Components of Neuronal Calcium Channels and their Interaction with GTP-Binding Proteins.

By

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IV List of Abbreviations

ARA-C          Cytosine β-D arabinofuranoside
AS             Antisense
ATP            Adenosine 5'-triphosphate
BayK 8644      2,6-dimethyl-3-nitro-5-methylcarboxylate-4-(2-tri-
                fluoromethylphenyl)-1,4-dihydropyridine
Co2            Carbon dioxide
CTX            Cholera toxin
DHP            1,4-dihydropyridine
DNA            Deoxynucleic acid
DRG            Dorsal root ganglion neurone
EDTA           Ethylenediamine-tetraacetic acid
EMBL           European Molecular Biology Laboratory
F14/HS         Hamm's F14 medium/horse serum
FITC           Fluorescein isothiocyanate
GABA           γ-aminobutyric acid
GDP            Guanosine 5'-diphosphate
G-Protein      GTP-binding protein
GTP            Guanosine 5'-triphosphate
GTPγS          Guanosine 5'-O-3'-thiotriphosphate
GVI            Grey value intensity
HVA            High voltage-activated current
IP3            Inositol 1,4,5-trisphosphate
KCl            Potassium chloride
Kd             Dissociation constant
Km             Michaelis constant
KH2PO4         Potassium dihydrogen orthophosphate
LVA            Low voltage-activated current
MgCl2          Magnesium chloride
mRNA           Messenger ribonucleic acid
NaCl           Sodium chloride
NaHCO3         Sodium bicarbonate
ODN            Oligodeoxyribonucleotide
PBS            Phosphate-buffered saline
PN200 110      2,6-dimethyl-3-isopropyl-carboxylate-5-carboxethoxy-4-(2,1,3-benzoxadiazole-4-yl)-1,4-dihydropyridine
PKA            Protein kinase A
PKC            Protein kinase C
PTX            Pertussis toxin
VDCC           Voltage-dependent calcium channel
**Abstract**

- Immunocytochemistry utilising antipeptide antibodies and confocal microscopy was used in this study to reveal the localisation of components of voltage-dependent calcium channels in cultured dorsal root ganglion neurones. The \( \alpha_1 \) subunits of the dihydropyridine-, \( \omega \)-Conotoxin GVIA-, and \( \omega \)-Agatoxin-IVA-sensitive calcium channels were found to have different localisations within the soma and neurite processes of dorsal root ganglion neurones. The calcium channel \( \beta \)-subunits were found to have an intracellular localisation, while the \( \alpha_2/\delta \) component was found to be wholly exofacial. Immunoreactivity associated with the \( \alpha \) subunits of the GTP-binding proteins \( G_\text{Q} \) and \( G_\text{I} \) was found to occur on the cytoplasmic surface of the somal membrane and neurite processes in these cells.

- Antisense oligonucleotides were used to transiently suppress the expression of calcium channel \( \beta \)-subunits or \( \alpha \)-subunits of the GTP-binding proteins \( G_\text{Q} \) and \( G_\text{I} \). The antisense oligonucleotides, complementary to a conserved sequence on the mRNA of calcium channel \( \beta \)-subunits or \( G_\text{Q}/G_\text{I} \) \( \alpha \)-subunits, were microinjected into the cytoplasm of dorsal root ganglion neurones and the effect on levels of the target proteins was monitored using confocal microscopy. The calcium channel \( \beta \)-subunit antisense oligonucleotide produced a maximal 90% decrease in \( \beta \)-subunit levels 110 hours after microinjection of the oligonucleotide. In contrast, the GTP-binding protein \( \alpha \) subunit antisense oligonucleotides produced an approximate 80% reduction in levels of \( G_\text{Q} \) and \( G_\text{I} \) 24 hours after microinjection.

- The dihydropyridine calcium channel agonist (-)-BayK 8644 was found to produce an enhancement of GTP hydrolysis in rat frontal cortex membranes, the maximal stimulation of GTPase activity being observed with 10 nM (-)-BayK 8644. Affinity purified anti-GTP-binding protein antibodies, raised against the \( \alpha \) subunit of either \( G_\text{Q} \) or \( G_\text{I} \), revealed that the stimulation of GTPase by (-)-BayK 8644 was due exclusively to activation of the intrinsic GTPase activity of the \( G_\alpha_\text{Q} \) subtype of GTP-binding protein. In contrast, the GABA\(_\text{B}\)-ergic stimulation of GTP hydrolysis was found to be coupled to both the \( G_\alpha_\text{Q} \) and \( G_\alpha_\text{I} \) GTP-binding protein subtypes in this preparation.

An antipeptide antiserum which recognises neuronal calcium channel \( \beta \)-subunits abolished the (-)-BayK 8644-stimulated GTP hydrolysis of \( G_\alpha_\text{Q} \) and also caused a 30% reduction in the dihydropyridine binding capacity of the cortical membranes. Furthermore, a peptide which mimics the \( \beta \)-subunit binding domain on the calcium channel \( \alpha_1 \) subunit, also abolished (-)-BayK 8644-mediated stimulation of GTPase.

The findings from this study suggest that dihydropyridine agonist binding is coupled to an increase in GTPase activity. The calcium channel \( \beta \)-subunit appears to be the principal component of the channel complex involved in linking dihydropyridine agonist binding to enhanced hydrolysis of
GTP by \( G\alpha_\text{Q} \). This provides a possible mechanism by which calcium channels can act to limit the duration of a GTP-binding protein modulatory signal.
Chapter 1

General Introduction
The influx of calcium ions (Ca\(^{2+}\)) into cells is a ubiquitous process that initiates a plethora of intracellular events in cell types ranging from bacteria to specialised neurones. Unlike many other second messenger molecules, Ca\(^{2+}\) is required for life, yet prolonged elevated intracellular Ca\(^{2+}\) levels lead to cell death. Cytosolic Ca\(^{2+}\) levels are therefore maintained at a low concentration (10-100nM) by sequestration of the ions in intracellular compartments such as the endoplasmic reticulum, chelation by Ca\(^{2+}\)-binding proteins, or active extrusion of Ca\(^{2+}\) ions by Ca\(^{2+}\)-ATPase pumps or Na\(^{+}\)-Ca\(^{2+}\) exchange (reviewed in Clapham 1995).

There are several mechanisms by which Ca\(^{2+}\) may enter the cytosol to initiate intracellular signalling pathways (reviewed in Clapham 1995). Ca\(^{2+}\) ions from the two largest Ca\(^{2+}\) pools, the extracellular space and the endoplasmic reticulum (ER), enter into the cytosol across either the plasma membrane or ER, through specialised pore proteins in the membrane called Ca\(^{2+}\) ion channels. In excitable cells the Ca\(^{2+}\) ion channels are voltage-dependent. Depolarisation from the resting membrane potential (approximately -70 mV) initiates a conformational change in the Ca\(^{2+}\)-selective ion channels via special voltage sensing regions of these molecules, which promotes the flood of Ca\(^{2+}\) across the membrane. Since the forces between the chemical and electrical balance for Ca\(^{2+}\) are equal at approximately +150 mV, Ca\(^{2+}\) flows into the cell at all physiological membrane potentials (-90 to +60 mV). Cytosolic Ca\(^{2+}\) levels may also be elevated by release of the ion from intracellular Ca\(^{2+}\) stores. Pools of internal Ca\(^{2+}\) stores are present in the ER, and the release of Ca\(^{2+}\) from these stores is mediated by two separate mechanisms. The first mechanism is by the binding of inositol trisphosphate (IP\(_3\)) to its receptor on the ER membrane. IP\(_3\) is a central regulator of intracellular free Ca\(^{2+}\) that is generated following exposure of cells to a variety of stimuli, including growth factors and neurotransmitters that act through GTP-binding protein-linked receptors. The second mechanism by which Ca\(^{2+}\) is released from internal stores is by Ca\(^{2+}\)-mediated Ca\(^{2+}\) release. In excitable cells Ca\(^{2+}\) entering through voltage-dependent Ca\(^{2+}\) ion channels may directly activate the ryanodine receptor (RynR), the excitable cell counterparts to the IP\(_3\) receptor, to release Ca\(^{2+}\) from intracellular stores. This system is prevalent in skeletal muscle where conformational changes induced by voltage in the Ca\(^{2+}\) ion channel protein result in Ca\(^{2+}\) influx and modulation of the RynR to release Ca\(^{2+}\) from intracellular stores (McPherson & Campbell 1993). Ca\(^{2+}\) may also enter the cytosol following activation of ligand-gated ion channels. Such types of channel include the N-methyl D-aspartate (NMDA) subtype of glutamate receptor. Activation of this receptor by release of glutamate following tetanic stimulation opens the channel allowing Na\(^{+}\) and Ca\(^{2+}\) to enter the cytosolic compartment (reviewed in Ghosh & Greenberg 1995).

As Ca\(^{2+}\) enters the cytosol, it encounters a number of proteins that regulate its biochemical effects. Central among them is calmodulin, a small Ca\(^{2+}\)-binding protein which acts as an
intracellular Ca\(^{2+}\) sensor (Pederson & Carafoli 1987, Hanson & Schulman 1992). Ca\(^{2+}\) -calmodulin binds to a number of enzymes and modulates their activity. These include CaM-dependent protein kinases, protein phosphatases, and adenylate cyclases. These enzymes either act locally by inducing modifications of pre-existing proteins or can mediate more long-term general cellular responses by activating molecules involved in the regulation of gene expression (reviewed in Ghosh & Greenberg 1995). In neurones Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) ion channels also regulates a diverse range of intracellular events, including neurotransmitter release from the pre-synaptic terminal, axonal outgrowth, modulation of synaptic strength and membrane excitability, and influence neuronal survival (Hille 1992).

1.1.1 Biophysical and Pharmacological Classification of Voltage-Dependent Calcium Channels

In many types of excitable cell there is more than one type of voltage-dependent calcium channel (VDCC) as classified by the characteristic properties of the single channel activity that are determined by electrophysiological patch clamp studies. There is a clear distinction between a low-conductance channel activated by moderate depolarisations (low-voltage-activated, LVA) and a high conductance channel activated by large depolarisations (high-voltage-activated, HVA) (Nilius et al 1985, Bean 1985, reviewed in Dolphin 1991). These have been termed T, meaning transient, and L, meaning long lasting, channels respectively, and are pharmacologically, as well as biophysically distinct (Bean 1985). There are several other types of HVA Ca\(^{2+}\) channel, namely N, P, Q and R, as well as a class E Ca\(^{2+}\) channel, which have been classified according to their differential molecular, pharmacological and biophysical properties (reviewed in Dolphin 1995- see Table 1.1)

1.1.2 Low-voltage-activated T-type Ca\(^{2+}\) channels

T-type Ca\(^{2+}\) channels have been observed in cell-attached patches in a variety of cell types (reviewed in Tsien et al 1988). Their biophysical properties are similar between cell types and are stable in excised patch recordings. The single channel conductance of T-type channels, using Ba\(^{2+}\) as the charge carrier, is about 8 pS, the channels show bursting behaviour and are rapidly inactivated by steady depolarisation (Droogmans & Nilius 1989). Inactivation of these channels may be significant at resting membrane potentials, and thus cells must be hyperpolarised to allow their observation.

A number of pharmacological agents have been shown to block T channels, these include octanol (Llinas 1988), amiloride (Tang et al 1988) and phenytoin (Coulter et al 1989).
However, none of these agents are sufficiently specific for T channels to be used as an aid for its purification and cloning.

**High-Voltage-Activated Ca\(^{2+}\) channels**

1.1.3  **High-voltage-activated L-Type Ca\(^{2+}\) channel**

In single-channel studies a large conductance channel of about 20-27 pS can be activated by step depolarisations to about +10 mV (Nilius et al 1985, Fox et al 1987). This channel has been recorded in cell-attached patches in many cell types, including neuronal, cardiac and smooth muscle and gland cells (Tsien et al 1988). The single channel current shows little voltage-dependent inactivation and is characterised by long openings in the presence of dihydropyridine (DHP) agonist drugs (Lacerda & Brown 1989). In contrast, DHP antagonists markedly increase long closures and are thought to promote channel inactivation (Hess et al 1984). Electrophysiological studies have also shown that DHP antagonist activity is promoted at depolarised membrane potentials, which results in an increase in the proportion of time channels remain in the closed state (Hess et al 1984). This has been interpreted as the binding of DHP antagonists to the inactivated state of the channel, possibly by interaction of the DHP with potential-sensitive structural components (Kokubun et al 1986), thereby promoting stabilisation of the channel in the inactivated conformation (Armstrong & Eckert 1987, Hymel et al 1988). In pituitary cells the increased binding affinity of DHP antagonists with depolarisation has been shown to result from an increased association rate; the dissociation rate was found to be independent of membrane potential (Lui et al 1994). That result contrasts with previous studies of the voltage-dependent binding of DHP antagonists to cardiac myocytes, where the increased affinity was reported to be derived from a decreased dissociation rate (Kokubun et al 1986, Wei et al 1989). These results suggest that different modes of action may exist for DHP antagonist interaction with L-type Ca\(^{2+}\) channels in pituitary cells and cardiac myocytes.

1.1.4  **High-voltage-activated N-type Ca\(^{2+}\) channel**

Evidence for a second class of HVA Ca\(^{2+}\) channels, the N-type (for neuronal), was initially obtained from chick DRG neurones, using macroscopic and single-channel current recordings (Fox et al 1987a,b). Evidence for N-type channels has been obtained in several neuronal cell types but not in heart for skeletal muscle. This DHP-insensitive channel corresponds to a component of whole cell current that inactivates more rapidly than the L-type current (Fox et al 1987). The channel also exhibits brief openings and has an intermediate single-channel conductance of 13 pS. Later work found a higher single channel
conductance for the N-type channel of 18 pS (Plummer et al 1989) and showed that it could switch between inactivating and non-inactivating modes, possibly by reorientation of an inactivating component (Plummer & Hess 1991).

The N-type Ca$^{2+}$ channel is proposed to be the major component of the whole cell HVA Ca$^{2+}$ current in sympathetic neurones (Plummer et al 1989) and can be irreversibly inhibited by ω-conotoxin GVIA (ω-CTX GVIA), a toxin derived from the cone shell mollusc *Conus geographus* (Fox et al 1987, Tsien et al 1991).

7.5

and Q-types of high-voltage-activated Ca$^{2+}$ channel

HVA Ca$^{2+}$ channel currents which are insensitive to both DHPs and ω-CTX GVIA have been reported in many tissues (Llinas et al 1989, Plummer et al 1989, Scroggs & Fox 1991). In the cerebellar Purkinje cell, where there is only a small proportion of N-and L-type Ca$^{2+}$ channels, the non-inactivating HVA is termed the P-type Ca$^{2+}$ channel (Llinas et al 1989). The P-type channel is sensitive to a peptide toxin, ω-agatoxin IVA (w-Aga IVA), which is derived from the venom of the funnel web spider *Agelenopsis aperta* (Mintz et al 1992). This toxin also inhibits a P-type component in hippocampal, cortical, spinal cord and sensory neurones although there is no effect in sympathetic neurones, where the principal component of the HVA current is the N-type channel (Mintz et al 1992).

An additional non-L, non-N and non-P type HVA Ca$^{2+}$ channel has been reported in cerebellar granule neurones (Zhang et al 1993). This channel current is termed Q-type, and is characterised by its sensitivity to ω-conotoxin MVIIC (ω-CTX MVIIC) from *Conus magus* (Hillyard et al 1992), its large conductance and rapid inactivation kinetics. It has however also been reported that ω-CTX MVIIC blocks N- and P-type Ca$^{2+}$ channel currents in sympathetic neurones and Purkinje cells respectively (Schwartz et al 1993). The Q-current is also sensitive to ω-Aga IVA, at concentrations > 100 nM (Wheeler et al 1994). Following blockade of the L, N, P and Q type Ca$^{2+}$ channel currents with their selective antagonist drugs and toxins, a proportion of HVA current persists in cerebellar granule neurones (Zhang et al 1993). This rapidly inactivating current has been termed the R-type.
Table 1.1 Biophysical and Pharmacological Properties of $\text{Ca}^{2+}$ channels

(Taken from Dolphin 1995)

<table>
<thead>
<tr>
<th>Functional Class</th>
<th>Type</th>
<th>Cloned $\alpha_1$ subunit</th>
<th>Pharmacology</th>
<th>Voltage-dependent Inactivation</th>
<th>Single Channel Conductance (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>HVA</td>
<td>$\alpha_1_S$ (skeletal muscle)</td>
<td>DHPs</td>
<td>None (Ca$^{2+}$ dependent)</td>
<td>20-24</td>
</tr>
<tr>
<td>N</td>
<td>HVA</td>
<td>$\alpha_1_B$ (brain, PC12 cells)</td>
<td>$\omega$-CTX GVIA</td>
<td>Intermediate</td>
<td>13-20</td>
</tr>
<tr>
<td>P</td>
<td>HVA</td>
<td>$\alpha_1_A$ ? (brain, heart)</td>
<td>$\omega$-Aga IVA</td>
<td>None</td>
<td>10-18</td>
</tr>
<tr>
<td>Q</td>
<td>HVA</td>
<td>$\alpha_1_A$ ? (brain)</td>
<td>$\omega$-CTX MVIIC</td>
<td>Intermediate</td>
<td>?</td>
</tr>
<tr>
<td>R</td>
<td>HVA/LVA</td>
<td>$\alpha_1_E$ ? (brain)</td>
<td>None known</td>
<td>Fast</td>
<td>?</td>
</tr>
<tr>
<td>T</td>
<td>LVA</td>
<td>$\alpha_1_E$ ? (brain)</td>
<td>Ni$^{2+}$, octanol, amiloride</td>
<td>Fast</td>
<td>7-9</td>
</tr>
</tbody>
</table>

1.2 $\text{Ca}^{2+}$ Channel Purification and Structure

1.2.1 Purification of the HVA L-type $\text{Ca}^{2+}$ channel

The $L$-type $\text{Ca}^{2+}$ channel was first purified from rabbit skeletal muscle since this preparation contains functional $L$-type channels (Sanchez & Stefani 1978) and a high density of DHP receptors (Gould et al 1984). Due to the ability of DHPs to affect channel activity, it was assumed that the DHP receptors were components associated with the channel. The high affinity of DHP binding provided a marker with which to follow purification of the DHP receptor. Skeletal muscle t-tubules were labelled with tritiated DHPs, solubilised and purified using wheat germ agglutinin chromatography, anion exchange chromatography and sucrose gradient centrifugation (Flockerzi et al 1986a, Striessnig & Glossmann 1991, Florio et al 1992, Schnieder et al 1992). The $\text{Ca}^{2+}$ channel activity of the purified DHP receptors was monitored by reconstitution of the receptors into lipid bilayers (Flockerzi et al 1986b). Three proteins were found to exist in the purified preparation, termed $\alpha$, $\beta$ and $\gamma$, although subsequent studies revealed the presence of two $\alpha$. 
subunits (Leung et al 1987, Vaghy et al 1987) - Figure 1.1. Azidopine photolabelling studies showed that the DHP binding subunit was a 165-190 kDa protein, whose mobility in SDS-PAGE gels was not affected by reducing agents. The other protein at 170 kDa, α2/δ, dissociates under these conditions, increasing its mobility to 142 kDa (α2) and 22-28 kDa (δ). In addition, the purified preparation contained a 55 kDa β subunit and a 32 kDa γ subunit. Purification of the cardiac L-type Ca\(^{2+}\) channel has revealed the presence of an α1 subunit (165-195 kDa), α2/δ which dissociate under reducing condition to α2 (140 kDa) and δ (20-30 kDa), and a β subunit (Chang & Hosey 1988, Schnieder & Hofmann 1988, Haase et al 1991, Hell et al 1993b).

1.2.2 Purification of the HVA N-type Ca\(^{2+}\) channel

The high affinity of ω-CTX GVIA for N-type Ca\(^{2+}\) channels was used as a tool for the purification of N-type channels from brain (McEnery et al 1991, De Waard & Campbell 1993). The N-type Ca\(^{2+}\) channel appears to have a subunit composition similar to that of the cardiac L-type Ca\(^{2+}\) channel, consisting of three components α, α2/δ and β, which co-purify with a 95-100 kDa subunit, whose identity remains as yet unknown (McEnery et al 1991, De Waard et al 1994, McEnery et al 1994). The N-type Ca\(^{2+}\) channel also co-purifies with the α subunit of the GTP-binding protein G\(_{0}\) (McEnery et al 1991) and with a number of proteins involved in the synaptic vesicle secretory process such as syntaxin and synaptotagmin (Leveque et al 1994)

1.3 Cloning of Ca\(^{2+}\) Channel subunits

1.3.1 Skeletal muscle L-type Ca\(^{2+}\) channel α1 subunit

Following purification of the DHP receptor from skeletal muscle, cDNA clones were obtained using primers derived from the amino acid sequence of proteolytic fragments (Tanabe et al 1987). The deduced sequence contains 1873 amino acids, and hydropathy plots indicated a structure consisting of twenty four transmembrane segments arranged as four repeated domains. Secondary structure analysis also predicts that the amino- and carboxy-termini are located inside the cell, and that there are long cytosolic loops that connect each domain. The proposed model for the membrane arrangement of the α1 subunit polypeptide of the skeletal muscle L-type Ca\(^{2+}\) channel (α1s) is presented in Figure 1.2. Expression of the skeletal muscle α1 subunit in mouse L-cells led to the appearance of functional channels which were sensitive to DHPs (Perez-Reyes et al 1989), providing evidence that the α1 subunit is capable of forming a functional ion pore and conferring DHP sensitivity.
Figure 1.1 Model of the subunit structure of the skeletal muscle L-type calcium channel. P denotes phosphorylation site.

Taken from Dolphin 1995
Figure 1.2 The putative membrane arrangement of the cloned calcium channel subunits. The green segments are the voltage-sensing S4 regions. The shaded areas on the intracellular domain between I and II represents the binding site for the β-subunit.
1.3.2 Other L-type Ca\textsuperscript{2+} channel \(\alpha 1\) subunits

Four additional types of Ca\textsuperscript{2+} channel \(\alpha 1\) subunit have been identified by homology cloning in a number of different tissues including brain (Hui et al 1991, Starr et al 1991, Dubel et al 1992, Collin et al 1993). Secondary structure analysis predicts all of these \(\alpha 1\) subunit polypeptides to have a similar membrane arrangement to the skeletal muscle L-type channel \(\alpha 1\) subunit (Figure 1.2). The two distinct clones that code for DHP-sensitive channels have been termed C and D (reviewed in Perez-Reyes & Schneider 1994). The C-type (\(\alpha 1C\)) is present in cardiac tissue, but also in excitable tissue including brain. Alternative splicing of a single gene produces distinct isoforms of the C-class channel. The D-type clone (\(\alpha 1D\)) has 70% homology with the C-clone but has a different tissue distribution, being predominantly expressed in neurosecretory tissue but also in kidney and in trace amounts in cardiac tissue. Both the C- and D-clones exhibit DHP sensitivity when expressed in *Xenopus* oocytes (Mikami et al 1989, Schultz et al 1993, Williams et al 1992b). However, the channel encoded by the D-clone was also partially blocked by a high concentration of \(\omega\)-CTX GVIA (Williams et al 1992b), although this may be due to block of an endogenous oocyte channel by \(\omega\)-CTX GVIA (Lacerda et al 1994).

1.3.3 N-type Ca\textsuperscript{2+} channel \(\alpha 1\) subunit

The neuron specific B-clone (\(\alpha 1B\)) (Williams et al 1992a, Dubel et al 1992) encodes for the N-type Ca\textsuperscript{2+} channel, since when the \(\alpha\) subunit of the B-clone was expressed in HEK-293 cells, dysgenic myotubes and *Xenopus* oocytes (Williams et al 1992a, Fujita et al 1993, Stea et al 1993) the resulting currents were sensitive to \(\omega\)-CTX GVIA. Each of the aforementioned expression systems produced currents with different activation kinetics, these discrepancies may be explained by differences in handling of post-translational modification processes in each of the expression systems, or single base mutations in cDNA.

