclase proteins, as no activity for this enzyme was detected in MEL cells either.

Functional heterologous expression of ANP-RA in HEK293 cells was very successful and no endogenous ANP-RA activity was detected in non-transfected HEK293 cells. A negligible amount of soluble guanylate cyclase activity was detected in non-transfected HEK293 cells (5pmoles cGMP/10^6 cells) while the level of activity in cells heterologously expressing ANP-RA ranged from 210pmoles cGMP/10^6 cells to 3600pmoles cGMP/10^6 cells following the addition of ANP. HEK 293 cells therefore appear to be very suitable for the study of heterologously expressed ANP-RA and, as this cell-line has already been used successfully for the electrophysiological analysis of heterologously expressed CNG-1 (Dhallan et al., 1992), HEK293 cells appear to be ideal for studying the interactions of heterologously co-expressed CNG-1 and ANP-RA.

However, attempts to express CNG-1 in both MEL and HEK293 cells failed, even after removal of an ATG upstream of the initiating ATG and the introduction of a 'Kozak' consensus sequence around the initiating ATG. Constructs were shown to have stably integrated into the transfected cell's genomic DNA but Western blots
using PMc ID1, an antibody designed to the carboxyl terminus of the channel protein, were unable to detect any CNG-1 protein product in either transfected MEL cells or transfected HEK293 cells. As ANP-RA was successfully heterologously expressed in HEK293 cells using the expression construct pcDNA3, the lack of expression is unlikely to have been caused by defects within the expression vector. This is also the case for the LCR vectors which were successfully employed, simultaneously with the experiments described here, to express inward rectifier K⁺ channels. The CNG-1 cDNA clone used in these experiments was found to contain a base pair difference which mutated the second amino acid from a lysine to an asparagine. It is possible that this cDNA clone contains further mutations, possibly resulting in the introduction of a stop codon, which would mean that the antibody PMc ID1, which binds to the channel's C-terminus, would be unable to detect the protein product. If time had allowed the CNG-1 sequence would have been sequenced in full to see whether mutations were responsible for the lack of detectable channel protein expression. Northern blot analysis would also be used to ascertain whether the lack of a detectable protein product was occurring at the transcriptional or translational level.
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6.0 Discussion

6.1 The Cardiovascular cGMP-Gated Cation Channel

6.1.1 Summary of Results

In this thesis a number of experiments have been described which demonstrate the existence of a cGMP-gated cation channel in cardiovascular tissue. Following observations that CNG-1 is expressed in rabbit sinoatrial node (Hundal et al., 1993), PCR-based experiments were carried out which showed that CNG-1 is expressed in many different areas of the heart and also in vascular smooth muscle tissue. Sequence encoding a full length cDNA for CNG-1 was retrieved from porcine coronary artery smooth muscle tissue by RT-PCR techniques. A CNG-1 channel was also detected in BAECs and HUVECs using RT-PCR methods, although it was not possible to examine the expression of CNG-1 in vascular smooth muscle cells as no suitable cell lines were available. Therefore, it is still not known whether vascular CNG-1 is expressed in both vascular smooth muscle and endothelial cells or just in endothelial cells. RT-PCR experiments also detected the expression of a cGMP-generating receptor molecule, ANP-RA, in HUVECs. The role of the cGMP-gated cation channel in cardiovascular tissue is still unknown. In the following sections hypothetical signalling pathways, involving cGMP-gated channels, are proposed based on data presented in this thesis and in recent publications.

6.1.2 Possible Roles for CNG-1 in Cardiac Tissue

Hundal et al., (1993) reported that a CNG-1 cDNA was present in a rabbit sinoatrial node cDNA library, which indicated that CNG-1 may be involved in pacemaking. However, results from a PCR-based screen, described in this thesis, showed that CNG-1 is expressed in all regions of the heart, which suggests that CNG-1 may have a wider function in cardiac tissue. Cardiac cGMP levels are elevated by agonists such as adenosine, acetylcholine and ANP resulting in a negative ionotropic effect. The mechanisms through which cGMP exerts its ionotropic effect are unknown, although it seems likely that inhibition of the cardiac L-type Ca$^{2+}$ channel current, $I_{Ca}$, is the net result (Lohmann et al., 1991). It is not clear how CNG-1, a channel which has a depolarising effect and allows Ca$^{2+}$ entry, could be directly involved in this ionotropic process.