1.3.4 Other Ca\textsuperscript{2+} channel \(\alpha 1\) subunits

Two additional clones have been identified in neuronal tissue, the A-clone and E-clone (Mori et al 1991, Starr et al 1991, Soong et al 1993). Expression of the A-type clone (\(\alpha 1A\)) in oocytes results in a current that can be inhibited partially by \(\omega\)-Aga IVA, and more potently by \(\omega\)-CTX MVIIC (Zhang et al 1993). The \(\alpha 1A\) clone may represent either the P- or Q-type Ca\textsuperscript{2+} channel current identified in cerebellar Purkinje (Mintz et al 1992) and granule neurones (Zhang et al 1993). The A-clone has about 33% homology with the L-type
The brain E-clone (Soong et al 1993) has a counterpart in the marine ray *Discopyge ommata*, termed doe-I (Horne et al 1993), and gives rise to a rapidly activating and inactivating current when expressed in oocytes that is insensitive to all Ca\(^{2+}\) channel antagonist drugs and toxins (Ellinor et al 1993, Soong et al 1993). Both doe-I and \(\alpha_{1E}\) exhibit sensitivity to Ni\(^{2+}\), and show activation at hyperpolarised potentials. Soong et al (1993) suggests that \(\alpha_{1E}\) encodes a low-voltage-activated channel (T-type). However, T-type channels activate at more hyperpolarised levels than \(\alpha_{1E}\) (Tsien et al 1988), and have a single channel conductance of 8 pS, while the \(\alpha_{1E}\) currents have a conductance of 14 pS (Schneider et al 1994). Zhang et al (1993) therefore propose that \(\alpha_{1E}\) is the electrophysiological counterpart to the residual HVA R-type current found in cerebellar granule neurones. The \(\alpha_{1E}\) clone is found in most regions of the central nervous system, and within the granule and Purkinje layers of the cerebellum (Soong et al 1993).

### 1.3.5 Structure of \(\text{Ca}^{2+}\) Channel \(\alpha\) subunit polypeptide

Conceptual translation of each of the VDCC \(\alpha\) subunit cDNAs has revealed a generalised secondary structure which is extremely similar to the proposed secondary structure of Na\(^{+}\) and K\(^{+}\) channels (reviewed in Catterall 1993). The amino acid sequence of the rabbit skeletal muscle L-type Ca\(^{2+}\) channel \(\alpha\) subunit has 55% similarity to the primary sequence of the Na\(^{+}\) channel \(\alpha\) subunit, and like the Na\(^{+}\) channel, the L-type VDCC \(\alpha\) subunit consists of four transmembrane motifs (I-IV), each motif being comprised of six hydrophobic segments (S1-S6). The N- and C-termini are located intracellularly (Tanabe et al 1987). The fourth hydrophobic segment (S4) of each transmembrane motif contains a positively charged amino acid in every third or fourth position. It has been demonstrated experimentally for Na\(^{+}\) and K\(^{+}\) channels that this S4 segment may be the voltage sensor for voltage-dependent channels (Stuhmer et al 1989). Computer modelling of the voltage-dependent ion channel \(\alpha\) subunits has identified a putative extracellular loop between S5 and S6 in all four transmembrane domains (Guy & Conti 1990). This region has been proposed to exist as an \(\alpha\)-helical hairpin loop (SS1) connected to a \(\beta\)-structure (SS2), bent back into the membrane, via strict consensus sequences that may form a bend structure in the SS2 segment (Varadi et al 1995). This region has been termed the SS1-SS2 or P-loop, and is thought to form the lining of the channel pore. Mutation of this conserved region in \(\text{Na}^{+}\) channels has suggested a role for SS1-SS2 in the regulation of ion conducting pathways (Heinemann et al 1992). By analogy to the Na\(^{+}\) channel, the SS1-SS2 region has suggested to have a similar role in Ca\(^{2+}\) channels. The strict alignment of negatively charged Glu residues in the SS2 segment of each transmembrane motif, indicates that these chains may impart selectivity for Ca\(^{2+}\) permeation. Mutation of individual Glu residues, particularly those occurring in transmembrane motif III, into either positively
charged or uncharged residues decreased the Ca$^{2+}$ selectivity of the L-type calcium channel ion conducting pathway (Tang et al 1993 Yang et al 1993). The latter authors suggested that the Ca$^{2+}$ selectivity was conferred by the four Glu residues located on the SS2 region, arranged on two close, but non-equivalent, planes and occupying trans positions. The proposed tertiary model of the $\alpha 1$ subunit suggests that the transmembrane segments (S1-S6) of each domain are arranged in the membrane to form a pore that is lined by the SS1-SS2 motifs that link the S5 and S6 regions of each domain (Guy & Conti 1990). The other segments are thought to form the outer part of the channel (Figure 1.3).

The differences between skeletal muscle and cardiac $\alpha 1$ subunits have been examined by the construction of chimeras (Tanabe et al 1990, Tanabe et al 1991). These results identified the large intracellular loop of the skeletal muscle channel, between domains I and II, as being involved in excitation-contraction coupling. The molecular determinants responsible for the activation kinetics of the Ca$^{2+}$ channel were assigned to domain I of skeletal and cardiac $\alpha 1$-subunits (Tanabe et al 1991). The critical activation region has since been narrowed down to the third segment of transmembrane motif I (IS3) and its associated connecting loop (Nakai et al 1994). More recently, Wang et al (1995) have reported that repeats III and IV of $\alpha 1_{\text{C}}$ from carp skeletal muscle possess structural determinants for slow activation of L-type Ca$^{2+}$ channels. It has also been shown that the sixth segment of transmembrane motif I (IS6) and its surrounding regions are critically important determinants of inactivation (Zhang et al 1994).

The binding sites for DHPs appears to be accessible from the extracellular side of the membrane based on photoaffinity labelling studies (Striessnig et al 1991). The study by those authors suggests the involvement of the SS1-SS2 hairpin between S5 and S6 of domain III, and also part of the transmembrane portion of S6 of domain III and, to a smaller extent, the same region in domain IV as sites of DHP interaction. In contrast, studies using chimeric Ca$^{2+}$ channels have shown that the S5 and S6 linker in motif IV is critical for DHP action, and that the SS2-S6 linker region of motif III, although not involved in DHP action, may play a role in the inactivation process of Ca$^{2+}$ channels (Tang et al 1993). The phenylalkylamine class of L-type Ca$^{2+}$ channel antagonists inhibit the channels by binding, in cationic form, to an intracellular receptor site (Hescheler et al 1982). This binding is greatly enhanced by repetitive activation of the channel (Streissnig et al 1990). Photoaffinity labelling studies show that the phenylalkylamine-binding site is associated only with the IVS6 region and an adjacent intracellular region on the C-terminus (Streissnig et al 1990), in agreement with these antagonists blocking from inside the channel.

The C-terminal tail of the cardiac $\alpha 1$ subunit has a role in controlling the probability of channel opening, since deletions in this region enhance current amplitude (Neely et al 1995). The C-terminal tail may normally inhibit a structural component of the channel, and this is relieved by phosphorylation or removal of the tail. Internal exposure to trypsin can
also cleave the tail and this results in an increase in current (Hescheler & Trautwein 1988). There is also a Ca$^{2+}$-binding domain at the beginning of the C-terminal tail, which may be responsible for Ca$^{2+}$-dependent inactivation (Babitch 1990). N-type Ca$^{2+}$ channels are modulated by G proteins (see Section 1.2-1.3 this Chapter) and it has also been suggested that the C-terminus of the $\alpha_{1B}$ polypeptide may stabilise the G protein-channel interaction or the conformational changes induced in the channel following binding of G protein (Zhou et al 1995).
Figure 1.3 A three-dimensional model for the calcium channel α1-subunit

Calcium ions
1.3.6 β-subunits

The skeletal muscle β-subunit (β1) was first cloned by Ruth et al (1989) and the deduced molecular weight is 55 kDa. Secondary structure analysis suggests that the protein contains four α-helical domains (I-IV). These helices contain many charged amino acid residues, indicating that they are probably not membrane spanning regions (Figure 1.2). The deduced amino acid sequence also contains heptad repeats within domains II, III and IV, in which most of the first and fourth residues of every seven are hydrophobic. Such heptad repeats of hydrophobic residues are thought to participate in interactions between cytoskeletal proteins (Fuchs & Hanakoglu 1983). These structures within the β-subunit may have a role in linking the α1-subunit onto the cytoskeleton. Each α-helical domain contains a similar stretch of eight amino acids, which contain a high proportion of negatively charged acidic residues that may function in the binding of divalent ions (Ruth et al 1989).

A number of different β-subunits have been cloned and the nomenclature (with most prevalent distribution in parentheses) is B1α (skeletal muscle; Ruth et al 1989), B1β (brain; Pragnell et al 1991), B2 (heart, lung, brain; Perez-Reyes et al 1992), B3 (brain, smooth muscle; Castellano et al 1993a) and B4 (brain, particularly cerebellum; Castellano et al 1993b).

Coexpression of β subunits with the α1 subunit leads to profound differences in the expression of functional channels and their biophysical behaviour. Coexpression of β1 with cardiac α1C leads to an increase in the number of DHP binding sites (Perez-Reyes et al 1992) and it increases the expressed current (Wei et al 1991). β1 was also found to accelerate activation and shift the voltage-dependence of activation to hyperpolarising potentials (Wei et al 1991). Coexpression of β1 with N-type α1B in oocytes also led to increases in the expressed current amplitude and alteration of kinetics (Stea et al 1993). However, no effect was observed on the voltage-dependence of activation. Studies in HEK-293 cells have shown that β1 increases the number of α-CTX GVIA receptors (Brust et al 1993). Similarly, coexpression of β2 with α1C increases the expressed current amplitude, shifts the voltage-dependence of activation and accelerates activation kinetics (Perez-Reyes et al 1992). The effects of β3 have been tested on α1C- and α1A-induced currents in oocytes (Castellano et al 1993b, Sather et al 1993). In both systems β3 affects the current in a similar manner as other Bs; there is an increase in the measured current, a hyperpolarising shift in the activation, and an acceleration of the kinetics of activation and inactivation. In addition to altering the inactivation kinetics, β3 decreased the plateau current attained during long pulses, suggesting that β3 alters the equilibrium between open and inactive states. β4 modulates α1C activity in a similar manner as β3 (Castellano et al 1993a). The increase in the expressed whole-cell current observed following coexpression of β subunits led to the conclusion that the Bs increase the number of functional channels on the cell
surface since the increase in current density correlated with an increase in the number of high affinity DHP binding sites (Lacerda et al 1991, Perez-Reyes et al 1992, Castellano et al 1993a). Studies of gating movement have reached an alternative conclusion (Neely et al 1993). Coexpression of β2 did not increase the amount of charge movement attributed to α1C, although it did increase the inward current. Based on the assumption that the measured gating current reflects the number of expressed channels Neely et al (1993) proposed that βs facilitate the conformational change that occurs between voltage sensing and channel opening. In a later study the same authors proposed that multiple modes of gating are encoded in the α1 subunit and that the β-subunit increases Ca²⁺ channel current by favouring a willing mode of gating in which the final transitions leading to channel opening are facilitated (Neely et al 1995).

1.3.7 Molecular Nature of α1-β Subunit Interaction

The allosteric interface which links the Ca²⁺ channel α1 subunit with the auxiliary β subunit has been identified as a sequence on the cytoplasmic loop between transmembrane domains I and II of the α1 subunit polypeptide (Pragnell et al 1994). This region is conserved in all six Ca²⁺ channel α1 subunits as a consensus sequence for binding of the β subunit. Mutations within this sequence of the α1A channel reduced the stimulation of peak current density by the β subunit and altered the inactivation kinetics and voltage-dependence of activation. A 30 amino acid domain of the β subunit, that is located in the N-terminus and is highly conserved for all known β subunits, was defined as being critical for the α1-β interaction (De Waard et al 1994). Modifications in this region interfered with the stimulation of Ca²⁺ currents by the β subunit. This domain was shown to be a prerequisite for β subunit-induced kinetic changes and the voltage-dependence of steady state inactivation (De Waard et al 1994). Olcese et al (1994) suggest that regulation of activation and inactivation of α1 by β are separable events and that the N-terminal region of β is a structural determinant important in determining the rate and voltage at which an α1 inactivates.

1.3.8 α2/δ Subunits

The α2 subunit is a large glycoprotein that binds to wheat-germ agglutinin affinity columns. It was this property, and the ability of digitonin to solubilise the Ca²⁺ channel as a complex, that allowed purification of α1.

The α2/δ subunit cloned from rabbit skeletal muscle and human brain has a deduced molecular weight of 125 kDa (Ellis et al 1988, Williams et al 1992b). The δ-subunit sequence is identical to the C-terminal region of the α2 cDNA, indicating that the α2- and δ
subunits are both the product of the same gene, and arise by post-translational processing (De Jongh et al 1990). Two models exist for the membrane arrangement of the α2/δ polypeptide (Ellis et al 1988, Jay et al 1991). The model proposed by Ellis et al (1988) suggest that this subunit contains two transmembrane regions, an intracellular loop containing two putative phosphorylation sites, and large extracellular N- and C-terminal domains. The second model (Jay et al 1991), suggests that the entire α2 polypeptide is entirely exofacial, being anchored to the membrane via disulphide interaction with the δ component which has a single transmembrane spanning domain. Immunocytochemical results presented in Chapter 3 of this Thesis provide evidence in agreement with the model of Jay et al (1991).

Most tissues appear to contain the same α2/δ protein, although a splice variant has been found in rat brain (Kim et al 1992). α2/δ has been shown to increase α1C currents with no apparent changes in the properties of the channel (Mikami et al 1989, Biel et al 1990). Coexpression of α2/δ with N-type α1B had little effect on the expressed currents in oocytes, while β1b increased the current amplitude and kinetics (Stea et al 1993). A synergistic increase in current was observed when the α1Bα2/δβ1b complex was expressed. Similarly, in HEK-293 cells, coexpression of all three subunits led to the largest currents and highest expression of α-CTX GVIA receptors (Brust et al 1993).

1.3.9 The γ subunit

The γ subunit was purified from skeletal muscle as a complex with the DHP receptor. The deduced amino acid sequence encodes a protein of 25 kDa, which contains four putative membrane spanning regions (Bosse et al 1990).

The γ-subunit does not appear to be present in any tissue other than skeletal muscle. Expression studies have not determined what role γ plays in the Ca^{2+} channel complex, although its exclusive expression in skeletal muscle suggests that it may have a role in excitation-contraction coupling.
1.4.1 Cellular Signalling

Cellular signalling requires both extracellular signalling molecules and a complementary set of receptor proteins in each cell that bind the signalling molecules and respond to them in a programmed and characteristic manner. Some small hydrophobic signalling molecules, such as the steroid and thyroid hormones and retinoids, diffuse across the plasma membrane of the target cell and activate intracellular receptor proteins, which directly regulate the transcription of specific genes. Some dissolved gases, such as nitric oxide, act as local mediators by diffusing across the plasma membrane of the target cell and activating an intracellular enzyme-usually guanylyl cyclase, which produces elevated cyclic GMP levels in the target cells. Most extracellular signalling molecules are hydrophilic and are able to activate receptor proteins only on the exofacial surface of the target cell; these receptors act as signal transducers, converting the extracellular binding event into intracellular signals that alter the behaviour of the target cell.

The receptors for many hormones, such as catecholamines, gonadotrophins, parathyroid hormone and glucagon, odorants and light consist of seven hydrophobic transmembrane domains (Dohlman et al 1991). Stimulation of these receptors activates a group of intracellular coupling proteins called GTP-binding proteins (G proteins) that, in turn, regulate a variety of enzymes and ion channels. The target enzymes or ion channels are called effectors since changes in their activity effect changes in the ionic composition or in cytosolic second messenger levels (such as cAMP or inositol phosphate levels) that ultimately lead to the cellular response.

1.4.2 The G Protein Cycle

G proteins consist of three polypeptides: an α subunit that binds and hydrolyses GTP, a β subunit, and a γ subunit. The β and γ subunits form a dimer that only dissociates when it is denatured and is, therefore, a functional monomer. In its GDP-bound form, the α subunit associates with the βγ subunit to form an inactive heterotrimer that interacts with the receptor with low affinity. The interaction becomes high affinity when the receptor binds agonist (Figure 1.4) (reviewed in Clapham & Neer 1993, Neer 1994). Both the α and βγ subunits can interact with the receptor. Following activation of a G protein linked receptor a conformation change occurring in the receptor molecule results in a conformational change in the G protein α subunit, causing a decrease in GDP affinity, so that GDP is released from the active site. Since the concentration of GTP in cells is much higher than that of GDP, the released GDP is replaced with GTP. Once GTP is bound the α subunit assumes its activated conformation and dissociates from both the receptor and from βγ. In the activated state the α subunit proceeds to interact with and activate effector molecules. Such effectors include
ion channels and enzymes that generate regulatory molecules or second messengers. Low
molecular weight second messengers such as cyclic adenosine monophosphate (cAMP) and
inositol trisphosphate, in turn, generate dramatic intracellular changes including selective
protein phosphorylation, gene transcription, cytoskeletal reorganization, secretion and
membrane depolarisation. The activated state lasts until the GTP is hydrolysed to GDP by
the intrinsic GTPase activity of the $\alpha$ subunit. All isoforms of $\alpha$ subunits are GTPases,
although the intrinsic rate of GTP hydrolysis varies from one type of $\alpha$ subunit to another
(Carty et al 1990, Linder et al 1990). The GTPase activity can be stimulated following
activation of a G protein-linked receptor. Such an enhancement of GTPase has been
observed following activation of the G protein linked dopamine D2 receptor in the rat
striatum (Onali & Olianas 1988), muscarinic M2 receptor in the rat brain (Hoss et al 1990)
and kainate receptor in the goldfish brain (Ziegra et al 1992). Activation of GABA$_B$ and
adenosine receptors has also been shown to increase GTPase activity in rat frontal cortex
(Sweeney & Dolphin 1992, Sweeney & Dolphin 1995). The latter authors reported that the
neurotransmitter-mediated stimulation of GTPase was due to the increased rate of guanine
nucleotide exchange since GABA$_B$ and adenosine receptor activation promoted an increase
in binding of the non-hydrolysable GTP analogue GTP$\gamma$S. Alternatively, the intrinsic
GTPase activity can be potentiated directly by an effector protein acting as a GTPase-
activating protein (GAP) and this is thought to represent a cellular mechanism to accelerate
G protein deactivation. Hydrolysis of GTP bound to isolated G proteins is slow when
measured in vitro. The $t_{1/2}$ for hydrolysis is 10-20 sec for most G proteins (reviewed in
Gilman 1987), 50 sec for Gq/11 (Strathmann & Simon 1990) and >7 min for Gz (Casey
et al 1990). Although the deactivation of Gs-stimulated adenylate cyclase following hormonal
stimulation, agrees with the measured rate of GTP hydrolysis by Gs (Brandt & Ross 1986),
the deactivation rates of other G protein-regulated effectors are considerably faster than the
rates at which their isolated G proteins hydrolyses GTP. The photoreceptor G protein,
transducin, hydrolyses GTP with a $t_{1/2}$ of 15 sec (Navon & Fung 1984), but the
deactivation of transducin-stimulated cGMP phosphodiesterase takes less than 1 sec
(Arshavsky et al 1991). Those authors suggest that cGMP phosphodiesterase directly
stimulates the intrinsic GTPase activity of the photoreceptor G protein transducin in
amphibian (Arshavsky & Bownds 1992) and bovine (Arshavsky et al 1994) rod outer
segments to deactivate the transducin modulatory signal. Moreover, the intrinsic GTPase
activity of the G proteins $G_{q/11}$ and $G_{\alpha_o}$ are stimulated by their respective effectors,
phospholipase C-$\beta$1 (Berstein et al 1994) and possibly the L-type Ca$^{2+}$ channel (Sweeney
& Dolphin 1992). Neither phospholipase C$\beta$1 or the activated L-type Ca$^{2+}$ channel promote
increased binding of GTP$\gamma$S (Bernstein et al 1992, Sweeney & Dolphin 1992). Those
authors suggest that the effector molecules increase GTP hydrolysis independently of GDP-
GTP exchange, rather, the effectors directly stimulate the intrinsic GTPase activity of the

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activated G protein by acting as GAPs. This would provide a mechanism to limit the temporal effectiveness of the G-protein modulating signal.

Once GTP is cleaved to GDP, the α subunit and βγ subunits reassociate, become inactive (i.e. unable to activate the effector) and return to the receptor.

Figure 1.4 Associations of three conformations of Qα with receptor, βγ, and effector during the GTPase cycle
R*, receptor; E*, effector.
1.5 Subtypes of G protein α subunits

Over 20 different G protein α subunits have been isolated from mammalian cells (16 gene products, some with alternatively spliced isoforms-Simon et al 1991). The proteins may be divided into several major classes according to the similarity of their sensitivity to bacterial toxins. Bacterial toxins from *Vibrio cholera* or *Bordetella pertussis* can covalently modify G proteins by the addition of an ADP-ribose group to the α subunit. Both toxins are enzymes which transfer ADP-ribose from NAD to specific acceptor sites on the G proteins (reviewed in Neer & Clapham 1988). Susceptibility to ADP-ribosylation divides the G proteins into three broad groups; substrates for cholera toxin (CTX) only, pertussis toxin (PTX) only, both toxins, or neither toxin. Initially the CTX substrates were thought to stimulate adenylyl cyclase and the PTX substrates to inhibit adenylyl cyclase. They were therefore named Gs and Gi respectively. The third category of G proteins, the transducins and gustducins, which are involved in light and taste transduction, are substrates for both toxins.

1.5.1 Gαs
Gαs is involved in hormonal stimulation of adenylyl cyclase (Cassel & Selinger 1977) and opening of cardiac and skeletal muscle L-type Ca\(^{2+}\) channels via both membrane delimited (Yatani et al 1987, Yatani et al 1988) and second messenger mediated pathways (Brum et al 1984, Tsien et al 1986). Gαs is a substrate for the bacterial cholera toxin (CTX) which ADP-ribosylates the α subunit of Gs at Arg-201 to promote persistent activation of adenylate cyclase by reduction of GTPase activity. Four Gαs cDNAs have been isolated from human brain (Bray et al 1986). The four Gαs clones are generated from alternative splicing during transcription of a single Gαs gene to produce two long forms (52 kDa) and two short forms (45 kDa) of Gαs.

Gαs is expressed in a variety of tissue and cell types including human liver (Mattera et al 1986), bovine brain (Harris et al 1985), lung (Merchen et al 1990), rat glioma cells (Itoh et al 1988) and lymphoma cells (Rall & Harris 1987)

1.5.2 Gαi
The classification of the Gi subtype of G protein is derived from the observation that the first member of this group to be isolated is responsible for coupling of inhibitory receptors to the adenylate cyclase system (Katada & Ui 1982, Hildebran et al 1983)

Studies of cDNA cloning of Gαi have revealed the existence of three distinct Gαi cDNAs, Gαi1, Gαi2 and Gαi3 (Itoh et al 1988, Jones & Reed 1987). Their amino acid sequences show more than 85% homology with each other. The three Gαi cDNAs are encoded by distinct genes. Two kinds of Gαi protein, 40 kDa (αi1) and 41 kDa (αi2), have been purified from porcine brain as substrates for pertussis toxin (PTX). PTX is a bacterial toxin
which catalyses a covalent ADP-ribose modification of the α subunit that leads to uncoupling of receptors from G proteins of the Gα₁₁-family. In Northern blot analysis, Gα₁₂ and Gα₁₃ mRNAs are expressed in almost all kinds of tissues and cells (Beal et al. 1987, Kim et al. 1988, Suki et al. 1987, Jones & Reed 1987). The expression of Gα₁₁ mRNA seems to be predominantly in brain (Bray et al. 1987, Brann et al. 1988). Among several neuronal cell lines, Gα₁₁ mRNA is detected in pheochromocytoma PC 12 cells and neuroblastoma cells, but not in glioma C6 cells and neuroblastoma-glioma hybridoma NG108-15 cells (Garibay et al. 1991).

In NG108-15 cells, the opioid-mediated-inhibition of adenylate cyclase is transduced specifically by Gα₁₂ (McKenzie & Milligan 1990). There is also evidence for the coupling of inhibitory receptors to phospholipase C in guinea pig neutrophils (Bokoch & Gilman 1984, Okajima & Ui 1984) and phospholipase A2 in rat mast cells (Nakamura & Ui 1985a, 1985b) via a PTX-sensitive G protein. More recently the antisense strategy has been used to demonstrate that Gα₁₁ couples the muscarinic receptor to activation of phospholipase C (Kasahara & Sugiyama 1994), and anti-Gα₁₂ antibodies have been employed to implicate Gα₁₁ in the ATP and thrombin receptor stimulation of phospholipase A2 (Winitz et al. 1994).