Carbachol has been shown to inhibit the spontaneous beating rate of cultured neonatal rat cardiac myocytes. This effect was reversibly blocked following the
addition of NO synthase inhibitors and was mimicked by cGMP analogues (Balligand et al., 1993). It appears, therefore, that carbachol is exerting a negative chronotropic effect through cGMP elevation. The NO synthase enzyme identified in myocytes appears to be a constitutively expressed, Ca\(^{2+}\)-calmodulin-activated isoform and CNG-1 could be involved in controlling the activation of this NO synthase. NO synthase may be attached to the plasma membrane by a myristylated N-terminus (see section 1.2.5). If such a membrane-attached NO synthase was present in close proximity to CNG-1, Ca\(^{2+}\) influx through the channel could activate the NO synthase. Production of NO would result in cGMP elevation, activating more CNG-1 channels and providing a positive feedback mechanism. In contrast, a negative feedback mechanism would result from the sensitivity of CNG-1 to Ca\(^{2+}\) and Ca\(^{2+}\)-calmodulin (see fig 6.1). The involvement of a cGMP-gated cation channel in NO synthase activation would provide the control mechanisms required for a signalling pathway which, if not tightly regulated, could have devastating effects.

Fig 6.1 Schematic diagram illustrating the possible mechanism by which CNG-1 maybe involved in the regulation of a myristylated NO synthase in cardiac myocytes. Activation of NO synthase by carbachol results in guanylate cyclase activation and a rise in intracellular cGMP. cGMP is then able to open CNG-1 in a positive feedback mechanism resulting in Ca\(^{2+}\) influx and further activation of NO synthase by Ca\(^{2+}\) (bound to calmodulin). A negative feedback mechanism results when intracellular Ca\(^{2+}\) rises to levels at which CNG-1 is inhibited. Balancing of the positive feedback mechanism against the negative feedback mechanism results in tight regulation of NO production.
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6.1.3 Possible Roles for CNG-1 in Vascular Endothelial Cells

The discovery of CNG-1 expression in vascular endothelial cell lines, described in this thesis, is of particular interest, as recently a number of non-specific cation channel currents have been measured from several vascular endothelial cell types for which the responsible channel protein remains unidentified. For instance, in cultured coronary endothelium, atrial natriuretic peptide (ANP), which activates a receptor with integral guanylate cyclase activity, causes fast depolarisation of the cell membrane (Seiss-Geuder et al., 1993). A cGMP analogue, 8-bromo-cGMP, also caused immediate depolarisation, suggesting that cGMP may be directly opening a cation channel in these cells.

Histamine has been shown to induce an inward mixed cationic conductance in human umbilical vein endothelial cells (HUVECs) (Nilius and Riemann, 1990; Groschner et al., 1994). Nilius and Reimann (1990) observed a non-selective cation current in HUVECs, following stimulation with histamine or thrombin, which was five times more permeable to Na+ and K+ than to Ca2+. They also observed, though much less frequently, a higher conductance non-selective cation current which was about 100 times more permeable to Ca2+ than Na+ or K+. Both of these channels rapidly disappeared following patch-excision, indicating that they may be second messenger-operated. Groschner et al. (1994) also observed histamine-activated, non-selective cation channel currents in HUVECs. First, this agonist causes opening of a calcium-activated potassium channel (KCa) followed by a delayed depolarisation through a mixed inward cation channel which allows a sustained period of calcium entry. The relatively slow time course for the development of this inward current following receptor stimulation implies that second messenger-activation of ion channels is required. The initial KCa current may be acting to increase driving force for subsequent calcium entry through the non-selective channel. However, use of strong calcium chelators in the intracellular solution did not prevent activation of the histamine-induced inward current. The histamine-induced inward Ca2+ current also activates endothelial NO synthase, resulting in intracellular cGMP elevations, so there is a possibility that this mechanism for calcium entry is through a cGMP-gated channel.