In rat pituitary cells Gα₁₃ has been shown to couple D2 dopamine receptor activation to stimulation of K⁺ currents (Lledo et al. 1992).

### 1.5.3 Gα₀

The classification of Gα₀ subtypes is derived from the original nomenclature as G "other", based on the observation of Sternweiss & Robishaw (1983) who noted the presence of a third type of G protein subunit, unlike Gα₅ or Gα₁ when purifying these proteins from bovine brain.

Three classes of Gα₀ cDNAs (Gα₀₁ and Gα₀₂) have been isolated from a number of different tissues including mouse brain (Strathmann et al. 1990), the insulin secreting cell line HIT-T15 (Hsu et al. 1990, Bertrand et al. 1990), bovine retina (Van Meurs et al. 1987) and rat brain (Itoh et al. 1986 Tsukamoto et al. 1991). They are derived from alternative splicing of the same Gα₀ gene. Two of these transcripts encode Gα₀ proteins with the same length (354 amino acids giving a calculated molecular weight of 40-40.5 kDa), which differ in 26 of the last 112 carboxy-terminal amino acids. These products were named Gα₀₁ and Gα₀₂ (Hsu et al. 1990). The third transcript is identical to Gα₁₁ in its coding region but differs in the 3' untranslated region 28 nucleotides downstream from the termination codon (Bertrand et al. 1990). Gα₀ is also a substrate for PTX catalysed ADP-ribosylation (Brabet et al. 1988) of a cysteine residue located four amino acid residues away from the carboxy terminus of the molecule (Hoshino et al. 1990).

Gα₀ is widely distributed in the brain (Worley et al. 1986) and is also present in most excitable cells including neuronal, neuroendocrine and endocrine cells (Asano et al. 1988, Heschler et al. 1987, Schultz et al. 1990, Toutant et al. 1990). In brain Gα₀ proteins are
particularly abundant and constitute approximately 1% of the total membrane protein (Bockaert et al 1990).

\( \Gamma \) is reported to have a role in coupling muscarinic receptors to the phospholipase C system, which causes inositol 1,4,5-trisphosphate (InsP3) to mobilise intracellular Ca\(^{2+} \) to evoke a Cl\(^- \) current (Moriarty et al 1990). There is now also a great deal of evidence to suggest that \( \Gamma \) is involved in mediating receptor control of Ca\(^{2+} \) channel currents (for review see Dolphin 1995). Several investigations have used purified \( \Gamma \) and \( \Gamma \) proteins to reconstitute the coupling of inhibitory receptors to voltage-sensitive Ca\(^{2+} \) channels of cells in which \( \Gamma /\Gamma \) proteins have previously been inactivated with PTX. These proteins were perfused into the cells with the patch pipette (Hescheler et al 1988, Toselli et al 1989). In those studies Go was able to reconstitute opioid and muscarinic inhibition of Ca\(^{2+} \) channel currents. An antibody raised against a peptide epitope common to both splice variants of \( \Gamma \) abolished the adrenaline-induced inhibition of N-type Ca\(^{2+} \) channel currents in NG108-15 cells; cells injected with an anti-\( \Gamma \) antibody responded normally (McFadzean et al 1989). Similarly, treatment of dorsal root ganglion neurones with an anti \( \Gamma \) antisera abolished the GABAergic (Menon-Johansson et al 1993) and opioid (Moises et al 1994) inhibition of neuronal Ca\(^{2+} \) channel currents, while anti-\( \Gamma \) antisera had no effect on the neurotransmitter-mediated inhibition of the currents. Antisense oligonucleotides have more recently provided a novel approach to investigate which subtypes of G protein have the principal role in coupling receptor activation to inhibition of Ca\(^{2+} \) currents. In pituitary GH3 cells the expression of G protein \( \alpha \) subunits was transiently switched off by an intracellular injection of antisense oligonucleotides complementary to a mRNA sequence common to all \( \Gamma \) subtypes. Only the transient suppression (Kleuss et al 1991) of Go1 and Go2 \( \alpha \) subunits, but not of Gi or Gs \( \alpha \) subunits, abolished the inhibition of L-type Ca\(^{2+} \) channel currents following activation of muscarinic and somatostatin receptors respectively. Similarly microinjection of antisense oligonucleotides complementary to a mRNA sequence common to \( \Gamma \) subtypes exclusively abolished the GABAergic inhibition of Ca\(^{2+} \) channel currents in DRG neurones (Campbell et al 1993). These findings suggest that \( \Gamma \) is the primary G protein subtype involved in coupling receptor activation to inhibition of Ca\(^{2+} \) currents.

1.5.4 \( \Gamma \) \( \Gamma \) has been described by Fong et al (1988) and Matsuoka et al (1988) as a \( \Gamma \)-like protein with minor modifications in the GTP-binding domain and, no cysteine residue near the carboxy terminus to act as an ADP-ribosylation site for PTX. The former modification suggests different GTP-binding and hydrolysis properties for this \( \alpha \) subunit, and these novel GTP-handling properties of Gz have been reported (Casey et al 1990).

\( \Gamma \) is predominantly expressed in neurons, platelets and spermatozoa, being absent or only expressed at very low levels in several other tissues (Casey et al 1990, Glassner et al 1991,
Gagnon et al. 1991). This restricted distribution may suggest an unique function of G\(\alpha_z\) in cellular processes of these cells. Cotransfection of \(\alpha_z\) with \(\alpha_2\)-adrenergic, D2 dopamine and A1 adenosine receptors in 293 cells leads to a PTX-insensitive inhibition of adenylate cyclase in response to receptor activation, demonstrating that G\(\alpha_z\) can convey an inhibitory signal of receptors to adenylate cyclase (Wong et al. 1992). Activated G\(\alpha_z\) has also been shown to inhibit adenylate cyclase activity in Sf9 cells (Kozasa & Gilman 1995).

1.5.5 G\(\alpha_q\) The recently described PTX-insensitive Gq family (with \(\alpha_q\), \(\alpha_{11}\), \(\alpha_{14}\), \(\alpha_{15}\) and \(\alpha_{16}\)) has been implicated in PTX-insensitive stimulation of \(\beta\)-isoforms of phospholipase C (PLC) (Strathmann et al. 1989, Pang & Stemweiss 1990). The widely expressed G proteins G\(\alpha_q\), G\(\alpha_{11}\) and G\(\alpha_{14}\) have been found to activate PLC-\(\beta\) in reconstituted cell free systems as well as in transfected cells (Smrcka et al. 1991, Waldo et al. 1991, Hepler & Gilman 1992). G\(\alpha_q\) has been demonstrated to regulate vasopressin activation of phosphatidylinositol-specific phospholipase C in rat liver (Wange et al. 1991). Antibodies raised against a peptide sequence of the \(\alpha\) subunit of Gq/11 have also been used to demonstrate that Gq/11 couples bradykinin receptor activation to stimulation of phospholipase C and activation of a K\(^+\) channel current in NG108-15 cells (Wilk-Blaszczak et al. 1994). G15 and G16, representing the murine and human form of the same protein (Wilkie et al. 1992), are only expressed in some haematopoietic cells with decreasing expression levels during differentiation (Amatruda et al. 1992). Both G15 and G16 can stimulate PLC (Lee et al. 1992, Schnabel et al. 1992) and have been demonstrated to couple chemoattractant receptors to PLC in transfected cells (Amatruda et al. 1993).

1.5.6 G\(\alpha_{olf}\) G\(\alpha_{olf}\) is expressed exclusively in olfactory sensory neurones and is therefore thought to be involved specifically in odorant signal transduction (Jones & Reed 1989). The rat G\(\alpha_{olf}\) cDNA codes for 382 amino acid residues with the calculated molecular mass of 44 kDa (Jones & Reed 1989). G\(\alpha_{olf}\) has 88% homology with G\(\alpha_s\) in amino acid sequence and is a substrate for ADP-ribosylation by CTX. G\(\alpha_{olf}\) has been found to stimulate adenylate cyclase when expressed in S49 cyc-kin- cells that lack G\(\alpha_s\) (Jones & Reed 1989).

1.5.7 G\(\alpha_t\) The transducin or G\(t\) subtype of G protein enables light-initiated signal transduction in the rods and cones of the retina. There are two distinct \(\alpha\) subunits within this group, G\(\alpha_{11}\) and G\(\alpha_{12}\), which are found exclusively in the rods and cones respectively (Lerea et al. 1986), and exhibit approximately 65% homology with G\(\alpha_t\) (Itoh et al. 1988). Both Gt1 and Gt2 couple the light-sensitive protein rhodopsin to cGMP phosphodiesterase. The resulting decrease in cGMP levels closes cGMP-gated ion channels, the cell membrane...
is hyperpolarised and nervous impulses from the retina are initiated (Hingorani & Ho 1990). Transducins are substrates for both CTX and PTX (Kaziro et al 1991).

1.5.8 Go\textsubscript{gust} The G protein involved in the taste transduction process (gustducin) has been cloned from rat taste cells (McLaughlin et al 1993). The primary sequence of gustducin shows similarities to the transducins in the receptor interaction domain and cGMP phosphodiesterase interaction site.

1.6 Structure of G protein \(\alpha\) subunits

1.6.1 Structure of p21\textsuperscript{ras} and EF-Tu The small GTP-binding superfamily play critical roles in the control of cell growth and differentiation, organisation of the cytoskeleton and intracellular membrane trafficking (reviewed in Boguski & McCormick 1993). The Ras superfamily of small GTP-binding proteins, including Ras, Rac/Rho and Rab proteins, are members of an extended family of GTPase, which include proteins involved in protein synthesis (elongation and initiation factors) and signal transduction (heterotrimeric G proteins). These proteins cycle between active and inactive states bound to GTP and GDP respectively. The GTP-bound form is slowly converted to the GDP-bound form by the protein's intrinsic ability to hydrolyse GTP, a process accelerated by associated GTPase activating proteins (GAPs) such as p120-GAP and neurofibromin. Activation in turn involves the replacement of GDP by GTP, an event mediated by guanine-nucleotide-exchange factors.

Until recently much of the information concerning the three dimensional structure of heterotrimeric G protein \(\alpha\) subunits has relied on conservation between the small GTP-binding protein p21\textsuperscript{ras} and bacterial elongation factor EF-Tu, since crystal structures exist for p21\textsuperscript{ras} and EF-Tu (Jurnack 1985, De Vos et al 1988, Schlichting et al 1989, Pai et al 1989). These proteins have a hydrophobic core composed of six strands of \(\beta\) sheet (\(\beta1-\beta6\)), which are connected by hydrophilic loops and \(\alpha\) helices (\(\alpha1-\alpha5\)). Five regions of the polypeptide chain, all associated with loops on one side of the protein are designated G1-G5 (G domains) and are consensus sequences involved in GTP/GDP binding and GTP hydrolysis (Bourne et al 1991). The G domains are also highly conserved among all identified heterotrimeric G protein \(\alpha\) subunits. Mutations within the various GDP-/GTP-binding regions have been performed in order to ascertain the functional role of each of these regions in heterotrimeric G protein function. These are summarised below.

G1 region Mutation of p21\textsuperscript{ras} Gly-12 to valine in the G1 region inhibits GTPase activity and promotes transformation (Gibbs et al 1984, McGrath et al 1984). The analogous mutation in \(\alpha_s\) also inhibits GTPase activity to cause persistent activation of
adenylate cyclase activity (Graziano & Gilman 1989, Johnson et al 1991). X-ray studies of p21\textsuperscript{ras} show this region as a loop which binds the $\alpha$- and $\beta$-phosphates of GTP or GDP (Bourne 1991).

**G2 region** Mg\textsuperscript{2+} is essential for proper binding of ligands, for the GTPase activity and for the structural integrity of small GTP-binding proteins, elongation factors and heterotrimeric G protein $\alpha$ subunits (Higashijima et al 1987). The G2 domain contains a highly conserved threonine residue is thought to interact with the Mg\textsuperscript{2+} ion bound to the $\beta$ and $\gamma$ phosphates of the guanine nucleotide. GTP binding alters the conformation of this loop, in part by changing the orientation of the critical threonine residue at position 35.

**G3 region** The G3 domain contains a sequence conserved among all $\alpha$ subunits and contains an invariant aspartate residue which binds the catalytic Mg\textsuperscript{2+} ion through an H\textsubscript{2}O molecule and a glycine (Gln-61) which forms a hydrogen bond with the $\gamma$ phosphate of GTP. A second glycine (Gln-227) functions as a pivot for conformational change induced by GTP. Mutations in the G3 region at Gln-61 of p21\textsuperscript{ras} and Gln-227 of $\alpha_s$ strongly inhibit GTPase activity, these modified proteins are unable to assume the correct conformation for binding of GTP and are therefore unable to activate their respective effectors (Osawa & Johnson 1991). Conformations of amino acids 60-63 within the G3 region and the downstream $\alpha$ helix ($\alpha_2$) differ dramatically in the GDP and GTP-bound forms of the protein indicating that these regions may represent a molecular switch during GDP/GTP exchange.

**G4 region** The G4 domain contains an aspartate residue which forms a hydrogen bond with the guanine ring and amides of asparagine and lysine to help stabilise GDP/GTP binding. Mutations in these regions of p21\textsuperscript{ras} and $\alpha_s$ decreased GDP and GTP affinity, but had no effect on GTPase activity (Osawa & Johnson 1991, Sigal et al 1986).

**G5 region** The G5 domain is less conserved but has a general consensus sequence in $\alpha$ subunits and is predicted to interact indirectly with the guanine nucleotide to stabilise its binding (Bourne et al 1991).

The recent revelation of the crystal structure of GTP- and GDP-liganded transducin and $\alpha_1$ (Noel et al 1993, Lambright et al 1994, Coleman et al 1994) has allowed further insight into the structural basis of heterotrimeric G protein function.

The heterotrimeric $\mathrm{G}_t$ and $\mathrm{G}_{11}$ $\alpha$ subunits consists of two domains; one, a GTPase domain that contains the guanine nucleotide binding pocket as well as sites for binding receptors, effectors, and $\beta\gamma$. The GTPase domain is similar to that of p21\textsuperscript{ras} and consists of a six-stranded $\beta$ sheet ($\beta_1$-$\beta_6$) surrounded by a set of five helices ($\alpha_1$-$\alpha_5$). A second highly $\alpha$-helical domain unique to heterotrimeric G proteins is also present, but its function is less clear. The highly helical domain comprises seven helices, (helix A-helix G), and is linked to the GTPase domain by two extended linker strands (Figure 1.5). Between the two domains
lies a deep cleft within which the guanine nucleotide is tightly bound. The helical domain, in particular the helix B-helix C region, may also contribute to the effector-binding site (Coleman et al 1994). There is a high level of amino acid sequence diversity between $G_{a_4}$ and $G_{a_{11}}$ at the helix B-helix C regions, such divergence may specify the differential effector interactions of these two subtypes of G protein. Substantial structural changes also occur in the helix B-helix C segment upon GTP hydrolysis (Coleman et al 1994).

**Figure 1.5** Three-dimensional crystal structure of $G_{a_4}$ in the GDP- (a) and GTP-bound (b) forms (Lambricht et al 1994)

1.6.2 Guanine-nucleotide Exchange  
$G_{a_4}$, Ras and EF-Tu differ in their affinity to bind guanine nucleotides. Exchange factors accelerate nucleotide exchange in both Ras and EF-Tu, but a significant basal exchange rate persists in their absence (reviewed in Boguski & McCormick 1993). Exchange does not occur for transducin in the absence of activated rhodopsin. The structural feature that distinguishes $G_{a_4}$ from both Ras and EF-Tu is the large helical domain that forms over one wall of the nucleotide-binding cleft and thereby occludes the nucleotide (Noel et al 1993). Activated rhodopsin may open the nucleotide binding cleft to facilitate GDP/GTP exchange. The stereocchemical links between the receptor-binding surface and elements that clamp the nucleotide are structurally interdependent so that a receptor-triggered structural change may initiate a cooperative unravelling of the interactions that secure the nucleotide. Following release of GDP, GTP binds to the open nucleotide-binding cleft. Crystal analysis studies reveal that the newly bound $\gamma$ phosphate initiates a conformational change, which potentially alters the structure of the region corresponding to amino acids 198-215 (comprising the $\alpha_2$ helix) (Figure 1.5), and leads to dissociation from the activated receptor and $B_{y}$ heterodimer (Noel et al 1993). Mutations in the corresponding region of $G_{a_5}$ also indicate that this region is critical in regulating affinity for $G_{B\gamma}$ (Lee et al 1992).
1.6.3 GTPase Activity

The p21\textsuperscript{ras} polypeptide has a low intrinsic GTPase activity ($k_{\text{cat}} < 0.03 \text{ min}^{-1}$) but in the presence of the GTPase activating protein (GAP) the GTPase activity is increased 200-fold (Trahey & McCormick 1987). In contrast, the α subunit of heterotrimeric G-proteins have a relatively high intrinsic GTPase activity ($k_{\text{cat}} = 2-3 \text{ min}^{-1}$) (Bourne et al 1990). The region in G protein α subunit polypeptides responsible for the high GTPase activity has putatively been mapped to a region that surrounds Arg-201. This site is located in the middle of the α\textsubscript{S} polypeptide and is the residue ADP-ribosylated by cholera toxin (Casey & Gilman 1988). ADP-ribosylation of Arg-201 results in inhibition of the intrinsic α\textsubscript{S} GTPase activity. Additionally, mutation of Arg-201 also results in inhibition of the intrinsic GTPase activity, despite the fact that this region is not involved in GTP/GDP binding (Casey & Gilman 1988). It has been postulated that this region of the polypeptide is controlling a GAP function which is intrinsic to G protein α subunit polypeptides (Landis et al 1989). Residues surrounding Arg-201 share homology with a putative GAP-binding site in p21\textsuperscript{ras} (McCormick 1989). The intrinsic GTPase activity of transducin and G\textsubscript{q/11} is stimulated directly by their respective effector proteins, cGMP phosphodiesterase and phospholipase-C\textsubscript{β} (Arshavsky et al 1991, Bernstein et al 1994). These effector proteins may also interact with a GAP-binding site on the α polypeptide of the heterotrimeric G proteins, in a similar manner as the p21\textsuperscript{ras} GAP, to promote GTP hydrolysis.

The highly helical domain of Gα\textsubscript{i1} and transducin, contain a key residue (Arg-174 in α\textsubscript{t}, Arg-178 in α\textsubscript{i1}) that may be needed for GTP hydrolysis and regulation of GTPase activity (Noel et al 1993, Coleman et al 1994). Arg-178, which is located on linker 2, and Gln-204, which is located on the α2 helix of the α\textsubscript{i1} subunit polypeptide are critical for GTP hydrolysis (Coleman et al 1994). Similarly, Arg-174 and Glu-203 on the α\textsubscript{t} polypeptide have also been implicated in GTP hydrolysis (Noel et al 1993). Mutations of the corresponding Arg residue in Gα\textsubscript{i} and Gα\textsubscript{S} dramatically reduces their respective GTPase activities and constitutively activates their respective target effectors (Landis et al 1989, Gibbs et al 1990). Also, this arginine on Gα\textsubscript{S} is the target for ADP-ribosylation by cholera toxin, which blocks GTP hydrolysis (Van Dop et al 1984).

The weak hydrolytic activity of p21\textsuperscript{ras}, which does not have an Arg residue at a position analogous to 178 suggests that Arg-178 provides the energy to stabilise the transition state for GTP hydrolysis. The higher GTPase activity rates in heterotrimeric G proteins α-subunits may reflect an inherently better-structured α2-helix, which provides a properly positioned general base without the aid of a GTPase-activating protein, and the presence of a transition-state-stabilising interaction with the side chain of a conserved arginine, corresponding to Arg-174 and Arg-178. Since α2 is structurally coupled to effector binding sites, Glu 203 and Glu 204 could be productively oriented by bound effectors to facilitate GTP hydrolysis (Arshavsky & Bownds 1992, Bernstein et al 1992).
1.6.4 Receptor and Effector Interaction  Pertussis toxin catalyses the ADP-ribosylation of $\alpha_i$ but not $\alpha_S$ (Gilman 1987). Within the $\alpha_{i1}$ polypeptide Cys-351, four amino acids from the C-terminus, is the residue ADP-ribosylated by pertussis toxin. Efficient pertussis toxin-catalysed $\alpha_i$ ADP-ribosylation also requires the presence of the $\beta\gamma$ subunit complex and sequences within the N-terminal moiety of the $\alpha$ subunit polypeptide (Woon et al 1988). Chimeric studies and specific point mutations have revealed the C-terminal moiety of heterotrimeric G protein $\alpha$ subunits to encode the effector activation and receptor selectivity domains (Russell & Johnson 1993). The G4 and G5 sequences within the GDP/GTP binding domains are also encoded within the C-terminal moiety. Consistent with the receptor selectivity site mapping to the extreme C-terminus, the $\alpha_i$ cysteine residues from the C-terminal end of $\alpha_i$-like polypeptides is the amino acid ADP-ribosylated by pertussis toxin. The ADP-ribosylation of this residue functionally uncouples receptor activation of the covalently modified G protein $\alpha$ subunit.

The extreme C-terminal region, the $\alpha_5$ helix and the N-terminal region are important sites of interaction with receptors (Conklin & Bourne 1993, Neer 1994). Mutations in the C-terminus (Hirsch et al 1991), its covalent modification by PTX (West et al 1985), and peptide-specific antibodies raised against it (Gutowski et al 1991) all uncouple G-proteins from their associated receptors. The region encoded by residues 311-328, which are located on the B strand ($B_6$) linking the 4th and 5th $\alpha$-helices of $\alpha_1$ (Lambright et al 1994) is also involved in receptor interaction. Like the C-terminal decapeptide, a peptide raised against this region blocks activation of Gt by its receptor photorhodopsin (Hamm 1991). An N-terminal $\alpha_t$ peptide also inhibited interaction of Gt with photorhodopsin (Hamm et al 1988). An activated receptor triggers the intracellular response by decreasing the affinity of the $\alpha$ subunit for GDP, perhaps by promoting a conformational change in the C-terminal $\alpha$ helix. This effect is mimicked by deletion of 14 amino acid residues from the C-terminus of $\alpha_o$ (Denker et al 1992). A conformational change occurring in the C-terminus of the $\alpha_5$ helix may be transmitted to the N-terminal loop. Mutations of amino acid residues in the N-terminal loop also decrease GDP affinity (Thomas et al 1993, liri et al 1994).

It would appear that the C-terminus of the $\alpha$ subunit polypeptide confers the specificity of receptor interaction. Use of $\alpha$ subunit chimeras has enabled mapping of certain $\alpha$ regions as receptor interaction sites. For example, when the $\alpha$ portion of the C-terminus of G$\alpha$Q is replaced by the corresponding region of G$i_2$, then GQ is able to couple with receptors normally associated with G$i_2$ (Conklin et al 1993). However, since many G protein $\alpha$ subunits are identical at the C-terminus but couple to different receptors, other regions of the $\alpha$ polypeptide may have a role in regulating receptor interaction specificity. Lee et al (1995) have shown that the region in the middle of the G$\alpha_{16}$ molecule (corresponding to residues 220-240), and some elements in the amino-terminus, in combination with the C-
terminus is critical in maintaining interaction specificity with the chemoattractant factor receptor.

The effector-binding region has been mapped only for the pairs $\alpha_s$/adenylate cyclase and $\alpha_i$/cGMP phosphodiesterase (reviewed in Conklin & Bourne 1993). Mutagenesis of $\alpha_s$ revealed three regions involved in coupling $G_s$ to its effector adenylate cyclase (Berlot & Bourne 1992). The key effector regions were found to be located on the loops $\alpha_2$-$\beta_4$, $\alpha_3$-$\beta_5$, $\alpha_4$-$\beta_6$. Of these, only the $\alpha_2$-$\beta_4$ loop exhibits a clearly identifiable structural change on nucleotide exchange (Lambright et al. 1994) (Figure 1.5). The effector binding region of $\alpha_s$ also partially overlaps the putative $\beta_\gamma$-binding surface (Lambright et al. 1994). It is therefore unlikely that the $\alpha$ subunit can simultaneously bind effector and $\beta_\gamma$.