LP-805, a releaser of NO in endothelial cells, was found to increase calcium influx into bovine pulmonary artery endothelial cells (BPAECs) through calcium permeant non-specific cation channels (Inazu et al., 1993). The LP-805-induced current was not inhibited by lowering intracellular calcium concentrations and in this case calcium influx resulted in delayed activation of KCa channels which hyperpolarize the membrane, possibly providing a driving force for calcium influx.
Similar non-selective cation channels have been reported to be activated in BAECs following application of bradykinin (Mendelowitz et al., 1992). Interestingly, in BAECs with Ca\(^{2+}\) as the permeant extracellular ion, this current was stable for 1-3 minutes and then declined whilst, when Na\(^{+}\) carried the current, it was sustained over a 10 minute period. This indicates that the channel responsible for this current may be switched off when intracellular Ca\(^{2+}\) concentrations reach a certain level. This kind of property is also displayed by CNG-1 channels.

![Schematic diagram illustrating a hypothetical model for positive feedback activation of the endothelial non-selective cation channel (NSCC) by K\(_{Ca}\). Ca\(^{2+}\) influx through the NSCC results in activation of a neighbouring K\(_{Ca}\) channel. Activation of a K\(_{Ca}\) current results in membrane hyperpolarisation which provides a driving force for Ca\(^{2+}\) entry through the NSCC. The Ca\(^{2+}\) entering the cell through the NSCC is also thought to activate NO synthase. If CNG-1 is responsible for the non-selective cation current observed in vascular endothelial cells further positive feedback would result following activation of the NO signalling pathway.](image)

Fig 6.2 Schematic diagram illustrating a hypothetical model for positive feedback activation of the endothelial non-selective cation channel (NSCC) by K\(_{Ca}\). Ca\(^{2+}\) influx through the NSCC results in activation of a neighbouring K\(_{Ca}\) channel. Activation of a K\(_{Ca}\) current results in membrane hyperpolarisation which provides a driving force for Ca\(^{2+}\) entry through the NSCC. The Ca\(^{2+}\) entering the cell through the NSCC is also thought to activate NO synthase. If CNG-1 is responsible for the non-selective cation current observed in vascular endothelial cells further positive feedback would result following activation of the NO signalling pathway.

A proposed function for these non-selective cation channels in vascular endothelial cells is to activate NO synthase by allowing the entry of Ca\(^{2+}\) into the cell. Ca\(^{2+}\) is an essential messenger in the NO release pathway as it is required for activation of NO synthase. Once synthesised NO diffuses from the endothelial cell to the smooth muscle cell where it activates soluble guanylate cyclases to produce cGMP which results in smooth muscle relaxation through unknown mechanisms. From the results obtained by Groschner et al. (1994) it appears that a soluble guanylate cyclase is also present in endothelial cells, as a cGMP rise is observed in
isolated HUVECs following histamine stimulation. CNG-1 could be responsible for the calcium influx required for NO synthase activation in endothelial cells especially if, as has been reported, an outward $K_{Ca}$ current provides driving force for calcium influx. The provision of such a driving force is an important factor to take into consideration where CNGCs are concerned as under normal conditions they have low conductances for calcium and can be blocked by this cation at positive membrane potentials. As described previously in section 6.1.2 (see fig 6.1), cGMP produced following NO activation of soluble guanylate cyclases could result in further CNGC switch-on providing a positive feed-back pathway. Electrophysiological experiments described in this thesis support this model by showing that the addition of NO donors to HUVECs activates a very small depolarising current which is sensitive to

![Diagram](image)

**Fig 6.3** Schematic diagram illustrating a model signalling pathway whereby the initial cGMP rise required to open CNG-1 is ANP-stimulated. This then results in a cycle of NO synthesis by a membrane-attached NO synthase and cGMP production through opening of CNG-1 similar to the model illustrated in fig 6.1.

intracellular $Ca^{2+}$ levels (Groschner, unpublished observations). However, the fact that CNGCs do have low calcium conductances under most conditions means this
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explanation of function is questionable, although if CNG-1 and a myristylated NO synthase were complexed together within the membrane the local rise in $Ca^{2+}$ may be sufficient to activate the NO synthase. Another problem with this model is that yet another signalling pathway is required for the initial $Ca^{2+}$ influx required to activate NO synthase, resulting in cGMP increases and subsequent opening of CNG-1.