1.6.4 $\beta_\gamma$ Subunit Interaction

The $\beta_\gamma$ subunits, effectors, and receptors appear to bind to different surfaces of $\alpha$ subunits. The first 25 amino acids of the $\alpha$ subunit are essential for $\beta_\gamma$ binding (Fung & Nash 1983, Denker et al. 1992), but their position in 3-D structure is unknown since they are mobile and therefore do not show in the crystal (Coleman et al. 1994). The $\beta_\gamma$-binding surface probably also includes the $\alpha_2$ helix of the GTPase domain since a cysteine residue on this helix (Cys-215 in $\alpha_o$) can be chemically crosslinked to $\beta_\gamma$ (Thomas et al. 1993). Since binding of $\beta_\gamma$ depends critically on the nucleotide bound to the $\alpha$ subunit, it makes sense that the $\beta_\gamma$ contact surface would include a region such as the $\alpha_2$ helix that is different in the GDP- and GTP-liganded states (Lambright et al. 1994). The N-terminal moiety of $\alpha_s$ has been shown to control both GDP dissociation and interaction with the $\beta_\gamma$ subunit (Conklin & Bourne 1993). Similarly, a monoclonal antibody directed against the N-terminus of $G_\gamma$ causes $G_\alpha$ to dissociate from $\beta_\gamma$ (Mazzoni et al. 1989). N-terminal myristoylation of $\alpha_o$ enhances its affinity for binding $\beta_\gamma$ (Linder et al. 1991), and an N-terminally myristoylated peptide competitively inhibits binding of $\alpha_\gamma$ to $\beta_\gamma$ (Kokame et al. 1992).

1.7 $\beta_\gamma$ Subunits of Heterotrimeric G proteins

The four known mammalian G protein $\beta$ subunits ($\beta_1$-$\beta_4$), each about 340 amino acids long, share more than 80% amino acid identity (reviewed in Simon et al. 1991, Clapham & Neer 1993). $\beta_1$, $\beta_2$, and $\beta_3$ are ubiquitously expressed, while $\beta_4$ is particularly abundant in brain and lung tissue. $\beta$ subunits are predicted to contain two types of structure: an N-terminal region thought to form an amphipathic $\alpha$ helix followed by seven repeating segments. Each segment shares a repetitive 40 amino acid sequence motif that is characterised by certain amino acid combinations, including tryptophan-aspartic acid and glycine-histidine pairs.

Seven different G protein $\gamma$ subunits are currently known (Simon et al. 1991). They are approximately 75 amino acids long and are much more heterogeneous than the $\beta$ subunits.
The $\gamma$ subunits are predicted to be $\alpha$-helical. $\gamma$ subunits also differ in their modification by prenyl groups. $\gamma_1$ is farnesylated, whereas $\gamma_2$ is geranyl geranylated (Spiegel et al 1991). Such modifications are necessary for membrane attachment and interaction with G protein $\alpha$ subunits (Muntz et al 1992, Simonds et al 1991, Inguez-Lluhi et al 1992). Similar lipid modifications have been reported for G protein $\alpha$ subunits (reviewed by Casey 1994). Control of processes such as palmitoylation may underlie some of the mechanisms responsible for desensitisation of the cell following agonist receptor activation.

The $\beta$ and $\gamma$ subunits associate tightly with each other and can only be separated by denaturants. The putative $\alpha$ helix at the N-terminus of $\beta$ contributes to the $\beta\gamma$ interaction site (Garritsen & Simonds 1994). The N-terminal portion of $\beta$ contains a cysteine residue which may cross-link to a cysteine residue in the $\gamma$ component (Bubis & Khorana 1990). Not all $\beta$ subunits are able to couple with all subtypes of $\gamma$ components. For example, $\beta_1$ can associate with both $\gamma_1$ and $\gamma_3$, whereas $\beta_2$ can associate with $\gamma_2$ but not with $\gamma_1$, $\beta_3$ is unable to associate with either $\gamma_1$ or $\gamma_2$ (Schmidt et al 1992, Pronin et al 1992). Selectivity between the $\beta$ and $\gamma$ subunits is conferred by multiple sites in the WD repeat region, particularly in repeat 5 (Pronin & Gautam 1992, Katz & Simon 1995).

Binding of the non-hydrolysable GTP analogue GTP$_\gamma$S to the G protein $\alpha$ subunit activates the $\alpha$ subunit which promotes dissociation of G$\alpha$ from the $\beta\gamma$ dimer. It is not certain whether dissociation is essential for G$\beta\gamma$ function and whether the subunits separate in the membrane or simply change conformation without physical separation. G$\alpha$ has been shown to assume an activated conformation while chemically crosslinked to $\beta\gamma$ (Yi et al 1991) G$\alpha$ and G$\beta\gamma$ remain active as long as GTP is bound to G$\alpha$. When GTP is hydrolysed to GDP, G$\alpha$ is not only inactivated itself, but also prevents further $\beta\gamma$ function by reassociation.

The first indication that the $\beta\gamma$ dimer could control effectors directly came with the report that G$\beta\gamma$ purified from bovine brain activated the cardiac K$^+$ channel normally regulated by the muscarinic acetylcholine receptor, independently of the G$\alpha$ subunit (Logothetis et al 1987, Codina et al 1987). The $\beta\gamma$ subunit has also been shown to activate phospholipase A2 in retinal membranes and phospholipase-C$\beta$ independently of G$\alpha$ (Jelsema et al 1987, Camps et al 1992). Nuclear microinjection of antisense oligonucleotides into GH3 cells shows that the somatostatin receptor inhibits the L-type Ca$^{2+}$ channel by G$\alpha_{o2}\beta_1\gamma_4$, whereas the M4 muscarinic receptor inhibits the same channel by G$\alpha_o\beta_3\gamma_4$ (Kleuss et al 1991, 1993a, 1993b). Since elimination of either the appropriate G$\alpha$ or G$\beta\gamma$ completely blocks the Ca$^{2+}$ channel inhibition, the two subunits may act independently of each other. Alternatively, different G protein $\beta\gamma$ dimers may specify which receptor activates which G$\alpha$ subtype, or each G$\alpha$ subtype may have a strict preference for a particular $\beta\gamma$. 
1.8 G Protein Modulation of Voltage-Dependent Ca\(^{2+}\) Channels

1.8.1 Ca\(^{2+}\) current modulation by Gs

In addition to the role of Gs in activating adenylate cyclase, it has been suggested that Gs activates L-type Ca\(^{2+}\) channels directly in cardiac ventricular cells in a process not requiring ATP or any second messenger regenerating system (Yatani et al 1988, Cavalie et al 1991). Hartzell et al (1991) dispute the direct action of Gs on Ca\(^{2+}\) currents and suggest that sympathetic regulation of cardiac Ca\(^{2+}\) channels is due exclusively to cAMP-dependent phosphorylation. An effect of G\(\alpha_S\) on skeletal muscle t-tubule Ca\(^{2+}\) currents also occurs independently of phosphorylation pathways.

Further evidence for a direct interaction of Gs and cardiac L-type Ca\(^{2+}\) channels comes from co-purification studies (Hamilton et al 1991) and experiments performed in lipid bilayers (Wang et al 1993). In rat sympathetic neurones N-type Ca\(^{2+}\) channels are positively modulated by secretin and vasoactive intestinal peptide (Zhu & Ikeda 1994). This modulation of Ca\(^{2+}\) current is reduced by anti-G\(\alpha_S\) antibodies, cholera toxin, and is also independent of cAMP.

1.8.2 Ca\(^{2+}\) current modulation by PTX-sensitive G proteins

In many neuronal and secretory cells Ca\(^{2+}\) channel currents are inhibited by a number of neurotransmitters and neuromodulators acting at the group of seven transmembrane-linked receptors coupled to the G proteins Gi and Go. These include noradrenaline acting on \(\alpha_2\)-adrenoreceptors (Holz et al 1986, Lipscombe et al 1989), opiates acting at \(\mu\)-, \(\kappa\)- and \(\delta\)-receptors (Hescheler et al 1987, Gross et al 1990, Seward et al 1991), GABA acting at GABAB receptors (Dolphin & Scott 1987), adenosine acting at A\(_1\) receptors (Dolphin et al 1986), somatostatin (Luini et al 1986), acetylcholine acting at M\(_2\) or M\(_4\) receptors (Caulfield & Brown 1991, Wanke et al 1994) and 5-HT (Dunlap & Fischbach 1981), among others. The involvement of Gi or Go in the modulation of Ca\(^{2+}\) currents was first indicated by the blockade of the responses by PTX (Holz et al 1986, Dolphin & Scott 1987), and the ability of the non-hydrolysable analogue of GTP, GTP\(\gamma\)S, to mimic the response (Scott & Dolphin 1990).

In most neuronal cells the Ca\(^{2+}\) channel current inhibited by these agonists is reduced by the N-type channel blocker \(\omega\)-CTX GVIA (Seward et al 1991, Menon-Johansson et al 1993), suggesting that N-type Ca\(^{2+}\) channels are the primary targets for modulation, although some effect on L-type Ca\(^{2+}\) channels has been reported (Scholz & Miller 1991, Elmslie et al 1992). The transient component of currents are abolished by GTP analogues, whereas sustained currents are less affected (Dolphin et al 1988). Single channel recordings
of N-type Ca\(^{2+}\) channels show that noradrenaline reduced the probability of opening (Lipscombe et al 1989, Delcour et al 1993), shifting the characteristics of channel opening to a lower open probability mode.

The P- and Q-type Ca\(^{2+}\) channels in cerebellar Purkinje and cerebellar granule neurones respectively have also been shown to be targets for modulation by a number of agonists (Mintz & Bean 1993, Randall et al 1993). In non-neuronal secretory cells it is the L-type Ca\(^{2+}\) channels that are modulated (Luini et al 1986, Schmidt et al 1991).

1.8.3 **Mechanism of G-protein inhibition of Ca\(^{2+}\) channel current**

Although some PTX-sensitive G proteins (Gi) inhibit the activation of adenylyl cyclase, the neurotransmitter-induced depression of Ca\(^{2+}\) current is not mediated by changes of cyclic-nucleotide metabolism. The effects of agonists on Ca\(^{2+}\) channel currents in DRG neurones could not be mimicked by various second messengers, nor inhibited by inhibitors of these systems (Dolphin et al 1989). Similar results were reported by McFadzean & Docherty (1989). Since none of the conventional second messengers have been implicated in the depression of current by PTX-sensitive G proteins, a direct action on the channel by the G protein is proposed, and this is known as a membrane-delimited regulatory pathway. Evidence for a membrane-delimited response is provided by experiments in which Ca\(^{2+}\) channel currents are recorded under cell-attached patch clamp mode. In this configuration, agonists applied to the bath would not have direct access to the channels located under the recording region of the electrode. With a messenger-mediated mechanism, agonist applied to the bath would act on receptors to make a messenger that diffuses to the channels in the patch and modulates their function. In a membrane-delimited pathway, there is no cytoplasmic messenger, and channels in the patch are not modulated by agonist applied to the bath. Using this protocol, responses to perfusion of the cell with the agonist noradrenaline are not observed, indicating that the signal is not carried any distance through the cytoplasm by a diffusible second messenger (Forscher et al 1986, Lipscombe et al 1989). The current general assumption is that a component of the activated G protein interacts directly with the Ca\(^{2+}\) channel to decrease its probability of opening.

The simplicity and directness of membrane-delimited mechanisms can make than faster then longer cascades that involve second messengers and protein phosphorylation. In rat sympathetic neurones, PTX-sensitive depression of Ca\(^{2+}\) currents by noradrenaline, or a muscarinic agonist, develops and recovers in less than 0.5 s (Bernheim et al 1991). In comparison to the rapid onset of modulation by the membrane-delimited mechanism, in sympathetic neurones the modulation of Ca\(^{2+}\) channels by angiotensin II and muscarinic agonists, via the PTX-insensitive pathway, proceeds through a second messenger pathway and therefore has a slower onset/offset, taking 2-60 s (Beech et al 1992, Shapiro et al 1994).
Formerly, it would have been supposed that the active component was the GTP-bound, PTX-sensitive α subunit, but with the clear demonstration that G protein βγ subunits act on inwardly rectifying K⁺ channels and other effectors (reviewed in Clapham & Neer 1993), the βγ hypothesis now also requires testing for Ca²⁺ channel modulation.

The biophysical changes that Ca²⁺ channels undergo during membrane-delimited modulation have been explored. A characteristic slowing of current activation by G protein activation (Scott & Dolphin 1986, Dolphin et al 1988) and neurotransmitters (Tsunoo et al 1986, Grassi & Lux 1989, Menon-Johansson et al 1993) is observed to underlie the depression of current. The slowing of activation is thought to be due to the voltage-dependence of inhibition, activated G protein being associated with the channel at hyperpolarised membrane potentials, and producing inhibition immediately the channel is opened by depolarisation. The inhibition is overcome with a slow time course upon continued depolarisation, a phenomenon attributed to the gradual dissociation of the G protein from the channel in its open state (Grassi & Lux 1989, Marchetti & Robello 1989). The inhibition is at least partially overcome by the application of a larger depolarising prepulse, immediately before the test pulse (Scott & Dolphin 1990) due to dissociation between the Ca²⁺ channels and their associated activated G protein. It has been hypothesised that the activated G protein interacts with the closed resting state of the Ca²⁺ channel to form a modified closed state (C*), which may be unavailable to open with depolarisation (reviewed in Dolphin 1991). This complex C*-G-GTPγS can dissociate with a slow rate constant and the free closed state C is then able to open normally. Depolarisation increases the rate of C→O, thus diminishing the pool of free C, and increasing the net rate of G-GTPγS-C*-→C. The finding that a brief prepulse to a large depolarising voltage reversed the GTPγS or agonist-induced slowing of current activation in a subsequent test pulse may be attributed to the equilibrium C*↔C↔O being shifted towards O during the prepulse. At the end of the prepulse O→C occurs rapidly, but the rate of C→C* is sufficiently slow that most of the channels remain as C at the time the test potential is delivered and consequently current activation due to the test pulse occurs rapidly. Kasai (1992) proposes that there is no dissociation between the channel and G protein, but the complex undergoes slower gating. It has also been proposed that an alteration in ion permeation pathway contributes significantly to G protein inhibition of N-type Ca²⁺ channels (Kuo & Bean 1993).
1.8.4 Evidence that inhibitory G protein is Go

Several lines of experimental evidence suggest the Go is the subtype of G protein which has a pivotal role in the neurotransmitter modulation of voltage-dependent Ca\(^{2+}\) channels. Following treatment with PTX in order to inactivate Gi/Go, the addition of purified Go is able to restore opiate inhibition of Ca\(^{2+}\) currents in NG108-15 cells (Hescheler et al. 1987) and dopamine-induced inhibition of Ca\(^{2+}\) currents in snail neurones (Harris-Warrick). Taussig et al. (1992) have developed a mutant NG108-15 cell line in which the Go1 α subunit is resistant to PTX. In this mutant cell line, opioids and noradrenaline inhibited the Ca\(^{2+}\) current in a PTX-insensitive manner, whereas inhibition by these transmitters in the wild type cells is sensitive to PTX, indicating that transduction occurs via Go1. The effect of somatostatin however remained sensitive to the toxin, indicating that transduction occurs either via Go2 or one of the Gi subtypes.

The production of antipeptide anti-G protein antibodies has proved useful for further understanding the transduction pathways involved in mediating the neurotransmitter inhibition of Ca\(^{2+}\) channels. An antibody raised against a C-terminal peptide common to the two splice variants of Go attenuated the adrenaline-induced inhibition of N-type Ca\(^{2+}\) channel currents when dialysed into NG108-15 cells via the patch pipette, while anti-Gi antibodies had no effect on the current modulation (McFadzean et al. 1989). Similar findings were made in snail neurones in which an affinity-purified antibody generated against Go interfered with the ability of dopamine to inhibit Ca\(^{2+}\) currents (Harris-Warrick et al. 1986).

The GABAergic inhibition of N-type Ca\(^{2+}\) currents in DRG neurones was abolished following treatment of the cells with an antiserum raised against a C-terminal decapeptide of Go, a region known to be involved in G protein-receptor interaction (Menon-Johansson et al. 1993). An antiserum raised against the N-terminus of Go or the C-terminus of Gi had no effect on the GABAergic inhibition of the Ca\(^{2+}\) current. This result provides evidence that C-terminus of Go is exclusively involved coupling the GABA receptor to the N-type Ca\(^{2+}\) channel.

Antisense oligonucleotide technology has provided a novel method with which to inhibit expression of a target protein transiently. In pituitary GH3 cells nuclear microinjection of antisense oligonucleotides complementary to a selective motif found in a G protein subunit encoding mRNA transiently switched off expression of G protein subunits (Kleuss et al. 1991). Only the suppression of Go subunits, but not of Gi or Go, abolished the muscarinic and somatostatin induced inhibition of Ca\(^{2+}\) channel currents (Kleuss et al. 1991). The muscarinic and somatostatin responses were found to be coupled to Go2 and G α1 respectively. The antisense strategy has also been used to reveal that somatostatin and muscarinic receptors are coupled to the inhibition of Ca\(^{2+}\) channel currents via differential variants of G protein β and γ subunits. Signal transduction by somatostatin and muscarinic
receptors has been shown to occur via $\beta_2\gamma_3$ and $\beta_3\gamma_4$, respectively (Kleuss et al 1992, Kleuss et al 1993). This finding indicates an additional means by which specificity of signalling may be achieved, despite the relatively small number of G protein $\alpha$ subunits available. The GABA$\beta$ergic modulation of $Ca^{2+}$ currents in DRG neurones (Campbell et al 1993) and the dopaminergic inhibition of $Ca^{2+}$ currents in pituitary lactotrophs (Baertschi et al 1992) was also exclusively abolished following treatment of the cells with antisense oligonucleotide complementary to the mRNA of Go$_O$.

There is also evidence to suggest that the activation of certain receptors in secretory cells enhances $Ca^{2+}$ currents via PTX-sensitive G proteins. In GH3 cells Go$_{i2}$ is the main PTX-sensitive G protein involved in the thyrotropin-releasing hormone $Ca^{2+}$ channel stimulation, although this pathway also requires concurrent protein kinase C activation mediated by PTX-insensitive G proteins (Gollasch et al 1993).

1.8.5 Regulation of voltage-dependent $Ca^{2+}$ channels by phosphorylation and second messengers

Although evidence suggests that $Ca^{2+}$ channels are modulated via a direct interaction with G proteins, in some systems the currents can be modulated by second messengers and phosphorylation pathways.

1.8.5.1 cAMP-dependent phosphorylation

Initial evidence for a neurotransmitter affecting $Ca^{2+}$ channel function was provided by noradrenaline, which increases cardiac L-type $Ca^{2+}$ channel current upon $\beta$-adrenoreceptor activation (Cachelin et al 1983, Bean et al 1984). This effect occurs via activation of adenyl cyclase by Gs, the resulting increase in cAMP levels causes activation of protein kinase A (PKA) which phosphorylates components of the $Ca^{2+}$ channel. The skeletal muscle L-type $Ca^{2+}$ channel $\alpha_1$-subunit has a PKA phosphorylation site on the intracellular loop between domains III and IV and also PKA phosphorylation two sites on the C-terminal domain (Rohrkasten et al 1988). Initial evidence suggests that the $\alpha_1$-subunit of the cardiac L-type $Ca^{2+}$ channel may not be phosphorylated in vitro (Chang & Hosey 1988). In intact cardiac myocytes phosphorylation induced by cAMP occurs primarily on the $Ca^{2+}$ channel $\beta$-subunit (Haase et al 1993). It has recently been shown that the neuronal $\alpha_1C$-subunit exists in two size forms, the shorter form resulting from proteolytic cleavage of part of the C-terminal tail. Only the C-terminus of the shorter form is a substrate for cAMP-dependent phosphorylation (Hell et al 1993). cAMP-dependent phosphorylation increases the $Ca^{2+}$ current by increasing the open probability of the channel (Cachelin et al 1983) and increasing the mean open time (Yue et al 1990).
Several reports suggest that neuronal \( \text{Ca}^{2+} \) currents are insensitive to cAMP (Shimahara et al 1987, McFadzean & Docherty 1989, Wanke et al 1994), although these results may reflect a high basal adenylyl cyclase activity in these cells which causes the channels to be fully phosphorylated and occludes any effects by exogenous cAMP. However, agents that increase cAMP are found to enhance sustained \( \text{Ca}^{2+} \) currents in rat DRG neurones, while agents that inhibit phosphorylation reduce sustained \( \text{Ca}^{2+} \) channel currents (Dolphin et al 1989). Similarly \( \beta \)-adrenoreceptor agonists increase \( \text{Ca}^{2+} \) currents in hippocampal neurones by a mechanism involving PKA (Gray & Johnston 1987). The N-type \( \text{Ca}^{2+} \) channel \( \alpha 1 \) subunit has also been found to be phosphorylated by protein kinase A and protein kinase C (Ahlijanian et al 1991)

### 1.8.1.2 Phosphorylation by protein kinase C

Protein kinase C (PKC) activity is promoted by phorbol esters to increase \( \text{Ca}^{2+} \) channel activity in cardiac cells and sympathetic neurones (Lacerda et al 1988, Lipscombe et al 1988). Cloned cardiac \( \text{Ca}^{2+} \) channels are also potentiated by PKC activators (Bourinet et al 1992, Singer-Lahat et al 1992). In contrast, it has been reported that PKC mediates the inhibition of \( \text{Ca}^{2+} \) currents by noradrenaline in sensory neurones (Rane et al 1987). The PKC mediated inhibitory modulation may involve N-type \( \text{Ca}^{2+} \) channels (Cox & Dunlap 1992), which are phosphorylated by PKC (Ahlijanian et al 1991b), while the PKC enhancement of \( \text{Ca}^{2+} \) current may involve L-type \( \text{Ca}^{2+} \) channels.
1.9 **GABA$_B$ Receptor**

$\gamma$-aminobutyric acid (GABA) acts as an inhibitory neurotransmitter in the mammalian brain. Two distinct types of GABA receptor are recognised in vertebrates, known as GABA$_A$ and GABA$_B$ (reviewed in Simmonds 1983). The GABA$_A$ receptor mediates a post-synaptic inhibitory effect by increasing the chloride permeability of the post-synaptic membrane, which has the effect of reducing the depolarisation produced by excitatory transmitter action. The GABA$_A$ receptor-mediated effects are sensitive to the antagonist bicuculline and can be mimicked by the agonist muscimol.

The ionic mechanisms underlying the GABA$_B$ receptor effect are different from the GABA$_A$ receptor. The GABA$_B$ receptor mediates a diminution in membrane Ca$^{2+}$ conductance (reviewed in Bowery 1993). In chick dorsal root ganglion neurones (DRGs) GABA$_B$ receptor activation reduced the Ca$^{2+}$ channel current in a PTX-sensitive manner, indicating a G-protein connection in the inhibitory process. Similarly, in rat DRGs the inhibition of Ca$^{2+}$ current induced upon activation of GABA$_B$ receptors by the GABA$_B$ agonist (-)-baclofen, was decreased by pretreatment of the cells with PTX (Dolphin & Scott 1987). Biochemical evidence also suggests that the GABA$_B$ receptor is coupled to the PTX substrates G$\alpha_1$ and G$\alpha_0$ (Sweeney & Dolphin, Morishita et al 1992), although it appears that only the coupling of the GABA$_B$ receptor to G$\alpha_0$ is involved in the GABA$_B$ergic inhibition of Ca$^{2+}$ channel currents (Menon-Johansson et al 1993, Campbell et al 1993).

(-)-Baclofen reduces release of the excitatory amino acid glutamate from the terminal of cerebellar granule neurones (Huston et al 1990). The inhibition of Ca$^{2+}$ conductance may account for the observed inhibition of excitatory neurotransmitter release upon GABA$_B$ receptor activation.

Activation of the GABA$_B$ receptor also promotes an increase in K$^+$ conductance to produce membrane hyperpolarisation (reviewed in Bowery 1993). The GABA$_B$ergic effects on Ca$^{2+}$ and K$^+$ conductances may therefore underlie the mechanism of GABA$_B$ergic post-synaptic inhibition.

The GABA$_B$ receptor has not yet been cloned since selective GABA$_B$ receptor antagonists have only recently become available (Bittiger 1993). The ability of the GABA$_B$ receptor to couple to G proteins indicates that this receptor may belong to the superfamily of receptors which contain seven transmembrane spanning domains and are coupled to their associated G proteins via regions of the third intracellular loop and C-terminus (Hedin et al 1993, Wagner et al 1995).
1.10 Dorsal Root Ganglia

Pain, pressure-tactile and thermal sensations from peripheral receptive elements are conveyed to the central nervous system via the dorsal roots, which consist of sensory (afferent) nerve fibres (Gilman & Winans Newman 1984). These afferents may be divided into several groups of nerve fibres, which are classified according to their different conduction velocities. The IA (A-α), IB and II-type (A-β) afferents, which relay information from touch and pressure receptors, are myelinated and have the fastest conduction velocities (14-30 ms⁻¹). The III (A-δ) and IV-type (C) fibers are involved in touch, pain and temperature sensation, and have slower conduction velocities (2--<1 ms⁻¹).