With ANP-initiated responses this is no longer a problem. Activation of ANP-RA by ANP results in activation of the guanylate cyclase domain of ANP-RA, providing the initial cGMP elevation required to open CNG-1 and propagate a cycle of NO synthesis and cGMP production (see fig 6.3). The production of a depolarising current following addition of ANP to cultured coronary endothelium (Seiss-Geuder et al., 1993) supports such a model. However, experiments to determine whether NO is being synthesised following this initial ANP-induced depolarisation of coronary endothelium need to be undertaken to provide further evidence to support this model.

As described in chapter 1 (section 1.2.4), particulate guanylate cyclases, such as ANP-RA, have been suggested to interact closely with the cGMP-gated cation channels expressed in retinal photoreceptor cells (Shyjan et al., 1992; Pardhasradhi et al., 1994). cGMP-gated channels expressed in rat retinal ganglion cells, which are highly homologous to CNG-1, have been shown to be activated following addition of NO donor molecules to isolated ganglion cell populations demonstrating the opening of a cGMP-gated cation channel following stimulation of soluble guanylate cyclases (Ahmad et al., 1994). The NO synthases, required for activation of the retinal ganglion cell soluble guanylate cyclase in vivo, were found to be expressed in neighbouring amacrine cells. Transcripts for ANP-RA were also found to be expressed in the ganglion cell layer of the rat retina although at lower levels than soluble guanylate cyclase transcripts (Ahmad and Barnstable, 1993). Therefore, in retinal ganglion cells there is a strong possibility that both particulate and soluble guanylate cyclases are regulating cGMP-gated cation channels. This may also be the case in vascular endothelial cells, such as HUVECs, where it appears that both particulate and soluble guanylate cyclases are expressed with a cGMP-gated cation channel.

Another possible function for the channel is to provide a fast, controllable mechanism for depolarising the cell membrane and thus allowing activation of voltage-dependent calcium channels. A negative feedback mechanism would be created by rises in internal calcium concentrations causing blockage of the CNGC. However, studies have shown that there are no voltage-dependent calcium channels in BAEcs, BPACs or HUVECs (Adams, 1994) although CNGC depolarisation could be activating other as yet unidentified voltage-dependent channels.
A third possibility is that CNG-1 is providing a mechanism for refilling of depleted calcium stores. Thapsigargin is a pharmacological agent which depletes InsP$_3$-sensitive calcium stores by specifically blocking the endoplasmic reticulum Ca$^{2+}$-ATPase. Thapsigargin-induced depletion of intracellular calcium pools has been shown to activate a non-selective cation channel in isolated HUVECs (Gericke et al., 1993). The characteristics of this current are consistent with the histamine-induced current described previously in that the time-course of current development is slow. In pancreatic acinar cells patch-clamp studies have shown that a sustained inward current of depletion-activated calcium entry is present which is blocked by LY83583, a guanyl cyclase inhibitor (Bahnson et al., 1993). The current can be reactivated following LY83583 inhibition by addition of 8-bromo-cGMP. It is, therefore, possible that a cGMP-gated cation channel is responsible for this replenishment of intracellular calcium stores in acinar cells. CNG-1 could have a similar involvement in refilling of calcium stores in vascular endothelial cells although this seems unlikely due, once again, to the relatively low calcium conductances of CNGCs and in particular CNG-1.

![Fig 6.4 Schematic diagram illustrating the possible mechanism for refilling of intracellular Ca$^{2+}$ stores through activation of a cyclic nucleotide-gated cation channel (CNGC). Binding of an agonist to its receptor (R) results in G-protein (G) activation and phospholipase C (PLC) activation resulting in an IP$_3$ signalling cascade and release of Ca$^{2+}$ from intracellular stores (I.S.). The increase in intracellular Ca$^{2+}$ results in cGMP increases opening a CNGC and allows refilling of Ca$^{2+}$ stores.](image-url)
6.1.4 The Production of Heterologously Co-expressing Models for the Study of CNG-1 Function.

As described above, the function of CNG-1 in cardiovascular tissue is, as yet, unknown, although several possible signalling pathways exist which could involve a cGMP-gated cation channel. There is a great need for heterologously co-expressing cell-lines which provide models for these hypothetical signalling pathways, although, unfortunately, attempts to produce such models, described in this thesis, could not be completed. Comparisons of the responses of cell lines co-expressing CNG-1, ANP-RA, NO synthase and a soluble guanylate cyclase with the responses of native vascular endothelial cells would allow considerable progress to be made in assigning a function to the cardiovascular CNG-1. The possible involvement of ANP-RA in the CNG-1 activation pathway is of particular interest. A recent paper reports the effect of ANP-RA gene knockout in mice (Lopez et al., 1995). Surprisingly, these transgenic mice displayed salt-resistant hypertension. This implies that ANP-RA must exert its major effects at the level of the vasculature, where ANP stimulation has been shown to result in vascular smooth muscle relaxation, and not in the kidney, where it was thought that ANP-RA might be involved in volume regulation through transport of salt into the collecting ducts. Approximately 50% of humans with essential hypertension display the salt-resistant variety of the disease and the results reported by Lopez et al., (1995) indicate that this may be a direct effect of mutations within the ANP-RA gene. If a cGMP-gated cation channel is directly involved in ANP signalling pathways in the vasculature, drugs targeted to this channel could alleviate the symptoms of salt-resistant hypertension. Therefore, future studies investigating the possible coupling of ANP-RA to CNG-1 in vascular endothelial cells will be of great importance in the search for effective treatments for hypertension.
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6.2 Functional Heterologous Co-expression of Inward Rectifier Potassium Channel Subunits

6.2.1 Summary of Results

Experiments to heterologously co-express the rat Kir 3.0 subfamily members GIRK1 and Kir3.4 (or rCKATP or CIR) were carried out, as described in this thesis, in an attempt to resolve conflicting data, published by Ashford et al. (1994) and Krapivinsky et al. (1995), concerning the function of Kir3.4 in cardiovascular tissue. Ashford et al. report that Kir3.4 encodes a subunit of the protein responsible for the K\textsubscript{atp} current in cardiovascular tissue, while Krapivinsky et al. have observed that co-expression of Kir3.4 with GIRK1 results in formation of a heteromultimeric channel showing the characteristics of the native atrial muscarinic K⁺ channel. Data presented in this thesis and summarised below leads us to believe that the protein encoded by Kir3.4 is not responsible for the K\textsubscript{atp} current observed in cardiovascular tissue but is in fact a second subunit of the atrial muscarinic K⁺ channel.

1. If Kir3.4 is a subunit of the atrial muscarinic K⁺ channel one would expect it to be expressed only in the atrial region of the heart. Western blot experiments, described in this thesis, using an antibody designed to the N-terminus of the rat Kir3.4 protein, demonstrated that the distribution of Kir3.4 in rat cardiac tissue was limited to the atria and no protein was detected in the ventricles. This indicated that Kir3.4 was more likely to be a subunit of the atrial muscarinic K⁺ channel than the K\textsubscript{atp} channel which is expressed throughout cardiac tissue.

2. Ashford et al. used HEK293 cells to heterologously express the Kir3.4 protein. However, we found that non-transfected HEK293 cells appeared to contain endogenous voltage-gated K⁺ currents and also barium-sensitive inward rectifier K⁺ currents meaning that HEK293 cells are unsuitable for the electrophysiological analysis of heterologously expressed K⁺ channel proteins.