The cell bodies of the dorsal root afferents are located in the spinal, or dorsal root, ganglia (DRG). Each ganglion cell possesses a single nerve process that divides in a pseudounipolar configuration, with a central branch running to the spinal cord and a peripheral branch coming from a sensory receptor organ. The majority of primary afferent inputs into the spinal cord synapse in the dorsal horn. Immunocytochemical studies suggest glutamic acid and the polypeptides substance P and somatostatin as candidate excitatory synaptic transmitters in the dorsal horn (Hokfelt et al 1976). Candidate inhibitory neurotransmitters include GABA and glycine (Game & Lodge 1975).

The dorsal root ganglion provides an ideal preparation with which to study the function and G-protein modulation of voltage-dependent Ca²⁺ channels. The ganglia are easily isolated from the spinal cord of neonatal rats and are thereafter viable in primary cell culture for up to 2-3 weeks. In culture, the DRGs possess several types of Ca²⁺ channel, including T-, L-, N-, and P-type Ca²⁺ channel currents (Dolphin & Scott 1989, Mintz et al 1992) which are modulated in a PTX-sensitive manner upon activation of various types of receptor, including GABA_B (Dolphin & Scott 1986), adenosine (Dolphin et al 1986), bradykinin and neuropeptide Y (Ewald et al 1989). The inhibition of Ca²⁺ currents by these neurotransmitters is thought to underlie the mechanism of neurotransmitter-mediated attenuation of substance P release from sensory neurones (Holz et al 1989). Since individual DRG neurones in culture are not electrically coupled to other cells, a good spacial clamp is obtained, thus optimising electrophysiological recording. The spherical morphology of the cells, and their large somal diameter (10-20 μm), facilitates microinjection of antisense deoxyoligonucleotides directly into the cell cytoplasm (Chapter 4). The cultured DRGs are also an ideal preparation with which to perform immunocytochemical studies. The cell somal plasma membrane, cytoplasm and neurites are easily distinguished, and this is useful in assessing the differential cellular immunolocalisation of certain Ca²⁺ channel and G-protein components within the cell (Chapter 3).
1.11 Aims of Study

The aims of this study are three-fold:

1) Immunocytochemical studies in combination with confocal microscopy have been employed in order to address the cellular localisation of the various subtypes of HVA Ca\(^{2+}\) channel (L-, N- and P/Q-types) in DRG neurones. The immunocytochemical localisation and membrane topography of the Ca\(^{2+}\) channel \(\alpha 2/\delta\) and \(\beta\) components, and \(\alpha\)-subunits of the G-proteins Go and Gi will also be presented.

2) The use of antisense deoxyoligonucleotides to produce a transient suppression of expression of the Ca\(^{2+}\) channel \(\beta\) subunit, and G-protein Go\(_{\alpha}\) and Go\(_{\xi}\) was examined. Antisense oligonucleotide sequences complementary to the mRNA of all known Ca\(^{2+}\) channel \(\beta\) subunits, or Gi/Go \(\alpha\) subunits, were microinjected into the DRG cell cytoplasm, and the efficiency of the antisense oligonucleotide in producing a selective depletion of the target protein was examined using immunocytochemical techniques.

3) Antipeptide anti-G protein antibodies were used in order to address which subtype of G protein couples the GABA\(_{B}\) receptor and L-type Ca\(^{2+}\) channel to increased GTP hydrolysis in the rat frontal cortex. Antipeptide anti-Ca\(^{2+}\) channel \(\beta\) subunit antiserum, anti-\(\alpha 2\) subunit antiserum, and a peptide which mimics the \(\alpha 1/\beta\) interaction site on the Ca\(^{2+}\) channel \(\alpha 1\) subunit, were also used in order to examine which component of the L-type Ca\(^{2+}\) channel couples the Ca\(^{2+}\) channel to the G protein.
Chapter 2

Materials and Methods
2.1 Primary culture of Dorsal Root Ganglion Neurones

Dorsal root ganglion neurones were cultured and plated out according to the procedure of Winter (1987) with some modifications (Campbell et al 1993)

2.1.1 Preparation of sterile coverslips

22 mm² glass coverslips (Chance Propper) were sterilised by an overnight incubation in 70% ethanol, after which the coverslips were oven dried at 200°C for 4 hours. The sterile coverslips were then incubated overnight at 37°C with poly-L-ornithine (250 µg/ml), washed in sterile water and dried. This was followed by a 2 hour incubation at 37°C with laminin (1 µg/ml) prior to the dissection.

2.1.2 Dissection

Male Sprague Dawley rats (2-day old) were killed by decapitation. An incision was made along the length of the dorsal surface of the body, and the skin pulled back in order to expose the vertebral column and the associated musculature. The entire vertebral column was removed from the animal and placed in a 50mm petri dish containing sterile phosphate buffered saline (PBS) (Table 2.1). The column was rinsed with three successive sterile PBS washes and excess tissue debris was removed. The vertebral column was then transferred to a 150 mm petri dish and kept moist with PBS. A lateral incision was made on both sides of the column, using fine spring loaded scissors, from the rostral end of the vertebral column to the caudal end, and the spinal cord tissue was removed. The dorsal root ganglia were removed from between the vertebrae with fine forceps and the spinal roots were stripped to leave only the ganglia. Approximately 30-40 dorsal root ganglia were obtained from each animal.

2.1.3 Dissociation Procedure

The ganglia were incubated with collagenase (1.25 µg ml⁻¹) for 13 min at 37°C and then for 6 min at 37°C with trypsin (2.5 µg ml⁻¹) in Hamm's F14 medium (Imperial, Andover, UK) containing 10% heat inactivated horse serum (Gibco BRL), glutamine (2mM), penicillin (50 i.u ml⁻¹) and streptomycin (50 µg ml⁻¹) (see Table 2.1). The ganglia were then mechanically dissociated with a fire-polished pipette in the presence of DNase (1600 kUnits ml⁻¹).
2.1.4 Final Plating of neurones

DRG neurones were plated out on the polyornithine/laminin coated 22 mm² glass coverslips. The coverslips were transferred to 35mm petri dishes and placed in an incubator at 37°C in air containing 5% CO₂, for 2 hours in order to allow the ganglia to adhere to the laminin. The petri dishes were then supplemented with 2 ml of the above pre-warmed F14/horse serum medium containing nerve growth factor (10 ng.ml⁻¹). After 2 days in culture at 37°C in air containing 5% CO₂, the cells were incubated with cytosine-arabino-furanoside (3 ng.ml⁻¹) for 24 hours in order to prevent further division of non neuronal post-mitotic cells and maintain a pure preparation of dorsal root ganglia. The medium was thereafter changed every 4-5 days. Cells were used after 10-15 days in culture (Figure 2.1).
**NaCl** 137 mM  
**KCl** 2.7 mM  
**KH$_2$PO$_4$** 1.5 mM  
**Na$_2$HPO$_4$.12H$_2$O** 3.2 mM

**The phosphate buffered saline (pH 7.4) was made up to a final volume of 200 ml, filter sterilised through 2 μm filters and kept at 4°C.**

**Table 2.1 Components of Phosphate Buffered Saline**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.5 mM</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.12H$_2$O</td>
<td>3.2 mM</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Table 2.2 Components of F14 with Horse serum (HS) culture medium**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Stock Solution</th>
<th>Final Conc</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham's Nutrient F14</td>
<td>Imperial</td>
<td>55 g of stock F14 powder in 500 ml sterile water</td>
<td>10%</td>
<td>50 ml aliquots of stock solution stored at -20°C</td>
</tr>
<tr>
<td>Heat-inactivated Horse serum</td>
<td>Gibco</td>
<td>Stock</td>
<td>10%</td>
<td>50 ml aliquots of stock stored at -20°C</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Sigma</td>
<td>200 mM in Sterile water</td>
<td>2 mM</td>
<td>Made up fresh</td>
</tr>
<tr>
<td>Penicillin + Streptomycin</td>
<td>Flow</td>
<td>5000 i.u + 5000 μg/ml</td>
<td>50 iu.ml$^{-1}$ + 50 μg ml$^{-1}$</td>
<td>3 ml aliquots of stock solution stored at -20°C</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sigma</td>
<td>Salt</td>
<td>14 mM</td>
<td>-</td>
</tr>
</tbody>
</table>

**The F14/HS medium was made up in sterile de-ionised water; filter-sterilized through 2 μm filters and kept at 4°C for a maximum of 21 days.**

**Table 2.2 Components of F14 with Horse serum (HS) culture medium**
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Stock Solution</th>
<th>Final Conc</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-ornithine</td>
<td>Sigma</td>
<td>25 mg/ml in sterile water</td>
<td>250 µg/ml</td>
<td>100 µl aliquots of stock sol\textsuperscript{m} in sterile water stored at -20°C.</td>
</tr>
<tr>
<td>Laminin</td>
<td>Sigma</td>
<td>0.1 mg/ml in PBS. Filter sterilised</td>
<td>1 µg/ml in PBS</td>
<td>10 µl aliquots of stock sol\textsuperscript{m} stored at -70°C.</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Sigma</td>
<td>12.5 µg/ml in sterile water. Filter-sterilised</td>
<td>1.25 µg/ml in F14 + HS</td>
<td>120 ul of stock stored at -20°C</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Sigma</td>
<td>25 µg/ml in sterile PBS. Filter-sterilised</td>
<td>2.5 µg/ml in PBS</td>
<td>120 µl of stock stored at -20°C</td>
</tr>
<tr>
<td>DNase</td>
<td>Sigma</td>
<td>1600 kunits/ml in sterile water. Filter-sterilised</td>
<td>70 µg/ml in F14 + HS</td>
<td>120 ul of stock stored at -20°C</td>
</tr>
<tr>
<td>Nerve growth factor (NGF)</td>
<td>Sigma</td>
<td>100 mg/ml in sterile water. Filter-sterilised</td>
<td>10 ng/ml in F14/HS medium</td>
<td>120 µl of stock stored at -20°C</td>
</tr>
</tbody>
</table>

Table 2.3 Reagents used in preparation of DRG cell culture
Figure 2.1  Dorsal root ganglion neurones in primary cell culture

DRGs at 10 days in primary cell culture. Scale bar = 20 µm
2.2 Immunocytochemistry

The immunocytochemistry protocol was essentially the same as that utilised by Menon-Johansson et al (1993), although in this study the biotin-Extravidin amplification procedure was applied in order to enhance the fluorescent signal.

The coverslips of cultured DRG neurones were removed from the petri dishes and placed on a raised platform in a humidified container. All incubations were performed by gently adding a 200 µl volume of solution across the coverslip.

Cells were washed with 154 mM NaCl containing 40 mM Tris pH 7.4 (TBS) and fixed with 4% paraformaldehyde in TBS for 30 minutes at room temperature. When permeabilisation was required the cells were incubated for 3 x 5 minutes with 0.02% Triton X-100 in TBS. Cells were then washed (3 x 5 min) with TBS containing 20% goat serum, 4% bovine serum albumin and 0.1% DL-lysine in order to reduce non specific binding of the primary antiserum/antibody. The DRGs were then incubated with a dilution in the same medium of the anti-G-protein or anti-calcium channel subunit antisera (see Table 2.4 and Figure 2.2/2.3), overnight at 4°C. The cells were washed again (4 x 5 min) in the 20% goat serum solution and then incubated with goat anti-rabbit IgG conjugated to biotin (diluted 1:200) for 120 min at 4°C. Following a further wash (4 x 5 min) in 20% goat serum the cells were incubated for 1 hour at room temperature with the fluorescent probe, Extravidin conjugated to fluorescein (diluted 1:50) (both reagents from Sigma). The DRGs were washed (5 x 5 min) with TBS and mounted in antifade mountant (Citifluor, City University, London) and viewed with a confocal scanning laser microscope (Biorad, Hemel Hempstead).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Location of immunising peptide</th>
<th>Immunising peptide sequence</th>
<th>Information</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Anti Go serum (OC2)</td>
<td>1:2000</td>
<td>C-terminal decapeptide of $G_{10}$</td>
<td>ANNLRCGGLY</td>
<td>Polyclonal antiserum</td>
<td>Dr G Milligan (Glasgow University)</td>
</tr>
<tr>
<td>Go antibody</td>
<td>50 µg/ml</td>
<td>As above</td>
<td>ANNLRCGGLY</td>
<td>Affinity purified antibody</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Gi antibody</td>
<td>50 µg/ml</td>
<td>C-terminal decapeptide of $G_{11}$ and $G_{12}$</td>
<td>KENLKDCGGLF</td>
<td>Affinity purified antibody</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel A clone</td>
<td>1:1000</td>
<td>amino acids 1374-1388 Region between III$\text{S}_5$/III$\text{S}_6$</td>
<td>YEKNEVKARDREWKK</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel B-clone</td>
<td>1:1000</td>
<td>amino acids 851-867 Intracellular loop between III$\text{S}_5$/III$\text{S}_1$</td>
<td>RHRHRDRTKTSASTPA</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
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<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel C-clone</td>
<td>1:1000</td>
<td>amino acids 799-812 Intracellular loop between III$\text{S}_5$/III$\text{S}_1$</td>
<td>EEEKIELKSITAD</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel D-clone</td>
<td>1:1000</td>
<td>amino acids 1437-1434 Region between IV$\text{S}_5$/IV$\text{S}_6$</td>
<td>KLCDDSDYNPGEYTC</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
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<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel $\beta$-subunit</td>
<td>1:1000</td>
<td>amino acids 65-79</td>
<td>SRPSDSDVSEEDRE ($\beta_{1,2,4}$)</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SRPSLSDVSEEDRE ($\beta_{1}$)</td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel $\alpha_2$ subunit</td>
<td>1:1000</td>
<td>amino acids 469-483 Putative intracellular loop</td>
<td>SLEDIKRLPRFTLC</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel $\alpha_2$-subunit</td>
<td>1:1000</td>
<td>amino acids 1-15 N-terminal peptide</td>
<td>EPFSAVIKSWVDKC</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
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<td>Dr K Brickley RFHSM</td>
</tr>
<tr>
<td>Calcium channel $\delta$-subunit</td>
<td>1:1000</td>
<td>amino acids 933-951 N-terminal peptide</td>
<td>EAADMEDDFTSMSKOSC</td>
<td>Polyclonal antiserum</td>
<td>Dr N Berrow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dr K Brickley RFHSM</td>
</tr>
</tbody>
</table>

All antibodies and antisera were stored at -70°C in 5-10 µl aliquots

**Table 2.4 Description of anti-G protein antibodies and anti-calcium channel antisera used in this study**
Figure 2.2  Recognition sites of the anti-Ca2+ channel antisera
Figure 2.3 Recognition sites for the VDCC $\alpha_2$-subunit antisera
2.3 Antibody production and Purification

The anti-peptide antibodies used in this study are described in Table 2.4 and Figures 2.2 and 2.3. All antibodies and antisera were manufactured by Dr N.S Berrow and Dr K Brickley (Royal Free Hospital School of Medicine), except for the anti-Go antiserum (OC2) which was kindly donated by Professor G. Milligan (Glasgow University). The procedures used for producing the antisera are outlined below.

2.3.1 Peptide synthesis and antibody production:

The antiserum and antibodies were raised, purified and characterised using previously described methods (Davies et al 1987, Berrow et al 1995, Brickley et al 1995). The immunising peptides, with an additional C-terminal cysteinyl residue, were generated using the Nα-fluorenylmethoxycarbonyl-polyamide solid-phase peptide synthesis method (Atherton & Sheppard 1985). High performance liquid chromatography (HPLC) revealed that the peptides had a purity of > 80%, so they were used without further purification. The peptides were coupled via their cysteine residues to ovalbumin using maleimidobenzoyl-N-hydroxysulphosuccinimide (Pierce Chemical Co. USA). The peptide conjugates were then emulsified in complete Freund’s adjuvant, in order to induce an immunogenic response when injected intramuscularly into female half-lop rabbits. An addition injection of immunising peptide was made in incomplete Freud’s adjuvant after 4 weeks. For the second injection the immunising peptide was conjugated to keyhole limpet protein in order to minimise the production of anti-ovalbumin antisera. Animals were bled after another 2 weeks. Antisera were treated at 60°C for 30 minutes in order to inactivate complement and then stored at -70°C. Control sera were obtained from the rabbits before the first injection.

2.3.2 Affinity Purification:

Peptide-specific antibodies were purified from crude serum by repeated passage of the serum samples through a column of immunising peptide (2 mg peptide/ml gel) immobilised on Sulfolink coupling gel. The column was then washed with phosphate-buffered saline (PBS; Table 2.1) in order to remove non-specifically bound protein. Bound IgG was then eluted in 5M MgCl₂, followed immediately by a 10-fold dilution in distilled water. The samples then underwent dialysis versus PBS, in order to remove any residual Mg²⁺, since the presence of Mg²⁺ has a denaturing effect on the antibody. Purified antibodies were then concentrated and stored at -70°C.
2.4 Confocal Microscopy

Confocal microscopy offers the advantage over conventional fluorescent techniques, of having the facility to selectively record a fluorescent image which is in focus at a set depth in the specimen. The confocal scanning microscope focuses a bright source of pinpoint illumination, supplied by the laser, onto a single point at a specific depth in the specimen. The fluorescent signal emitted from the focal point of the illuminated material is collected and brought to an image at the photomultiplier. A pinhole aperture is placed at the photomultiplier, at the site that is confocal with the illuminating pinhole, where the rays emitted from the illuminated point in the specimen come to a focus. Thus the light from this point in the specimen converges on this aperture and enters the photomultiplier. In contrast, the light from regions out of the plane of focus of the illuminating laser is excluded from the photomultiplier. The laser moves through the specimen and the images from each point in the plane of focus are collected and digitised in order to yield a two-dimensional image. Since this system can dissect the fluorescent signal from discrete regions throughout the cell, it offers the potential to study the localisation of the immunoreactivity of certain proteins, extra- and intracellularly.

Immunostained cells were viewed with an MRC-600 series laser scanning confocal imaging system (Biorad) using an Argon ion multi line laser (15Mwatt) and an Olympus upright microscope. Laser illumination of the specimen was filtered at 494-520 nm through a filter selective for the fluorochrome, fluoroscein isothiocyanate (FITC). A second channel was used to record the corresponding phase contrast image.

2.4.2 Data acquisition and analysis

Confocal images were taken across the specimen in the X and Y plane, images throughout the depth (Z plane) of the specimen were also recorded in 1 μm or 2 μm sections. Each fluorescent image in the XY plane was scanned 6 times for each Z level and the resulting mean image was digitised and recorded onto an optical disk cartridge. Images were taken under conditions of constant illumination of the laser, without prior laser exposure. To determine the level of immunofluorescence associated with each cell, all images were enhanced equally to make best use of the the total range of grey values (0-255). Using the image analysis facility supplied with the confocal Biorad system, the average grey value intensity (GVI) was obtained for each fluorescent image. The image analysis procedure involved manually selecting the fluorescent region associated with a single 2 μm section that had been recorded midway through the cell. The fluorescent image was merged with the corresponding phase contrast image of the cell in order to
differentiate the boundary of the cell and an outline of the section was made using a cursor on the computer screen. When the immunostaining produced a fluorescent signal which was distributed diffusely throughout the section, the whole of the confocal section was selected and the average GVI value for that section of the cell was obtained. When the fluorescent signal was localised, for example at the plasma membrane, then, using a merged fluorescent/phase contrast image, the average GVI value for the discrete region in which the fluorescence was localised was obtained, an average GVI value was then assigned for that region. In order to limit inaccuracy in the measurement of GVI values the initial data acquisition process was performed with all data acquisition parameters (aperture, gain, grey value ramp) constant for each cell. The fluorescent data are expressed as mean ± sem (GVI-arbitrary units)

2.5 Microinjection

To identify DRGs following microinjection, cells were grown on coverslips with a 2 mm circle etched in the centre, all cells within the circle were injected. In some experiments the DRGs were plated out on CELLlocate coverslips (Eppendorf, Germany). These coverslips contain a 175 μm square numerically labelled grid in order to record the location of the injected cells. Prior to microinjection the cells were removed from the culture incubator and placed in warmed CO₂-independent medium (Gibco BRL) containing penicillin (50 i.u ml⁻¹). This medium avoids fluctuations in the cells' ambient pH following withdrawal from the 5% CO₂ incubator. Cells were microinjected using an automated Eppendorf microinjector and Femtotip pipettes (Zeiss/Eppendorf). This apparatus was attached to a Nikon microscope in order that the injection procedure could be monitored using phase contrast optics. The cells were microinjected with approximately 20 fl of oligonucleotide, representing about 5 x 10⁶ copies, using pipettes of diameter 0.5 μm, an injection time of 0.1 s and an injection pressure of 100 hPa. This gives a estimated internal oligonucleotide concentration of 3 μM, assuming a cell volume of 4 pl, which is comparable to the oligonucleotide concentration used to dialyse lactotrophes in the study of Baertschi et al., (1992). The injection target was the cell cytoplasm since oligonucleotides accumulate rapidly in the nucleus following cytoplasmic microinjection (Leonetti et al., 1991). The success of intracellular injections was verified in several experiments by the injection of fluorescein isothiocyanate-conjugated dextran.

Following microinjection the cells were transferred to sterile 35 mm petri dishes containing 1 ml of pre warmed Hamm's F14/heat inactivated horse serum medium supplemented with nerve growth factor (10 ng.ml⁻¹) and returned to the incubator until required.
2.6 Oligonucleotides

2.6.1 G-protein oligonucleotides

Phosphorothioate deoxyoligonucleotide 20-mers (British Biotechnology, Oxford or Oswel, Edinburgh) were used in this study. The oligonucleotides purchased from British Biotechnology had a purity of 85-90%. Oligonucleotides purchased from Oswel were purified by the manufacturers using high performance liquid chromatography (HPLC) giving an enhanced purity of > 95%. All oligonucleotides were stored at 4°C at a concentration of 500 μM in distilled water.

The Go antisense oligonucleotide had the sequence: 5'ATCGCCTTGCTCGCTCGAG3' and will hybridize with oligonucleotides 37-56 inclusive of the mRNA for both Go1 and Go2 (Hsu et al., 1990) and all other published Go sequences. The Gi antisense oligonucleotide had the sequence: 5'AGGTTCCTGTCGATCATCTT3' and will hybridize with nucleotides 51-70 inclusive of the mRNA for Gi1, Gi2 and Gi3 (Jones and Reed, 1987). The nonsense oligonucleotide used as a control in these experiments had the sequence: 5'GAAGTACCTTGTTGGTGG3'. The nonsense oligonucleotide has a target sequence without any significant homology to any sequence within the Genbank/EMBL database, and no other sequences with significant homology to Go and Gi antisense target sequences were found.

2.6.2 Calcium channel β-subunit oligonucleotides

The oligonucleotides used for the β-subunit study were 26-mers (Oswel, Edinburgh). The oligonucleotides were purified by the manufacturers using HPLC to yield a purity of > 95%. The oligonucleotides were stored at a concentration of 500 μM in distilled water at 4°C.

The VDCC β-subunit antisense oligonucleotide had the sequence: 5'ACCAGCCTTCCGTCCACCAGTCATT3', this is 96% complementary to nucleotides 487-511 of the B1β mRNA (Pragnell et al., 1991), and 92% complementary to nucleotides 676-701 of the B2 mRNA (Perez-Reyes et al., 1992), 394-419 of the B3 mRNA (Castellano et al., 1993b) and 397-422 of the B4 mRNA (Castellano et al., 1993a). The only sequences found within the Genbank/EMBL databases to have significant homology to the VDCC β-subunit antisense target sequence were those encoding other VDCC β-subunits.

The nonsense oligonucleotide initially used as a control in these experiments had the same sequence as the 20-mer nonsense oligonucleotide described above, which had previously
been used as the control in the G-protein study. Subsequently, a 26-mer oligonucleotide with a scrambled β-subunit antisense sequence was used as the control. This control scrambled oligonucleotide had the sequence:

\[ 5' \text{AACGCTTACGTCCACCTACTTACCCG3'} \]

and was of the same length and contained the same base sequence composition as the VDCC β-subunit antisense oligonucleotide. The control scrambled oligonucleotide has no target sequence of significant homology within the Genbank/EMBL database.