3. When we heterologously expressed the human isoform of Kir3.4, hcK\textsubscript{ATP}, in the MEL/LCR system fairly small but definitely inward rectifying, barium-sensitive K⁺ currents were observed, but only following the addition of the non-hydrolysable GTP analogue GTP-γ-S. Heterologous expression of rat Kir3.4 also resulted in very small inward rectifying K⁺ currents. This suggested that Kir3.4 was being activated by endogenous MEL cell G-proteins and it is therefore highly likely that Kir3.4, together with GIRK1, forms the G-protein activated, atrial muscarinic K⁺ channel.
4. Previous experiments carried out within the group showed that GIRK1 could be functionally expressed in MEL cells, although the currents observed were small. Further attempts to heterologously express GIRK1 in MEL cells, reported in this thesis, have been unsuccessful. These data support observations made by Krapivinsky et al. (1995) in which they show that GIRK1 can be expressed on its own in *Xenopus* oocytes but not in mammalian cell lines. They suggest that this is because GIRK1 requires the second subunit, supplied by Kir3.4, for functional expression and an isoform of this subunit is expressed endogenously in *Xenopus* oocytes but not in mammalian cell lines. Krapivinsky et al. (1995) also report that Northern blot analysis detects the presence of a mRNA very similar to Kir3.4 in *Xenopus* oocytes. Therefore in oocytes GIRK1 is forming heteromers with this endogenous Kir3.4-like subunit to produce functional channels.

5. When we heterologously co-expressed rat cardiac Kir3.4 with GIRK1 in MEL cells clear inward rectifying K+ currents were produced during whole-cell patch-clamp recordings when GTP-γ-S was present in the pipette solution. These currents were very similar to those reported by Krapivinsky et al. (1995) when they heterologously co-expressed Kir3.4 and GIRK1 in CHO cells. The channel currents produced following heterologous co-expression of these two subunits are far more like native atrial muscarinic K+ channel currents than native K\textsubscript{ATP} channel currents which are inhibited by ATP.

Since these experiments were undertaken cDNA clones belonging to a new subfamily of inward rectifier K+ channels, the Kir 6.0 subfamily, have been isolated. Kir 6.1 was shown to display the characteristics of the native K\textsubscript{ATP} channel but was not expressed in pancreatic β cells where K\textsubscript{ATP} is important in the control of insulin secretion (Inagaki et al., 1995a). Later a cDNA encoding another member of this subfamily, Kir 6.2, was cloned from glucose-responsive insulin-secreting cells (Inagaki et al., 1995b). When Kir 6.2 was heterologously co-expressed with the sulphonylurea receptor, also recently cloned from glucose-responsive insulin-secreting cells (Aguilar-Bryan et al., 1995), a protein complex displaying the main features of the ATP-sensitive K+ current, I\textsubscript{KATP}, was produced (see section 1.3.4). These reports confirm that members of this new inward rectifier subfamily, Kir 6.0, and not Kir 3.4, encode the channel proteins responsible for I\textsubscript{KATP}.

\* Before submission of this thesis the paper published by Ashford et al. (1994) was retracted (Nature 378, 1995, pg 792).
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6.2.2 The Heteromultimeric Nature of Ion Channels

The data presented in this thesis and summarised above clearly show that the atrial muscarinic K⁺ channel is a heteromultimeric channel consisting of at least two subunits, GIRK1 and Kir3.4, both of which belong to the inward rectifier subfamily Kir3.0. An important future experiment would be to produce a cell line heterologously co-expressing both Kir3.4 and GIRK1 and also a muscarinic M2 receptor to demonstrate gating of the channel complex through activation of the muscarinic receptor. Recently, several other ligand-gated ion channels have been shown to exist as heteromultimers. For example, the cGMP-gated cation channel has been shown to consist of an α and β subunit. Expression of the α subunit alone produces a channel which is cGMP-activated but otherwise very different to the native cGMP-gated cation channel. Heterologous co-expression of the α and β subunits in mammalian cell lines produced a channel which was much more like the native channel (see section 1.1.5). More recently the cation-selective P2X receptor channel expressed in sensory neurons was also found to be a heteromultimer consisting of the P2X2 and P2X3 subunits (Lewis et al., 1995). It appears that many native ion channels may actually exist as heteromultimeric proteins. The development of heterologous expression systems in which channel subunits can be co-expressed and subsequently analysed with ease is therefore of considerable importance. The MEL/LCR system has been shown to be very effective for the heterologous co-expression of inward rectifier K⁺ channels. However, this system is not so suitable for the expression of other ion channel families, such as the cGMP-gated cation channel family.

A new system of ‘position independent’ expression, which also employs an LCR-type element, is under development within the group. The LCR element used in this system is the liver-enriched activator protein (LAP) LCR, which is found in the 5' flanking region of the rat LAP gene (Talbot et al., 1994). In this new expression system a 3.5Kb fragment, containing the LAP LCR, has been cloned just upstream of the LAP promoter to create a novel expression vector which also includes an ampicillin-resistance gene and a G418-resistance gene. cDNAs, cloned downstream of the LAP promoter, can be expressed in a position-independent manner in rat cell lines expressing the correct transcription factors. As LAP mRNA has been detected in all rat tissues examined, being expressed constitutively and most abundantly in liver and lung, a number of rat-cell lines may be suitable for heterologous expression of proteins using this system. Each cell-line may be appropriate for the expression of different ion channel families and reconstitution of different signal-transduction pathways. This is a distinct advantage over the β-globin LCR expression system,
where only MEL cells may be used. Another advantage of this new system is that, as LAP is expressed constitutively, no induction procedures are required. Preliminary experiments have shown that Kir2.1 can be expressed successfully, using the LAP LCR expression system, in a rat epithelial cell line (Dr. P. Shelton, unpublished observations). There is no doubt that mammalian expression systems employing LCR elements are by far the most effective. Development of more expression vectors utilising LCRs will increase the versatility of this approach to heterologous co-expression in mammalian cell lines and allow multiple components to be expressed with ease. As it is becoming clear that many receptors and ion channels exist as heteromultimeric proteins, the availability of these techniques is essential in furthering our understanding of these complex signalling molecules.
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Cyclic nucleotide-gated channels are expressed in retinal photoreceptor cells[1] and olfactory neurons[2] and have been shown to play key roles in sensory signal transduction. It is now emerging that a family of CNGCs exists with members also expressed in non-sensory tissues such as kidney, sperm, heart and bone[3,4,5,6]. Recently[7] we cloned from a rabbit sinoatrial node cDNA library a cDNA which is highly related to the rod photoreceptor CNGC. Following this we designed PCR experiments to establish whether channel expression is confined to this pacemaker region or occurs universally throughout heart tissue. PCR primers were designed to the 5' end of the bovine rod photoreceptor CNGC cDNA[1] spanning two intron/exon junctions[8] and used to screen a sinoatrial node cDNA panel.

A CNGC cDNA has been cloned and expressed from rabbit atrial tissue which is highly homologous to the olfactory channel[9]. whereas sequence data from bovine aorta and BAEC PCR fragments show these sequences to be strongly related to the rod photoreceptor channel. This is also the case for a CNGC membrane protein which has been cloned from PCASM. The following primers were designed to the human rod photoreceptor channel's 3' end forward cG-A and reverse primers cG-B, corresponding to bases 469-494 and 794-819 of the published cDNA[1], gave a low yield of amplification product of the size predicted (350bp) from the photoreceptor sequence in each sample. Re-amplification of the product with nested forward and reverse primers cG-C and cG-D, corresponding to bases 505-522 and 740-757 of the published cDNA, gave a 252bp band, as predicted from the photoreceptor sequence, in each sample from the cDNA panel.

A chain of experiments show that a CNGC, probably encoded by the same gene as the rod photoreceptor CNGC, is commonly expressed in cardiovascular tissue. cGMP is an important messenger in cardiovascular tissue. cGMP is an important messenger in cardiovascular tissue.