2.7 GTPase Assay

2.7.1 Preparation of frontal cortex membranes for GTPase assay and \[^3^H\] PH200 110 binding

200-250g male Wistar rats (BK Universal, Hull, UK) were sacrificed by cervical dislocation and decapitated. The scalp was removed with scissors in order to expose the cranium. A scissor blade was then placed into the foramen magnum and a cut was made along the dorsolateral border of the calvarium from caudal to rostral. The dorsal calvarium was lifted up and removed. A scissor cut was made between the olfactory bulb and the cerebral cortex, transecting the optic nerves. The brain was gently pulled out of the cranial vault and placed on filter paper which had been moistened with homogenising buffer (see below) and placed on a large petri dish lid on ice. The olfactory bulb, dura and blood vessels were removed. An incision was made through the corpus callosum in order to separate the cerebral hemispheres. A coronal slice was made with a cold scalpel, rostral to the occipital and parietal cortex in order to obtain the frontal cortex (Figure 2.4).

Figure 2.4 Dissection of rat brain frontal cortex (coronal view)
Underlying regions of brain were removed and the frontal cortex was placed in ice cold homogenising buffer
(10mM Tris-HCl/1mM EDTA, pH 7.4 containing the following protease inhibitor cocktail: 5 mM benzamidine, 5 mM dithiothreitol, 100 μM chlorpromazine, 50 μM leupeptin and 0.25 U/ml soybean trypsin inhibitor ) and homogenized (7 stokes) in a glass tube with a Teflon pestle. Following centrifugation at 1,000 x g for 10 minutes at 4°C, the supernatant was centrifuged at 40,000 x g for 10 minutes at 4°C. The resulting membrane pellet was washed and resuspended in the homogenizing buffer.

2.7.2 GTPase Assay

The protocol for the GTPase assay was essentially the same as that described by Koski & Klee (1981), except for a few minor modifications. 20 μl of membrane suspension were added in triplicate to 5 ml glass tubes containing 30 μl test solution (H2O or drug or 100 μM GTP) and 50 μl of GTPase assay mix. The latter solution contained approximately 80,000 cpn [γ32P]GTP (NEN) and the following reagents such that their final concentration in 100 μl assay was: 20 mM Tris-HCl (pH 7.4), 0.1mM EDTA, 100mM NaCl, 5 mM MgCl2, 2 mM dithiothreitol, 1 mM oubain, 1 mM ATP, 1 mM creatine phosphate, 5U creatine phosphokinase and 0.5 μM cold GTP. The creatine phosphate and creatine phosphokinase were included in the assay in order to replenish ATP levels. Oubain acts as an inhibitor of the Na+/K+ ATPase pump, the presence of oubain in the assay therefore limits the occurrence of free phosphate nucleotides resulting from activation of the Na+/K+ ATPase pump. The contribution of a high Km GTPase to total GTP hydrolysis is routinely corrected for by subtraction of the amount of 32P released in the presence of 100 μM unlabelled GTP. [32P]GTP hydrolysis at 37°C for 10 minutes was terminated by the addition of 900 μl of ice cold 2 % charcoal in 20 mM phosphoric acid, pH 2.2. The tubes were then immediately centrifuged at 1,500 x g for 10 minutes at 4°C, allowing bound phosphate to be retained in the charcoal pellet and free phosphate to remain in the supernatant. 200ul of the resulting supernatant were counted for [32P] content in a liquid scintillation counter (Beckman LS 6000 SC) in a total volume of 2 ml scintillation fluid (Hionic-Fluor, Packard).

2.7.3 Analysis of GTPase assay

The basic equation of enzyme kinetics is the Michaelis-Menten equation (Michaelis & Menten 1913):

$$v = V_{max} \frac{[S]}{[S] + K_m} \quad \text{Equation 1}$$
This equation is based on the theory that \( v \), the rate of product formation, is directly proportional to the concentration of enzyme, \([E]\). This equation also assumes that \( v \) generally follows saturation kinetics with respect to the concentration of substrate, \([S]\), such that at low \([S]\), \( v \) increases linearly with \([S]\). However, as \([S]\) is increased, this relationship begins to break down and \( v \) increases less rapidly than \([S]\) until, at a saturating \([S]\), \( v \) tends towards a limiting value termed \( V_{\text{max}} \). \( K_m \) refers to the substrate concentration at which the reaction rate is half of its maximal value.

The Michaelis-Menten equation can be transformed into a linear version in the form of an Eadie-Hofstee plot (Eadie 1942, Hofstee 1959). *Equation 1* may be rearranged to give:

\[
v = V_{\text{max}} - \frac{K_m v}{[S]}
\]

*Equation 2*

Plotting \( v \) against \( v/[S] \) gives an intercept of \( V_{\text{max}} \) on the y-axis. The slope of this line is equal to \(-K_m\). The intercept on the x-axis is \( V_{\text{max}}/K_m \). Thus, \( V_{\text{max}} \) and \( K_m \) values for the enzymatic reaction can be calculated.

### 2.8 \(^{3}\text{H}\) PN 200 110 Binding

#### 2.8.1 \(^{3}\text{H}\)PN 200 110 Binding

The \(^{3}\text{H}\)PN200 110 binding protocol used in this study was based upon the procedures used by Rampe *et al* (1986) and Ferrante *et al* (1989).

200 µl of membrane suspension and 200 µl of either H₂O or 10 µM nicardipine (to assess non-specific binding) were added in duplicate to a 5 ml tube containing 500 µl of binding buffer of the following composition (mM) NaCl, 132; KCl, 5; CaCl₂, 1.2; MgCl₂, 1.3; glucose, 10; Tris-hydroxymethylaminomethane, 25; adjusted to pH 7.4 with HCl. This binding buffer also contained \(^{3}\text{H}\)-PN200 110 (Amersham) at a range of concentrations (nM): 0.5, 1.0, 2.0, 5.0, 10.0 in order to determine \( B_{\text{max}} \) and \( K_d \) using Scatchard transformation analysis.

Following incubation at 25°C for 60 minutes the reaction was terminated by filtration over GF/B filters. The filters were washed with 3 x 3 ml volumes of ice cold Tris-HCl buffer (50 mM, pH 7.4) and then counted for \(^{3}\text{H}\) content in a liquid scintillation counter in a 2 ml volume of scintillation fluid (Hionic-Fluor).
2.8.2 Analysis of [3H]PN200 110 Binding

The initial step in the action of a drug on specific receptors is the formation of a reversible drug-receptor complex. This reaction obeys the Law of Mass Action, which states that the rate of a chemical reaction is proportional to the product of the concentration of reactants.

If a piece of tissue contains a total number of receptors \(N_{\text{tot}}\), for an agonist such as adrenaline, when the tissue is exposed to adrenaline at concentration \(x_A\), and allowed to reach equilibrium, then a certain number \((N_A)\) of receptors will become occupied and the number of vacant receptors will be reduced to \(N_{\text{tot}}-N_A\). The magnitude of the resulting physiological response will be proportional to the number of receptors occupied.

The reaction can be represented by:

\[
A + R \xrightleftharpoons[k_{-1}]{k_{+1}} AR
\]

Where \(A\) is the drug concentration \((x_A)\), \(R\) is the amount of free receptors \((N_{\text{tot}}-N_A)\), and \(AR\) is the drug-receptor complex \((N_A)\).

If the Law of Mass Action is applied, then:

Rate of forward reaction \(= k_{+1}x_A(N_{\text{tot}}-N_A)\) \(\text{Equation 3}\)

Rate of backward reaction \(= k_{-1}N_A\) \(\text{Equation 4}\)

At equilibrium the two reaction rates are equal:

\[
k_{+1}x_A(N_{\text{tot}}-N_A) = k_{-1}N_A \\
\text{Equation 5}
\]

The proportion of receptors occupied, \(p_A = N_A/N_{\text{tot}}\), which is independent of \(N_{\text{tot}}\) by rearranging \text{Equation 5} is:

\[
p_A = \frac{x_A}{x_A + \frac{k_{-1}}{k_{+1}}} \\
\text{Equation 6}
\]

The equilibrium constant, \(K_A\), is characteristic of the agonist, and is numerically equal to the concentration of agonist required to occupy 50% of the sites at equilibrium. Defining the equilibrium constant for the binding reaction:

\[
K_A = \frac{k_{-1}}{k_{+1}}, \\
\text{Equation 7}
\]

\(\text{Equation 6}\) can now be rewritten:
In a ligand binding experiment, the relationship between the amount of radiolabelled ligand bound (B) and the ligand concentration (x), substituting B/B_max for P_A, and x for x_A is:

\[ B = \frac{B_{\text{max}}x}{x + K_D} \quad \text{Equation 9} \]

B_max is the total number of binding sites in the preparation and K_D is the equilibrium constant. Equation 9 may be rearranged to give:

\[ \frac{B}{x} = \frac{B_{\text{max}}}{K_D} - \frac{B}{K_D} \quad \text{Equation 10} \]

A plot of B/x against B is known as a Scatchard plot. This plot gives a straight line from which the binding parameters K_D and B_max can be calculated. B_max is the intercept of the x-axis and K_D is calculated as the negative gradient of the line.

2.9 Incubation of membranes with antisera

Frontal cortex membranes were incubated at 30°C for 1 hour with either anti G-protein or anti calcium chanel antiserum or affinity purified antibodies (see Table 2.3). Preimmune serum of a corresponding dilution was used as the control when antisera were used, and rabbit IgG, of a corresponding concentration, was the control when affinity purified antibodies were used.

2.10 Incubation with VDCC β-subunit site peptide

The VDCC β-site peptide had the sequence : QQLEEDLKGYLDWI which is homologous to the VDCC β-α1 interaction site described by Pragnell et al (1994). This peptide sequence corresponds to amino acids 428-441 in the rat brain α_1C and α_1D calcium channel clones, which are located on the cytoplasmic loop linking transmembrane domains I and II. The control scrambled peptide had the sequence : GYLLEDEKWILDQQ, this peptide has no significant homology with any other peptide sequence in the Genbank/EMBL databases. The frontal cortex membranes were incubated with the peptides (20 μg/ml;10 μM) for 1 h at 30°C prior to the experiment.
2.11 Depolarisation of DRG neurones with $K^+$

In order to depolarise DRG neurones, the cells were incubated in Krebs medium where Na$^+$ was replaced by either 30 mM or 50 mM K$^+$. Non-depolarised cells were incubated in basal maintaining Krebs medium, containing 3.7 mM K$^+$ (Table 2.5).

The coverslips of DRG neurones were removed from their petri-dishes containing F14/HS culture medium and replaced in new petri dishes containing 1 ml of prewarmed Krebs medium containing either 3.7, 30 or 50 mM K$^+$. The petri dishes containing the cells were then returned to the incubator. The cells were maintained in the Krebs medium for 20 sec or 2 min at 37°C in order to induce depolarisation. The cells were then immediately fixed with 4% paraformaldehyde and prepared for immunocytochemistry.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[K$^+$]</td>
</tr>
<tr>
<td>Basal Krebs</td>
</tr>
<tr>
<td>High $K^+$ Krebs</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

All Krebs solutions were adjusted to pH 7.4 at 37°C, filter sterilised and stored at 4°C.

*Table 2.5 Composition of the Krebs medium used to $K^+$-depolarise DRG neurones*
2.12 Drugs and Reagents

<table>
<thead>
<tr>
<th>Drug</th>
<th>Source</th>
<th>Stock</th>
<th>Storage</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Baclofen</td>
<td>Dr W Bencze</td>
<td>30 mM in distilled H₂O</td>
<td>-20 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIBA-GEIGY Pharmaceuticals, Horsham UK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-BayK 8644</td>
<td>Research Biochemicals Inc</td>
<td>15 mM in 70 % ethanol. Drug dissolved after brief sonication in ice cold H₂O.</td>
<td>-20 °C</td>
<td>Final conc of EtOH in assay was 10 nl/ml</td>
</tr>
<tr>
<td>(-)-BayK 8644</td>
<td>Natick, USA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicardipine</td>
<td>Sigma</td>
<td>10 mM in distilled H₂O</td>
<td>-20 °C</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 Information and source of drugs used in this study

Experiments using dihydropyridines were performed under dimmed sodium illumination. All other chemicals were of analytical grade and were obtained from Sigma (Poole, Dorset, UK).
Chapter 3

Localisation of Components of the Voltage-Dependent Calcium Channel in Dorsal Root Ganglion Neurones
This Chapter is concerned with the localisation of the α1, α2/δ and β-subunit components of voltage-dependent Ca\(^{2+}\) channels (VDCC) in dorsal root ganglion (DRG) neurones. The various subunits are discussed in different sections of this Chapter. The α1-subunits are discussed in Section 3.1, the α2/δ subunit in Section 3.2 and the β-subunit in Section 3.3. The localisation of the G-proteins, G\(_{o}\) and G\(_{i}\), is discussed in Section 3.4.

### 3.1 Localisation of Ca\(^{2+}\) channel subtype α1-subunits in DRG neurones

#### 3.1.1 Introduction

The characterisation of voltage-dependent calcium channels (VDCC) has been made by virtue of their various biophysical and pharmacological profiles (MacDonald et al. 1994, Spedding & Paoletti 1992, Tsien & Tsien, 1990). These studies have indicated that several types of high-voltage activated VDCCs exist, namely L, N, P/Q, and R (Zhang et al. 1993). Biochemical and molecular techniques have revealed that the skeletal muscle calcium channel is a multisubunit complex (Catterall et al. 1988), consisting of an α1 subunit (165-190 kDa), which forms a functional ion pore and contains the main drug/toxin interaction sites, and ancillary α2-δ (170 kDa), β (55 kDa) and, in skeletal muscle γ (30 kDa) subunits, which each serve to modulate the properties of the α1-subunit (Perez-Reyes & Schneider 1994). Molecular cloning techniques have identified six genes which encode the α1-subunit, four genes which encode the β-subunit, one α2 gene and one γ-subunit gene (Perez-Reyes & Schneider 1994). These components of VDCCs are discussed in Chapter 1. In this study, immunocytochemical techniques utilising antipeptide anti-VDCC α1 subunit antisera have been used to address the localisation of each of the α1\(_A\), α1\(_B\), α1\(_C\) and α1\(_D\) cloned Ca\(^{2+}\) channel subtypes in cultured dorsal root ganglion (DRG) neurones. The anti-VDCC antisera recognition sites are described in Figure 3.1. The anti-α1\(_A\) antiserum was raised against amino acids 1374-1388 of the deduced rat brain Ca\(^{2+}\) channel α1\(_A\) amino acid sequence (Starr et al. 1991). This peptide sequence is also conserved in the isoforms of rabbit brain α1\(_A\) (Mori et al. 1991). The anti-α1\(_A\) antiserum has been shown previously to recognise a 155 kDa protein in a rabbit t-tubule preparation (K. Brickley, unpublished observations). The anti-α1\(_B\) antiserum was raised against amino acid residues 851-867 of the deduced rat brain Ca\(^{2+}\) channel α1\(_B\) amino acid sequence (Dubel et al. 1992). This peptide sequence bears 100 % sequence homology to the human brain isoforms of α1\(_B\) (Williams et al. 1992). The anti-α1\(_C\) antiserum was directed to amino acid residues 799-812 of the rat brain DHP-sensitive Ca\(^{2+}\) channel amino acid sequence (Snutch et al. 1991), and this sequence was conserved in all other cloned α1\(_C\) subunits (Mikami et al. 1989, Koch et al. 1990, Biel et al. 1990). The anti-α1\(_D\) antiserum was raised against amino acid residues 1417-1434 of the deduced rat brain Ca\(^{2+}\) channel α1\(_D\) subunit sequence (Hui et al. 1991).
and was conserved in the human pancreatic and neuronal $\alpha_1D$ sequences (Seino et al 1992, Williams et al 1992).
Figure 3.1 Recognition sites of the anti-Ca2+ channel antisera
3.1.2 Results

3.1.2.1 Localisation of the Class A subtype of Ca\textsuperscript{2+} channel
In order to localise the \( \alpha_1 \) subunit of the Class A subtype of Ca\textsuperscript{2+} channel in DRG neurones the cells were fixed with paraformaldehyde, detergent-permeabilised and incubated with the antipeptide anti-\( \alpha_1 \text{A} \) antiserum. Confocal microscopy revealed immunostaining associated with \( \alpha_1 \text{A} \) around the somal plasma membrane (Figure 3.2A). Preincubation of the anti-\( \alpha_1 \text{A} \) antiserum with its immunising peptide (100 \( \mu \)g/ml, 1h at 37\( ^\circ \)C) abolished the \( \alpha_1 \text{A} \) immunoreactivity (Figure 3.2B).

3.1.2.2 Localisation of the Class B subtype of Ca\textsuperscript{2+} channel
In order to localise the \( \alpha_1 \) subunit of the Class B subtype of Ca\textsuperscript{2+} channel in DRG neurones the cells were fixed, detergent-permeabilised and incubated with the antipeptide anti-\( \alpha_1 \text{B} \) antiserum and prepared for immunocytochemistry. Immunostaining associated with \( \alpha_1 \text{B} \) was detected around the somal plasma membrane and neurites (Figure 3.3A). The specific \( \alpha_1 \text{B} \) immunostaining was abolished following pretreatment of the anti-\( \alpha_1 \text{B} \) antiserum with its immunising peptide (100 \( \mu \)g/ml 1h at 37\( ^\circ \)C) (Figure 3.3B).

3.1.2.3 Localisation of the Class C subtype of Ca\textsuperscript{2+} channel
In order to localise the \( \alpha_1 \) subunit of the Class C subtype of Ca\textsuperscript{2+} channel in DRG neurones the cells were fixed, detergent-permeabilised and incubated with the antipeptide anti-\( \alpha_1 \text{C} \) antiserum. Immunolocalisation of \( \alpha_1 \text{C} \) was detected primarily around the somal plasma membrane, although weak immunostaining was also observed in the neurites (Figure 3.4A). Preadsorption of the anti-\( \alpha_1 \text{C} \) antiserum with its corresponding immunising peptide abolished \( \alpha_1 \text{C} \) immunoreactivity (Figure 3.4B).

3.1.2.4 Localisation of the Class D subtype of Ca\textsuperscript{2+} channel
In order to localise the \( \alpha_1 \) subunit of the Class D subtype of Ca\textsuperscript{2+} channel in DRGs the cells were fixed, permeabilised and prepared for immunocytochemistry utilising the antipeptide anti-\( \alpha_1 \text{D} \) antiserum. \( \alpha_1 \text{D} \) immunostaining was detected around the somal plasma membrane and neurites, the neurite immunostaining exhibited a punctate pattern (Figure 3.5A). Preadsorption of the anti-\( \alpha_1 \text{D} \) antiserum with its corresponding immunising peptide (100 \( \mu \)g/ml 1h at 37\( ^\circ \)C) abolished \( \alpha_1 \text{D} \) immunostaining (Figure 3.5B).
Figure 3.2 Immunolocalisation of the VDCC $\alpha_1A$ subunit

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the anti-$\alpha_1A$ antiserum (1:1000 dilution). A, immunostaining associated with $\alpha_1A$ was observed exclusively around the plasma membrane (pm). B, preadsorption of the anti-$\alpha_1A$ antiserum with its immunising peptide (100 $\mu$g/ml 1h at 37°C) abolished $\alpha_1A$-immunoreactivity. Fluorescent images are 2 $\mu$m confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 $\mu$m.
DRGs were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the anti-\(\alpha_1\)B antiserum (1:1000 dilution).
A, immunostaining associated with \(\alpha_1\)B was observed around the plasma membrane (pm) and at the neurites (n)
B, preadsorption of the anti-\(\alpha_1\)B antiserum with its immunising peptide (100 \(\mu\)g/ml 1h at 37°C) abolished \(\alpha_1\)B-immunoreactivity.
Fluorescent images are 2 \(\mu\)m confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 \(\mu\)m.
Figure 3.4 Immunolocalisation of the VDCC $\alpha_{1C}$ subunit

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the anti-$\alpha_{1C}$ antiserum (1:1000 dilution). A, immunostaining associated with $\alpha_{1C}$ was observed exclusively around the plasma membrane. B, preadsorption of the anti-$\alpha_{1C}$ antiserum with its immunising peptide (100 $\mu$g/ml 1h at 37°C) abolished $\alpha_{1C}$-immunoreactivity. Fluorescent images are 2 $\mu$m confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 $\mu$m.
Figure 3.5 Immunolocalisation of the VDCC $\alpha_{1D}$ subunit

DRGs were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the anti-$\alpha_{1D}$ antiserum (1:1000 dilution).
A, immunostaining associated with $\alpha_{1D}$ was observed around the plasma membrane and at the neurites (n).
B, preadsorption of the anti-$\alpha_{1D}$ antiserum with its immunising peptide (100 $\mu$g/ml 1h at 37°C) greatly reduced $\alpha_{1D}$-immunoreactivity.
Fluorescent images are 2 $\mu$m confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 $\mu$m.
3.1.2.5 VDCC α1 subunit immunoreactivity is reduced following preadsorption of the VDCC antisera with their respective immunising peptides

DRGs were fixed, detergent-permeabilised and incubated with the anti-α1A (Figure 3.6A), anti-α1B (Figure 3.6B), anti-α1C (Figure 3.6C) or anti-α1D antisera (Figure 3.6D). The degree of VDCC α1 subunit immunoreactivity associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the fluorescent region associated with each cell, and using the corresponding phase contrast image to differentiate the boundary of the cell, an average grey value intensity (GVI) value was ascribed to each DRG. GVI values were obtained for cells with had been incubated with each of the antipeptide anti-α1 subunit antisera, +/- preincubation of the antisera with its immunising peptide (100 µg/ml, 1h at 37°C).

Figure 3.6 shows the decrease in the immunofluorescent signal (grey value intensity) measured following preadsorption of the VDCC antisera with their respective immunising peptides (100 µg/ml, 1h at 37°C). The immunofluorescent signal observed following incubation of the cells with the corresponding preimmune sera is also shown.

3.1.2.6 In the absence of permeabilisation no α1A, α1B, α1C or α1D immunostaining is observed

In intact DRG neurones, in the absence of detergent-permeabilisation, immunostaining associated with the anti-α1A, anti-α1B, anti-α1C and anti-α1D antisera was absent (Figure 3.7).
Figure 3.6 Analysis of VDCC α1 subunit immunoreactivity following preadsorption of the anti-VDCC α1-subunit antisera with their respective immunising peptides

The degree of VDCC α1-subunit immunofluorescence associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the fluorescent region associated with each cell, and using the corresponding phase contrast image to differentiate the boundary of the cell, an average grey value intensity (GVI) value was ascribed to each DRG. GVI for DRG neurones which were incubated with the anti-α1A (A), anti-α1B (B), anti-α1C (C) and anti-α1D (D) antisera (■). The GVI for cells incubated with the anti-α1A (A), anti-α1B (B), anti-α1C (C) and anti-α1D (D) antisera which had been preincubated with their respective immunising peptides (100 µg/ml, 1h at 37°C) (□). The immunofluorescent signal observed following incubation of the cells with the corresponding preimmune sera is also shown (■■■). Results are expressed as mean GVI ± sem. *p<0.05, compared to the GVI of cells incubated with the anti-VDCC α1 subunit antisera in the absence of immunising peptide, n=5.
In the absence of permeabilisation no $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$ or $\alpha_{1D}$ immunoreactivity was observed.

DRG neurones were fixed with 4% paraformaldehyde and incubated with either the anti-$\alpha_{1A}$ (A), anti-$\alpha_{1B}$ (B), anti-$\alpha_{1C}$ (C) or anti-$\alpha_{1D}$ (D) antisera (all at 1:1000 dilution).