Fig. 1. The translated protein sequence of 586 amino acids from the porcine coronary artery smooth muscle cGMP-gated cation channel. Primer-detected sequence is indicated by type in bold.

Abbreviations used: CNGC, cyclic nucleotide-gated channel; BAEC, bovine aorta endothelial cell; PCASM, porcine coronary artery smooth muscle.

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Staphylococcal plasmids of the pT181 family replicate via a rolling circle mechanism, initiated by one of the nicking-closing Rep proteins [1]. These Rep proteins become covalently attached to the displaced DNA strand during replication, and following termination have been observed to retain a small oligonucleotide covalently linked to the protein. This Rep adduct has been suggested to be incapable of further initiation of replication [2].

Using the RepD protein variant [3] and a synthetic oligonucleotide corresponding to the replication origin, we have generated this covalent adduct in vitro. Contrary to previous observations with RepC/C*, the RepD/D* adduct appears capable of a topoisomerase-like nicking-closing activity, indicative of potential replication initiation function in vivo.

The generation of RepD/D* in vitro is outlined in Figure 1. A 24-base oligonucleotide, corresponding in sequence to the unique site of phosphodiester bond cleavage, is combined with RepD. Oligonucleotide cleavage is accompanied by covalent attachment of part of the substrate to one subunit of the protein dimer via the active site tyrosine (residue 191) and release of the 5' part of the substrate as a free oligonucleotide. The protein monomer covalently attached to DNA migrates anomalously on SDS-polyacrylamide gel electrophoresis (Figure 2). The reaction is reversible; addition of the 5' 13-base oligonucleotide to the RepD/D* heterodimer regenerates the RepD homodimer.

Purification of the RepD/D* heterodimer (Figure 2) ensures none of the observed activities are attributable to unreacted RepD homodimer. These include the ability of RepD/D* to relax negatively supercoiled pC221, and its non-covalent binding to the replication origin as detected by a gel-shift assay.

RepD homodimer is capable of type I topoisomerase activity with plasmid substrates containing the cleavage site. In the reverse reaction, the 13-base oligonucleotide can be added to RepD/D* to regenerate the RepD homodimer

Figure 1: Cleavage of 24 base oligonucleotide and covalent attachment of one monomer of RepD to the 11 base, 3' part of the substrate. The 5' end is released as a free 13 base oligonucleotide. In the reverse reaction, the 13 base oligonucleotide can be added to RepD/D* to regenerate the RepD homodimer.

In conclusion, RepD/D* has reduced but nonetheless detectable activity in vivo. In vivo the implications are for a modified protein that will have less affinity for its cognate specificity sequence (Figure 4), the calculated binding affinity being at least an order of magnitude lower than that for RepD homodimer. The primary effect of the RepD* modification can therefore be attributed to reduced affinity of the heterodimer for the replication origin, modulating the subsequent cleavage process that initiates replication in vivo or topoisomerisation in vitro.

The topoisomerase activity thus demonstrates the processes required for initiation of replication, namely the cleavage and religation of a specific phosphodiester bond at the origin of replication. Using this assay it is seen that the RepD/D* heterodimer retains some topoisomerase activity, but at a reduced rate of approximately 30-fold less than RepD homodimer (Figure 3). This rate is intermediate between the rates of relaxation of cognate and non-cognate plasmids by RepD homodimer. Regeneration of RepD homodimer from RepD/D* by addition of the 13-base oligonucleotide results in full restoration of activity.

The difference in relaxation rate of cognate and non-cognate plasmids by RepD reflects the difference in affinity for the specificity sequence described above. Preliminary gel shift analysis also shows that RepD/D* has reduced affinity for its cognate specificity sequence (Figure 4), the calculated binding affinity being at least an order of magnitude lower than that for RepD homodimer. The primary effect of the RepD* modification can therefore be attributed to reduced affinity of the heterodimer for the replication origin, modulating the subsequent cleavage process that initiates replication in vivo or topoisomerisation in vitro.

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