In intact cells no $\alpha_{1A}$ (A), $\alpha_{1B}$ (B), $\alpha_{1C}$ (C) or $\alpha_{1D}$ (D) immunoreactivity was observed. Fluorescent images are 2 $\mu$m confocal sections taken midway through the cell. Scale bar=20 $\mu$m.
3.1.3 \textit{K+} depolarisation of DRG neurones reveals the respective recognition sites for the anti-\(\alpha_{1A}\) and anti-\(\alpha_{1D}\) antisera

The anti-\(\alpha_{1B}\) and anti-\(\alpha_{1C}\) Ca\(^{2+}\) channel antisera were raised against putative intracellular regions on the \(\alpha_1\)-subunit of the rat brain class B subtype of Ca\(^{2+}\) channel (Dubel \textit{et al} 1992) and rat brain DHP-sensitive class C subtype of Ca\(^{2+}\) channel (Snutch \textit{et al} 1992) respectively. Immunostaining associated with these antisera could therefore only be observed following detergent-permeabilisation of the membrane (Figure 3.3, Figure 3.4 respectively). In contrast, the anti-\(\alpha_{1A}\) and anti-\(\alpha_{1D}\) antisera were raised against putative extracellular regions on transmembrane domain III of the rat brain A-clone of Ca\(^{2+}\) channel (Starr \textit{et al} 1991) and transmembrane domain IV of the D-clone Ca\(^{2+}\) channel (Hui \textit{et al} 1991). Immunostaining associated with these antisera was observed only following detergent-permeabilisation of the membrane (Figure 3.2, Figure 3.5 respectively). This immunocytochemical result was surprising since both the anti-\(\alpha_{1A}\) and anti-\(\alpha_{1D}\) antisera should interact with their respective putative extracellular recognition sites (see Figure 3.1) in intact cells.

In order to assess if depolarisation of the membrane was sufficient to expose the anti-\(\alpha_{1A}\) and anti-\(\alpha_{1D}\) antisera recognition sites in non-permeabilised intact DRG neurones, the immunolocalisation of \(\alpha_{1A}\) and \(\alpha_{1D}\) was examined in DRG neurones which had been depolarised with K\(^{+}\) prior to preparation of the cells for immunocytochemistry.

3.1.4 Resting membrane potential: The concentration of certain key ions vary on the inside and outside of cells. On the outside, Na\(^{+}\), Ca\(^{2+}\) and Cl\(^{-}\) exist in much higher concentrations; by contrast, K\(^{+}\) ions and membrane impermeant anions are concentrated on the inside. Since ions are unequally distributed across the membrane, they tend to diffuse down their concentration gradients through specific ion channels. Ions are electrically charged molecules, and therefore, their movement across the membrane is dictated by the voltage difference across the membrane, as well as the concentration gradients. If a membrane is made permeable to K\(^{+}\) ions by the opening of K\(^{+}\) channels, then the K\(^{+}\) ions would leave the cell to diffuse down their concentration gradient. The removal of the positively charged K\(^{+}\) ions would cause the inside of the cells to have a net negative charge and consequently this would tend to attract the K\(^{+}\) ions and slow down the rate of flow out of the cell. The voltage difference at which the rate of K\(^{+}\) flow out of the cell is neutralised by the attraction of the intracellular negative charge, is termed the equilibrium potential (\(E\)), and is different for each ionic species. The equilibrium potential for any given ion can be calculated from its intracellular and extracellular concentrations using the Nernst equation:
Where \( E \) is the equilibrium potential in volts, \( C_o \) and \( C_i \) is the outside and inside concentrations of the ion, respectively, \( R \) is the gas constant, \( T \) is the absolute temperature, \( F \) is Faraday's constant, \( z \) is the valency of the ion.

The postulation that the \( K^+ \) ion is the primary ion involved in maintaining the resting membrane potential in excitable cells was first proposed by Bernstein (1902). In later experiments it was shown that the membrane potential of squid axons (Curtis & Cole 1942) and isolated frog muscle fibres (Hodgkin & Horowicz 1959) was dependent upon the extracellular \( K^+ \) concentration. At rest the cellular membrane is dominated by its permeability to \( K^+ \) due to the presence of \( K^+ \) leak channels. The \( K^+ \) permeability draws the membrane potential of the cell to approximately \(-100 \) mV. At rest neuronal membranes are also slightly permeable to \( Na^+ \) and \( Cl^- \) ions, such that the membrane potential is also pulled towards \( E_{Na^+} \) (\(+40 \) mV) and \( E_{Cl^-} \) (\(-75 \) mV). The point at which the movements of these varied ions come into equilibrium such that there is no net current flow across the membrane corresponds to the resting membrane potential and is typically between \(-60 \) to \(-80 \) mV.

3.1.5 Activation of an Action Potential: Brief changes in membrane potential induce signalling in nerve cells and this allows communication between the soma and terminals of a cell's axon. Action potentials result from a rapid depolarisation of the membrane potential from a threshold level, to positive to \( 0 \) mV. The action potential was first identified in the squid giant axon (Hodgkin & Huxley 1952) and those observations underly the basic changes in membrane ionic permeability occurring as part of the action potential. The voltage clamp technique was used to reveal that the rapid upswing of the action potential is mediated by a regenerative increase in a transient \( Na^+ \) current (\( I_{Na} \)). This current was found to be rapidly activated by depolarisation, and is itself a depolarising influence to act as a positive feedback loop. Depolarization of the membrane increases the number of \( Na^+ \) ions flowing down their electrochemical gradient into the cell. This transfer of charge further increases \( Na^+ \) permeability by opening voltage-sensitive \( Na^+ \) channels, so that more \( Na^+ \) ions cross the membrane and the membrane potential approaches the sodium equilibrium potential (\( E_{Na^+} \)). The \( Na^+ \) channels then begin to inactivate causing \( Na^+ \) inflow to decline. Subsequent activation of the delayed rectifier channel increases \( K^+ \) conductance to potentiate \( K^+ \) outflow. The membrane potential thus begins to fall, repolarization reduces \( K^+ \) permeability and the membrane potential is brought back to its resting level. At this point the \( K^+ \) permeability is still greater then normal and this causes the membrane
potential to pass the resting level and move even nearer to $E_{K^+}$. Finally, as the $K^+$ permeability returns to its normal value the membrane potential returns to its resting level. The depolarisation caused by entry of $Na^+$ ions into localised regions of membrane spreads to neighbouring membrane regions by electrotonic current flow. The depolarisation of the new region of membrane may then activate the same regenerative mechanism and thus bring about the propagation of the action potential along the axon. The triggering of an action potential occurs when the membrane potential of the neuron is depolarized sufficiently to reach an action potential threshold. The action potential threshold is the membrane potential at which the regenerative activation of inward depolarizing $Na^+$ currents is strong enough to overcome the inactivation of these currents, as well as the activation of other currents that hyperpolarize the neuron towards rest.

Membrane depolarisation is both a physiological and pathological stimulus for voltage-dependent $Ca^{2+}$ channels (VDCCs). Under physiological conditions depolarisation serves to permit $Ca^{2+}$ entry for stimulus-coupling processes. Under pathological conditions prolonged channel opening may cause excess $Ca^{2+}$ entry, cellular $Ca^{2+}$ overload, and cell damage and death. The subtypes of VDCC activated during a depolarising change in the membrane potential have different biophysical and pharmacological properties, which has allowed the various VDCC currents to be differentially observed in whole-cell current recordings in mouse sensory neurones (Bean 1985, Fox et al 1987). The subtypes of VDCC were found to be in the activated and inactivated states under different membrane potentials. The low-voltage-activated T-type calcium channels show bursting behaviour under single channel recording and are rapidly inactivated by steady depolarisation (Droogmans & Nilius 1989). Inactivation of these channels may be significant at resting membrane potentials, and thus the cells must have a hyperpolarised membrane potential in order to allow their observation. In contrast the high-voltage-activated calcium channels, which include the L, N, P, Q and R subtypes of calcium channel (Dolphin 1995), have a higher threshold of activation to produce a long lasting current of large conductance -see Introduction, Chapter 1.

### 3.1.6 Depolarisation of DRG neurones with 50 mM K⁺:

In order to depolarise the DRG cell membrane, the cells were placed in Kreb's buffer containing 50 mM K⁺. Control cells were maintained in 3.7 mM K⁺-containing Krebs medium. The permeability of the membrane to $K^+$ causes the $K^+$ ions to enter the cell via diffusion down the $K^+$ electrical gradient. Assuming an intracellular $K^+$ concentration of 150 mM, according to the Nernst equation, the increase in extracellular $K^+$ from 3 mM to 30 mM or 50 mM would depolarise the cell by 40 mV and 60 mV respectively. In cultured rat DRG neurones the resting membrane potential has been reported to be approximately -58 mV (Zang et al 1994), a 40 or 60 mV depolarising step would therefore adjust the membrane potential to -20 and 0 mV
respectively, and consequently the high-voltage-activated calcium channels would be activated and partially inactivated.
3.1.7 Results

3.1.7.1 K⁺ depolarisation for 2 min reveals distinct anti-α₁A and anti-α₁D recognition sites on the somal membrane

DRG neurones were incubated for 2 min at 37°C with Krebs solution (without Ca²⁺) containing either 3.7 mM K⁺ (Figure 3.8A, C) or 50 mM K⁺ (Figure 3.8B, D). No α₁A or α₁D immunoreactivity was observed when the cells were incubated with 3.7 mM K⁺-containing Krebs medium (Figure 3.8 A, C respectively). However, following depolarisation of the cells with Krebs medium containing 50 mM K⁺, α₁A (Figure 3.8 B) and α₁D (Figure 3.8 D) immunoreactivity was detected around the somal plasma membrane. In some cells a punctate distribution of α₁A and α₁D was observed.

3.1.7.2 Preadsorption of the anti-α₁A and anti-α₁D antisera with their respective immunising peptides abolished the α₁A and α₁D immunoreactivity observed following depolarisation.

DRG neurones were incubated for 2 min at 37°C in Krebs medium containing either 3.7 mM or 30 mM K⁺. When the cells were incubated in basal Krebs medium containing 3.7 mM K⁺ (Figure 3.9A) no α₁A immunoreactivity was observed. Following depolarisation of the DRG neurones with 30 mM K⁺ (Figure 3.9B), α₁A immunoreactivity was observed around the somal plasma membrane. Preadsorption of the anti-α₁A antiserum with its corresponding immunising peptide (100 µg/ml, 1h at 37°C) (Figure 3.9C) abolished the α₁A immunoreactivity detected around the somal plasma membrane following depolarisation of the cells with 30 mM K⁺. No α₁D immunoreactivity was observed following incubation of the cells in basal Krebs medium containing 3.7 mM K⁺ (Figure 3.9D). Following depolarisation of the DRG neurones with 30 mM K⁺ (Figure 3.9E), α₁D immunoreactivity was observed around the somal plasma membrane with a punctate distribution. The α₁D immunoreactivity associated with the somal plasma membrane following K⁺-induced depolarisation was abolished following preadsorption of the anti-α₁D antiserum with its corresponding immunising peptide (100 µg/ml, 1h at 37°C) (Figure 3.9F).
Figure 3.8 Depolarisation with 50 mM K⁺ reveals the anti-α₁A and anti-α₁D antisera recognition sites

In order to depolarise the DRGs, the cells were incubated for 2 min in Krebs medium containing 50 mM K⁺. Control cells were incubated in basal maintaining Krebs containing 3.7 mM K⁺. The cells were then fixed with 4% paraformaldehyde and prepared for immunocytochemistry utilising either anti-α₁A (A, B) or anti-α₁D antisera (C, D) (both at 1:1000 dilution).

A, no α₁A immunostaining was observed in non-depolarised control cells.

B, following K⁺-depolarisation for 2 min, α₁A immunoreactivity was observed around the plasma membrane with a punctate distribution.

C, no α₁D immunostaining was observed in non-depolarised control cells.

D, following K⁺-depolarisation for 2 min, α₁D immunoreactivity was observed around the plasma membrane.

Fluorescent images are 2 μm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 μm.
Figure 3.9 Depolarisation of DRG neurones with 30 mM K⁺ reveals the anti-α₁ᴬ and anti-α₁ᴰ antisera recognition domains

DRG neurones were either maintained for 2 min at 37°C in basal Krebs solution containing 3.7 mM K⁺ (A, D), or depolarised for 2 min at 37°C in Krebs solution containing 30 mM K⁺ (B, C, E, F). The DRG neurones were then fixed with 4% paraformaldehyde, incubated with the anti-α₁ᴬ (A, B) or anti-α₁ᴰ (D, E) antisera and prepared for confocal microscopy.

α₁ᴬ immunoreactivity was absent from the DRG neurones which had been incubated in the 3.7 mM K⁺-containing Krebs medium (A). α₁ᴬ immunoreactivity was observed around the plasma membrane with a punctate distribution in the DRG neurones which had been depolarised in the 30 mM K⁺-containing Krebs medium (B). Preadsorption of the anti-α₁ᴬ antisera with its immunising peptide (100 µg/ml, 1h at 37°C) (C) abolished α₁ᴬ immunostaining observed following depolarisation of the cells with 30 mM K⁺.

α₁ᴰ immunoreactivity was absent from the DRG neurones which had been incubated in the 3.7 mM K⁺-containing Krebs medium (D). α₁ᴰ immunoreactivity was also observed around the plasma membrane with a punctate distribution in the DRG neurones which had been depolarised in the 30 mM K⁺-containing Krebs medium (E). Preadsorption of the anti-α₁ᴰ antisera with its immunising peptide (100 µg/ml, 1h at 37°C) (C) abolished α₁ᴰ immunostaining observed following depolarisation of the cells with 30 mM K⁺.

Fluorescent images are 2 µm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 10 µm.
3.1.7.3  **K**⁺ depolarisation for 20 sec reveals distinct anti-α₁ₐ and anti-α₁ₒ recognition sites on the somal membrane

Decreasing the K⁺ depolarisation incubation time to from 2 min to 20 sec had no effect on the distribution of α₁ₐ (Figure 3.10 B) and α₁ₒ (Figure 3.10 C) immunoreactivity in DRG neurones. α₁ₐ and α₁ₒ immunoreactivity were still observed around the somal membrane following depolarisation for 20 sec with Krebs medium containing 50 mM K⁺.

3.1.7.4  **K**⁺ depolarisation in the presence of 1.8 mM **Ca**²⁺

In order to examine if the revelation of the anti-α₁ₐ and anti-α₁ₒ antibody recognition sites following K⁺ depolarisation was affected by Ca²⁺-dependent processes, the DRG neurones were depolarised with 50 mM K⁺ for 2 min in the absence (Figure 3.11 A,C) and presence (Figure 3.11 B,D) of 1.8 mM **Ca**²⁺. Immunoreactivity associated with α₁ₐ (Figure 3.11 A, B) and α₁ₒ (Figure 3.11 C, D) was observed around the plasma membrane in cells which had been depolarised in the absence or presence of **Ca**²⁺. There was no difference in the distribution of α₁ₐ and α₁ₒ immunostaining when the cells were depolarised in media containing 1.8 mM **Ca**²⁺.

3.1.7.5  **VDCC** β-subunit immunoreactivity is not revealed following K⁺ depolarisation

In order to assess if K⁺ induced depolarisation caused disruption of the plasma membrane, immunoreactivity associated with the intracellular VDCC β-subunit was examined in K⁺-depolarised cells. DRG neurones were depolarised for 2 min with 50 mM K⁺ and prepared for immunocytochemistry utilising the anti-β subunit antiserum. No VDCC β-subunit immunoreactivity was observed in control cells which had been incubated in basal Krebs medium for 2 min containing 3.7 mM K⁺ (Figure 3.12 A). Similarly, no β-subunit immunofluorescence was observed in cells which had been depolarised for 2 min in Krebs medium containing 50 mM K⁺ (Figure 3.12 B).
Figure 3.10 Depolarisation of DRG neurones with 50 mM K⁺ for 20 sec reveals the anti-α₁A and anti-α₁D antisera recognition domains

DRG neurones were either maintained for 20 sec at 37°C in basal Krebs solution containing 3.7 mM K⁺ (A, C), or depolarised for 20 sec at 37°C in Krebs solution containing 50 mM K⁺ (B, D). The DRG neurones were then fixed with 4% paraformaldehyde, incubated with the anti-α₁A (A, B) or anti-α₁D (C, D) antisera and prepared for confocal microscopy. α₁A immunoreactivity was absent from the DRG neurones which had been incubated in the 3.7 mM K⁺-containing Krebs medium (A). α₁A immunoreactivity was observed around the plasma membrane with a punctate distribution in the DRG neurones which had been depolarised in the 50 mM K⁺-containing Krebs medium (B). α₁D immunoreactivity was absent from the DRG neurones which had been incubated in the 3.7 mM K⁺-containing Krebs medium (C). α₁D immunoreactivity was also observed around the plasma membrane with a punctate distribution in the DRG neurones which had been depolarised in the 50 mM K⁺-containing Krebs medium (D). Fluorescent images are 2 µm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 10 µm.
Figure 3.11 Depolarisation of DRG neurones with 50 mM K$^+$ in the presence of 1.8 mM Ca$^{2+}$ reveals the anti-$\alpha_1\Lambda$ and anti-$\alpha_1\Delta$ antisera recognition domains

DRG neurones were depolarised for 2 min at 37°C in Krebs solution containing 50 mM K$^+$, either in the absence (A, C) or presence (B, D) of 1.8 mM Ca$^{2+}$. The DRG neurones were then fixed with 4% paraformaldehyde, incubated with the anti-$\alpha_1\Lambda$ (A, B) or anti-$\alpha_1\Delta$ (C, D) antisera and prepared for confocal microscopy. $\alpha_1\Lambda$ immunoreactivity was observed around the plasma membrane in the DRG neurones which had been depolarised in the 50 mM K$^+$-containing Krebs medium in the both the absence (A) and presence (B) of 1.8 mM Ca$^{2+}$.

$\alpha_1\Delta$ immunoreactivity was observed around the plasma membrane in the DRG neurones which had been depolarised with 50 mM K$^+$-containing Krebs medium in the both the absence (C) and presence (D) of 1.8 mM Ca$^{2+}$.

Fluorescent images are 2 µm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 µm.
Figure 3.12 Depolarisation of DRG neurones with 50 mM K\(^+\) does not reveal the anti-VDCC β subunit antiserum recognition domain

DRG neurones were either maintained for 2 min at 37°C in basal Krebs solution containing 3.7 mM K\(^+\) (A), or depolarised for 2 min at 37°C in Krebs solution containing 50 mM K\(^+\) (B). The DRG neurones were then fixed with 4% paraformaldehyde, incubated with the anti-VDCC β subunit antiserum and prepared for confocal microscopy. VDCC β-subunit immunostaining was absent from the DRG neurones which had been incubated in the 3.7 mM K\(^+\)-containing Krebs medium (A). VDCC β-subunit immunoreactivity was also absent from the DRG neurones which had been depolarised in the 50 mM K\(^+\)-containing Krebs medium (B). Fluorescent images are 2 μm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 10 μm.
3.1.8 Discussion of the VDCC subtype α1-subunit localisation

The distribution of the calcium channel subtypes, α1A, α1B, α1C and α1D, was examined in cultured dorsal root ganglion neurones using immunocytochemical techniques.

3.1.8.1 Localisation of α1A

The α1A calcium channel subtype, when expressed in Xenopus oocytes, is sensitive to ω-Aga-IVA, from the crude venom of the funnel-web spider Agelenopsis aperta (Mori et al. 1991). This pharmacological result, along with the abundant distribution of α1A in cerebellar Purkinje neurones (Llinas et al. 1992), suggested that α1A corresponded to the P-type calcium channel current. However, expression of α1A in oocytes resulted in a current that could be partially inhibited by ω-Aga-IVA and more potently inhibited by ω-CTX MVIIC (Zhang et al. 1993). In contrast, the P-type current in Purkinje cells was found to be 100-fold more sensitive to inhibition by ω-Aga-IVA, and 10-fold less sensitive to inhibition by ω-CTX MVIIC, although the latter finding has since been refuted (Zhang et al. 1993). Those authors therefore proposed that α1A corresponded to the cerebellar granule neurone Q current, which was found to have a similar biophysical and pharmacological profile to the current observed following expression of α1A in oocytes. α1A may thus represent the P or Q-type current identified in cerebellar Purkinje (Mintz et al. 1992) and granule (Zhang et al. 1993) cells respectively.

In DRG neurones, the anti-α1A antiserum, which was raised against amino acid residues at a region N-terminal to the SS1/SS2 pore lining domain of transmembrane domain III, labelled the plasma membrane of the cell body, following detergent-permeabilisation of the cells. The immunostaining associated with α1A was abolished by pretreatment of the antiserum with its immunising peptide indicating that the antiserum was interacting with its recognition site in a peptide-specific manner. The corresponding preimmune serum produced no detectable immunoreactivity.

P-type channels have been shown to contribute to 23% of the total high-voltage activated Ca²⁺ channel current in DRG neurones (Mintz et al. 1992). The distribution of the P-type channel in the mammalian central nervous system has been previously demonstrated immunohistochemically using a polyclonal antibody raised against the P-type Ca²⁺ channel (Hillman et al. 1991). The study by these authors identified immunolabelling associated with P-type Ca²⁺ channel antibody throughout the molecular layer of the cerebellum and on Purkinje cell dendrites, although relatively little immunostaining was observed in the Purkinje cell soma. Electron microscopy revealed P-type Ca²⁺ channel immunoreactivity associated with patches of somatic plasma membrane and dendrites; portions of the smooth endoplasmic reticulum were also labelled. P-type Ca²⁺ channel immunostaining was also observed in many other regions of the brain, including the brainstem, cortex, CA1 layer of
the hippocampus and spinal nucleus. A polyclonal antiserum raised against the rat brain Ca$^{2+}$ channel $\alpha_1A$ subunit, was found to exhibit specific immunostaining at the mammalian neuromuscular junction, and in the granule cell layer of the cerebellum in the region where mossy fibres synapse with granule cell dendrites, although no localisation of $\alpha_1A$ was observed in the Purkinje cells (Ousley & Froehner 1994). The P-type calcium channel blocker, $\omega$-Aga-IVA has been shown to inhibit neurotransmitter release at the neuromuscular junction (Usowicz et al 1992) and in cultured cerebellar granule neurones (Huston et al 1995). The localisation of $\alpha_1A$ to synaptic sites within the cerebellar glomeruli (Ousley & Froehner 1994) corresponds to the inhibition of neurotransmitter release observed by $\omega$-Aga-IVA. However, the finding that $\alpha_1A$ was not localised in Purkinje cell soma and dendrites (Ousley & Froehner 1994), where P-type channels have been localised using electrophysiological techniques (Usowicz et al 1992), suggests that the $\alpha_1A$ subunit may be part of a calcium channel complex distinct from the P-type calcium channel expressed in Purkinje cells. The differential distribution of the P-type calcium channel and $\alpha_1A$ subunit may reflect alternative splicing of the class A $\alpha 1$ subunit transcript, or expression of a distinct but closely related gene. In contrast to the absence of $\alpha 1A$ in Purkinje cells reported by Ousley & Froehner (1994), the study by Stea et al (1994) used in situ localisation with antisense deoxyoligonucleotides and RNA probes to show that mRNA for $\alpha 1A$ was in fact distributed in Purkinje and granule cells. That study also showed that co-expression of the rat brain Ca$^{2+}$ channel $\alpha 1A$ subunit with $\beta 1b$ and $\beta 3$ subunits in oocytes caused a significant acceleration of inactivation of expressed $\alpha 1A$ currents, resulting in a biophysical profile consistent with the Q-type Ca$^{2+}$ current observed in cerebellar granule neurones (Zhang et al 1993). Co-expression of $\alpha 1A$ with the $\beta 2$ subunit dramatically slowed the inactivation of the $\alpha 1A$ current to that seen typically for P-type currents (Usowicz et al 1992). While P- and Q-type Ca$^{2+}$ channels may be encoded by distinct $\alpha 1$ subunit genes, the results presented by Stea et al (1994) suggest a significant structural and functional relationship between $\alpha 1A$ and both channel types. The differences in properties may be explained by alternative splicing of the $\alpha 1A$ gene, differential modulation by ancillary subunits or post-translational modification.

The finding in this study suggets that the VDCC $\alpha 1A$ subunit is exclusively localised to the somatic plasma membrane of detergent permeabilised DRG neurones, with no distribution in the neurites. It would be of interest to compare $\omega$-Aga IVA sensitivity of Ca$^{2+}$ channel current in axotomized DRGs and in intact DRGs in order to ascertain if the functional $\omega$-Aga IVA-sensitive channels were localised exclusively to the soma. Localisation of $\alpha 1A$ exclusively to the plasma membrane suggests that this Ca$^{2+}$ channel subtype may be involved to coupling cell surface electrical signals to intracellular processes. Elevation of intracellular Ca$^{2+}$ via L-type calcium channels has been reported to activate Cl$^-$ and K$^+$ channels, modulate the activity of other proteins via Ca$^{2+}$-dependent
phosphoprotein phosphatases and protein kinases (Kennedy 1989) and regulate gene expression (Sheng & Greenberg, Murphey et al 1991). $\text{Ca}^{2+}$ influx through $\alpha_1A$-type calcium channels may therefore have a role in the regulation of such processes, in a similar manner to the L-type calcium channel.

3.1.8.2 Localisation of $\alpha_1B$

In DRGs the anti-$\alpha_1B$ antiserum, which was raised against a domain on the intracellular loop linking transmembrane domains II and II, was found to be localised around the plasma membrane and neurite processes in detergent-permeabilised cells. Preincubation of the anti-$\alpha_1B$ antiserum with its corresponding immunising peptide abolished the immunostaining. The preimmune serum failed to produce any detectable immunoreactivity. Expression of $\alpha_1B$ in *Xenopus* oocytes produced a detectable current with biophysical and pharmacological properties consistent to those of the high-voltage activated N-type calcium channel current (Williams et al 1992). In acutely axotomized DRGs the high-voltage activated current was irreversibly inhibited approximately 55% by $\omega$-CTx-GVIA (Menon-Johansson et al 1993). In contrast, the high-voltage activated current in DRG neurones with intact neurites was inhibited approximately 70% by $\omega$-CTx-GVIA (Campbell et al 1995). The increase in $\omega$-CTx-GVIA sensitivity in DRG neurones containing their neurite processes suggests that functional N-type $\text{Ca}^{2+}$ channels are present in the neurites as well as at the soma and is consistent with the immunolocalisation of $\alpha_1B$ at the neuritic and somatic regions in DRG neurones. A similar $\alpha_1B$ localisation was been reported in other studies (Westenbroek et al 1992). A site-directed antipeptide antibody which recognized the rat brain $\alpha_1B$ subunit, was found to predominantly label dendrites, cell bodies and nerve terminals of cortical, hippocampal and cerebellar neurones. Localization at neuronal terminals suggests that $\alpha_1B$ may be involved in the regulation of neurotransmitter release. In DRG neurones electrically evoked release of substance P was inhibited by the N-type calcium channel blocker $\omega$-CTx-GVIA (Holz et al 1988). Furthermore, the excitatory synaptic responses induced in cultured spinal neurones upon stimulation of DRG neurones was eliminated by a selective non-NMDA receptor antagonist and $\omega$-CTx-GVIA (Gruner & Silva 1994). The result of that study suggests that N-type $\text{Ca}^{2+}$ channels play a dominant role in glutamatergic sensory neurotransmission and that modulation of N-type $\text{Ca}^{2+}$ channel activity may underlie presynaptic inhibition of synaptic transmission between DRG neurones and their targets in the spinal cord. The release of newly synthesized glutamate from cultured cerebellar granule neurones was also found to be blocked following $\omega$-CTx GVIA treatment (Huston et al 1994), thereby indicating a role for the N-type calcium channel in presynaptic modulation of transmitter release in central neurones.
3.1.8.3 Localisation of $\alpha 1_C$

The anti $\alpha 1_C$ antiserum was raised against an epitope on the intracellular loop linking transmembrane domains II and III on the $\alpha 1$ subunit of the rat brain class C subtype of $\text{Ca}^{2+}$ channel. In detergent-permeabilised DRGs $\alpha 1_C$ immunostaining was distributed primarily around the plasma membrane of the soma, although a weaker degree of immunostaining was associated with the neurites. The anti-$\alpha 1_C$ antiserum bound in a peptide-specific manner since pretreatment of the antiserum with its immunising peptide abolished the immunoreactivity. Expression of $\alpha 1_C$ in oocytes produced a detectable current which could be blocked by DHPs (Mikami et al 1989, Wei et al 1991, Singer et al 1991, Schultz et al 1993). In acutely axotomized DRG neurones, which had been maintained in primary cell culture for 3-days, the high-voltage activated calcium channel current was unaffected by the DHP antagonist nicardipine, suggesting that the cell body contained no functional DHP-sensitive $\text{Ca}^{2+}$ channels (Menon-Johansson et al 1993). However, $\text{Ca}^{2+}$ channel currents in non-axotomized DRG neurones, which had been maintained for 10 days in primary cell culture, were found to exhibit DHP sensitivity (Berrow et al 1995). The differences in $\text{Ca}^{2+}$ channel DHP sensitivity between axotomized and non-axotomized DRG neurones suggests that $\alpha 1_C$ may be abundant in the neurites, thereby causing DHP sensitivity to be greater in the DRG neurones with intact neurites. However, since the immunocytochemical result presented in this study describes $\alpha 1_C$ immunoreactivity primarily in the cell body, developmental processes may occur which direct $\alpha 1_C$ to the cell body in older cultures of DRG neurones. In cortical, hippocampal and cerebellar preparations immunoreactivity associated with $\alpha 1_C$ has also been reported to occur in neuronal cell bodies and proximal dendrites, with weaker immunoreactivity occurring at the distal dendrites (Hell et al 1993).

The distribution of $\alpha 1_C$ at the cell bodies of DRG neurones suggests that this subtype of $\text{Ca}^{2+}$ channel exerts a greater influence on cytosolic calcium levels in the cell body than in the distal dendrites or presynaptic nerve terminals. The localisation of the L-type calcium channel at cell bodies suggests their involvement in general cellular functions such as regulation of cellular signalling pathways and gene expression. Activation of L-type calcium channels has been shown to directly activate $\text{K}^+$ and $\text{Cl}^-$ channels and modulate the activity of other proteins via $\text{Ca}^{2+}$-dependent phosphoprotein phosphatases and protein kinases (Kennedy 1989). In addition, L-type calcium channel activity has been shown to modulate the expression of c-fos and several other immediate early genes in the nervous system (Murphy et al 1991, Sheng & Greenberg 1991). The neuronal $\alpha 1_C$ L-type calcium channel was found to undergo facilitation by positive pre-depolarisation (Bourinet et al 1994). The voltage-dependent facilitation of $\alpha 1_C \text{Ca}^{2+}$ channels is likely to contribute to $\text{Ca}^{2+}$ entry into the post-synaptic site which may regulate the above $\text{Ca}^{2+}$-dependent processes.
3.1.8.4 Localisation of $\alpha 1_D$

The anti-$\alpha 1_D$ antiserum was directed toward the region C-terminal to the putative SS1/SS2 pore lining region of transmembrane motif IV. The deduced amino acid sequence of $\alpha 1_D$ was found to have a high level of homology with the cardiac DHP-sensitive channel ($\alpha 1_C$) (Seino et al 1992). $\alpha 1_D$ was also found to be expressed predominantly in neuroendocrine tissue (Seino et al 1992, Hui et al 1991). Co-expression of $\alpha 1_D$ with $\alpha 2/\delta$ and $\beta$-subunits in oocytes produced a functional DHP-sensitive current, which, unlike $\alpha 1_C$, was reversibly inhibited by $\omega$-CTx-GVIA (Williams et al 1992).

Immunostaining associated with the anti-$\alpha 1_D$ antiserum was detected around the somal plasma membrane and neurites in detergent-permeabilised DRG neurones. In some cells a punctate distribution of $\alpha 1_D$ immunoreactivity was observed. Pretreatment of anti-$\alpha 1_D$ with its respective immunising peptide abolished $\alpha 1_D$ immunoreactivity, thereby providing evidence that the anti-$\alpha 1_D$ antiserum bound to its recognition site in a peptide-specific manner.

In central neurones $\alpha 1_D$ has been detected in the cell bodies and proximal dendrites in a variety of cell types, although no clustering of $\alpha 1_D$ channels was observed (Hell et al 1993). The punctate distribution of $\alpha 1_D$ in DRG neurones, particularly along the neurites, may indicate discrete clustering of $\alpha 1_D$ channels. These channels may be available to participate in localised accumulation of intracellular Ca$^{2+}$, which may have an important impact on local Ca$^{2+}$ concentrations in the neurites. Given the abundance of $\alpha 1_D$ in brain areas responsible for neuroendocrine function, such as the hypothalamus and pituitary gland (Chin et al 1992), $\alpha 1_D$ may have an important role in excitation-secretion coupling and in modulating neuroendocrine functions. In DRG neurones the release of substance P has been shown to exhibit DHP sensitivity due to the involvement of DHP-sensitive Ca$^{2+}$ channels in the neurosecretory process (Holz et al 1988). Hormone secretion from a variety of neuroendocrine cells has also been inhibited by blocking L-type Ca$^{2+}$ channels (Malaisse-Lagae et al 1984, Reisine 1990, Fakunding & Catt 1980). The L-type Ca$^{2+}$ channel encoded by $\alpha 1_D$ may therefore regulate hormone secretion from neurosecretory cells.

3.1.8.5 Voltage-dependence of interaction of the anti-$\alpha 1_A$ antiserum with its recognition domain on the $\alpha 1$ subunit of the class A subtype of VDCC

The anti-$\alpha 1_B$ and anti-$\alpha 1_C$ antisera were raised against amino acid residues located on the second putative intracellular loop of the $\alpha 1$-subunit on the rat brain class B and class C subtypes of Ca$^{2+}$ channel. Immunoreactivity associated with these antisera could only be
detected following detergent-permeabilisation of the DRG neurones. In contrast, the anti-α1A antiserum was raised against the region N-terminal to the putative SS1/SS2 pore lining region on transmembrane domain III on the α1-subunit of the rat brain class A subtype of Ca2+ channel. In the model for the proposed membrane arrangement of the α1A subunit polypeptide (Mori et al 1991), the anti-α1A antiserum recognition domain is reported to exist on the exofacial surface of the membrane. The inability of the anti-α1A antiserum to recognise its binding domain in the absence of permeabilisation suggests that the antiserum recognition domain may have been occluded by a membrane or protein component in resting cells.

Interestingly, depolarisation of the DRG neurones with elevated K+ prior to fixation, allowed α1A immunoreactivity to be detected around the somal plasma membrane, even in the absence of permeabilisation. Assuming an intracellular K+ concentration of 150 mM, an increase in extracellular K+ from 3 mM to 50 mM would depolarise the cell by +60 mV, according to the Nernst equation. The resting membrane potential of DRG neurones is approximately -58 mV (Wang et al 1994) and a 60 mV depolarising step would therefore clamp the membrane potential at 0 mV. The Ca2+ channel encoded by the α1A gene (Moru et al 1991, Starr et al 1991) has been shown to inactivate rapidly in response to positive potentials, with the peak current being reduced by 50% after a 400 msec depolarising pulse (Stea et al 1994). Expression of α1A in Xenopus oocytes produced a current with an inactivation time constant of 50-100 msec (Zhang et al 1993). Inactivation of the P-type channel current has also been reported to be dependent upon intracellular Ca2+ levels (Tareilus et al 1994). Following K+-depolarisation of the membrane for 2 min, a significant proportion of the Ca2+ channels encoded by α1A may therefore be in an inactivated state.

Chimeric Ca2+ channels in which portions of domain III or domain IV of a DHP-insensitive brain Ca2+ channel, were introduced into the DHP-sensitive L-type Ca2+ channel, revealed that the SS2-S6 linker in domain III played a critical role in the inactivation process of Ca2+ channels and therefore represented part of the gating structure (Tang et al 1993). In a similar manner, slow C-type inactivation of some types of K+ channel is thought to be mediated by hydrophobic amino acid residues in the extracellular end of the S6 region (Hoshi et al 1991). The anti-α1A antiserum has a recognition domain near to the region on transmembrane domain III which is critical for inactivation. The ability of the anti-α1A antiserum to bind to amino acid residues 1374-1388 of the A class Ca2+ channel α1 subunit following K+-depolarisation may therefore be attributed to a conformational change occurring in the α1-subunit, as a result of voltage-dependent channel inactivation, such that the anti-α1A antiserum recognition site is revealed to externally applied antisera (Figure 3.13 A). Alternatively, depolarisation may induce a conformation change which removes the occlusion of the antisera recognition sites by a lipid or protein particle. Depolarisation by K+ had no adverse affect on the integrity of the cell membrane since immunostaining
associated with the VDCC intracellular β-subunit was not revealed following the depolarisation protocol.

3.1.8.6 Voltage-dependence of interaction of the anti-α1D antiserum with its recognition domain on the α1 subunit of the class D subtype of VDCC

The anti-α1D antiserum was raised against a recognition domain C-terminal to the SS1-SS2 pore lining region on transmembrane domain IV on the α1-subunit of the rat brain D class of DHP-sensitive Ca^{2+} channel (Hui et al 1991). The proposed membrane arrangement of the α1D subunit polypeptide suggested that the anti-α1D antiserum recognition site was located on the exofacial surface of the membrane. Immunoreactivity associated with the anti-α1D antiserum was surprisingly only observed following detergent-permeabilisation of the membrane in resting cells.

However, when the cells were depolarised with elevated K^{+}, α1D immunoreactivity was observed around the somal membrane in the absence of permeabilisation. Assuming that application of 50 mM K^{+} produced a 60 mV depolarising step which clamped the cell membrane at 0 mV, high-voltage-activated calcium channels would be activated and partially inactivated. Although L-type calcium channels exhibit relatively little inactivation over a 200 msec depolarising pulse, when Ba^{2+} is used as the charge carrier (Fox et al 1987), with an inactivation constant of > 500 msec (Hille 1992). Inactivation of L-type channels is predominantly regulated by the concentration of intracellular Ca^{2+}, although this process is also voltage-dependent (Matsuda et al 1990, Jmari et al 1986, Kass & Sanguinetti 1984). After a relatively long K^{+}-depolarisation period of 20 sec or 2 min, differential proportions of the L-type channels may therefore exist in the activated or inactivated states.

In L-type Ca^{2+} channels the linker between the fifth (S5) and sixth (S6) α-helices on transmembrane motif IV has been identified as the major site for DHP action (Tang et al 1993). The DHP agonist and antagonist molecules interact with distinct sites on the α1 subunit of the L-type channel. The study of Tang et al (1993) constructed chimeric calcium channels in which the SS2-S6 region in motif IV of the L-type Ca^{2+} channel was replaced by the corresponding region of a DHP-insensitive channel (α1A). The resulting chimeric channel retained L-type sensitivity to the DHP antagonist, but lost sensitivity to DHP agonists. This suggests that the SS2-S6 region of motif IV is an essential site for DHP agonist action rather than for antagonist action. The DHP antagonist interaction site has been proposed to reside in other regions of motif IV. Binding experiments have shown that DHP antagonists exhibit increased binding affinity to Ca^{2+} channels in PC12 cells following K^{+}-depolarisation (Greenberg et al 1986). Electrophysiological studies have also shown that DHP antagonist activity is promoted at depolarised membrane potentials, which
results in an increase in the proportion of time channels remain in the closed state (Hess et al 1984). This has been interpreted as the binding of DHP antagonists to the inactivated state of the channel, possibly by interaction of the DHP with potential-sensitive structural components (Kokubun et al 1986), thereby promoting stabilization the channel in the inactivated conformation (Armstrong & Eckert 1987, Hymel et al 1988). In pituitary cells the increased binding affinity of DHP antagonists with depolarisation has been shown to result from an increased association rate; the dissociation rate was found to be independent of membrane potential (Lui et al 1994). That result contrasts with previous studies of the voltage-dependent binding of DHP antagonists to cardiac myocytes, where the increased affinity was reported to be derived from a decreased dissociation rate (Kokubun et al 1986, Wei et al 1989). These results suggest that different modes of action may exist for DHP antagonist interaction with L-type Ca\(^{2+}\) channels in pituitary cells and cardiac myocytes.

In this Thesis it has been shown that membrane depolarisation is a prerequisite for binding of the anti-\(\alpha_1D\) antiserum to its recognition site on the L-type Ca\(^{2+}\) channel in DRG neurones. Following K\(^+\)-depolarisation of the DRGs, the \(\alpha_1\)-subunit of the L-type Ca\(^{2+}\) channel may undergo a conformational change in transmembrane motif IV. The conformation change may relocate amino acid residues 1417-1434, corresponding to the C-terminal region of the SS2-S6 domain in transmembrane motif IV, to an exofacial site which is more accessible to recognition by the externally applied anti-\(\alpha_1D\) antiserum (Figure 3.13 B). Since DHPs have been shown to interact with transmembrane domain IV on the VDCC \(\alpha_1\)-subunit, possibly at a site close in proximity to the putative SS1-SS2 pore-lining domain, the conformational change which occurs in the VDCC \(\alpha_1\)-subunit to expose the anti-\(\alpha_1D\) recognition site may also underlie the molecular mechanism for DHP voltage-sensitivity.

Computer modelling of the voltage-dependent ion channel \(\alpha_1\) subunits identified a putative extracellular loop between S5 and S6 in all four transmembrane domains (Guy & Conti 1990). This region has been proposed to exist as an \(\alpha\)-helical hairpin loop (SS1) connected to a \(\beta\)-structure (SS2), bent back into the membrane, via strict consensus sequences that may form a bend structure in the SS2 segment (Varadi et al 1995). This region has been termed the SS1-SS2 or P-loop, and is thought to form the lining of the channel pore. Mutation of this conserved region in K\(^+\) and Na\(^+\) channels have suggested a role for SS1-SS2 in the regulation of ion conducting pathways (Heinemann et al 1992). By analogy to the Na\(^+\) channel, the SS1-SS2 region has been suggested to have a similar role in Ca\(^{2+}\) channels. The strict alignment of negatively charged Glu residues in the SS2 segment of each transmembrane motif, indicates that these side chains may impart selectivity for Ca\(^{2+}\) permeation. Mutation of individual Glu residues, particularly those occurring in transmembrane motif III, into either positively charged or uncharged residues decreased the Ca\(^{2+}\) selectivity of the L-type calcium channel ion conducting pathway (Tang et al 1993).
Yang et al (1993). The latter authors suggested that the Ca\(^{2+}\) selectivity was conferred by the four Glu residues located on the SS2 region, arranged on two close, but non-equivalent, planes and occupying trans positions. Depolarisation may induce a conformational change within the pore lining region of the Ca\(^{2+}\) channel \(\alpha_1\)-subunit polypeptide, resulting in a conformational transition of the region C-terminal to the SS2 domain. Such a conformation change following depolarisation may allow the anti-\(\alpha_1\)D antiserum access to its recognition binding site on the \(\alpha_1\)-subunit of the class D subtype of Ca\(^{2+}\) channel (Figure 3.13 B). Alternatively, since structural determinants located in transmembrane domain III and IV of the Ca\(^{2+}\) channel \(\alpha_1\) subunit have been implicated in activation of the L-type Ca\(^{2+}\) channel (Wang et al 1995), depolarisation of the cell membrane may promote a conformational change within the activation region which exposes the anti-\(\alpha_1\)D antiserum recognition site.

3.1.8.7 The immunolocalisation of \(\alpha_1A\) and \(\alpha_1D\) following depolarisation is unaffected by Ca\(^{2+}\)

The class A and class D subtypes of Ca\(^{2+}\) channel exhibit Ca\(^{2+}\)-dependent inactivation (Tareilus et al 1994, Matsuda et al 1990, Jmari et al 1986). The finding that both anti-\(\alpha_1A\) and anti-\(\alpha_1D\) immunostaining were identical in the absence or presence of Ca\(^{2+}\) suggests that revelation of the anti-\(\alpha_1A\) and anti-\(\alpha_1D\) antisera recognition sites following depolarisation was independent of a Ca\(^{2+}\)-dependent process of inactivation. In rat pituitary cells and cerebellar granule neurones down-regulation of L-type Ca\(^{2+}\) channels has been reported to occur via Ca\(^{2+}\) entry and activation of a calmodulin-dependent process in response to membrane depolarisation by K\(^{+}\) (Liu et al 1994). The study by those authors reported that L-type channels began to internalise 30 min after depolarisation by K\(^{+}\), a maximal internalisation being observed after 90 min. Since the distribution of \(\alpha_1A\) and \(\alpha_1D\) was comparable following depolarisation induced in both the presence and absence of Ca\(^{2+}\), it is unlikely that Ca\(^{2+}\)-dependent internalisation of the channel complex had any effect on the conformational state of the channel which revealed amino acid residues 1374-1388 of the rat brain A-type of Ca\(^{2+}\) channel and residues 1417-1434 of the rat brain class D Ca\(^{2+}\) channel to their respective extracellularly applied antisera. \(\alpha_1A\) and \(\alpha_1D\) immunoreactivity would have been absent from the somal membrane if the Ca\(^{2+}\) channels had been internalised by a Ca\(^{2+}\)-dependent mechanism during K\(^{+}\)-depolarisation.
Figure 3.13  Depolarisation reveals the anti-α1A and anti-α1D antisera recognition sites
3.2 Topography of the Voltage-dependent calcium channel α2-subunit

3.2.1 Introduction

The purification of the dihydropyridine receptor from skeletal muscle t-tubule membranes revealed the presence of at least three proteins, termed α, β and γ (Flockerzi et al 1986, Striessnig & Glossmann 1991). The existence of two α-subunits was uncovered (Sharp et al 1987, Leung et al 1987). Azidopine photolabelling studies showed that the α-subunit of molecular mass 165-190 kDa, had the capacity to bind DHPs. The mobility of this subunit on SDS-PAGE gels was not affected by reducing agents. In contrast, the other protein at ~170 kDa was found to be extensively glycosylated and unable to bind DHPs. This protein dissociated under reducing conditions (Chang & Hosey 1988), decreasing its motility to 142 kDa and 22-28 kDa, giving rise to a new nomenclature for the α-subunit. The DHP binding subunit which was unaffected under reducing conditions was termed the α1-subunit, while the subunit which dissociated under reducing conditions was termed α2/δ. The α2/δ subunit dissociated to α2 (142 kDa) and δ (22-28 kDa) components upon reduction.

The α2/δ primary transcript is encoded by a single gene, the α2 and δ subunit arise as a result of postranslational proteolytic cleavage of the C-terminal end of the α2/δ polypeptide (De Jongh et al 1990 Jay et al 1991) and the two components are linked by disulfide bond interaction (Takahashi et al 1987). In contrast to the varying protein sizes and primary sequences of α1 and β subunits in different tissues that contain L-type calcium channels, the α2/δ component is conserved in a wide range of organs. mRNA transcripts that hybridise with α2 cDNA probes are detectable in many tissues, including cardiac muscle, aorta, ileal smooth muscle, lung and brain (Ellis et al 1988 Kim et al 1992).

The cloning of α2/δ in 1988 allowed the proposal of a model for its putative arrangement in the membrane, based upon hydrophatic analysis of the deduced amino acid sequence (Ellis et al 1988). Those authors reported that although α2/δ is substantially hydrophilic, it does contain three hydrophobic regions that may represent transmembrane domains. The proposed topographical model predicted that the α2/δ component consisted of three membrane spanning domains. The large hydrophilic N-terminal domain was reported to be extracellular, based on the abundance of potential glycosylation sites. A large hydrophilic domain was proposed to exist on the cytoplasmic side of the membrane serving as a linker between the transmembrane regions. The shorter C-terminal domain was subsequently predicted to be intracellular (Figure 3.14 A). In contrast, a more recent model predicts that the entire α2 subunit is extracellular and is bound to the membrane via disulfide interaction with the δ component, which has a single putative membrane-spanning region, an exofacial N-terminus and an endofacial C-terminus (Jay et al 1991) (Figure 3.14 B).
In order to provide direct evidence for the arrangement of the \(\alpha2/\delta\) protein in the membrane, and thus allow discrimination between the two models, polyclonal antipeptide antisera, raised against highly conserved regions of the rabbit skeletal muscle \(\alpha2/\delta\) subunit cDNA sequence, have been used as immunological probes. The antisera correspond to amino acid residues 1-15, 469-483 and 933-951 of the \(\alpha2/\delta\) polypeptide sequence. The sequence 469-483 contains a potential consensus sequence for cAMP-dependent phosphorylation, which is located on the putative intracellular loop of the model of Ellis et al (1988), while being exofacial in the model of Jay et al (1991). The antisera have been used as membrane impermeant probes to study the orientation of these regions of both the \(\alpha2\) and \(\delta\) subunits in intact and detergent-permeabilised, cultured DRG neurones.
Figure 3.14 Alternative models for the membrane topography of the VDCC $\alpha 2$ subunit
3.2.2 Results

3.2.2.1 Localisation of the putative intracellular loop of the α2 subunit
DRGs exhibited plasma membrane and neurite associated immunofluorescence, following incubation with the α2 subunit antiserum, which had been raised against amino acid residues 469-483 of the α2 polypeptide, in detergent-permeabilised (Figure 3.15A) and non-permeabilised cells (Figure 3.15 B). The corresponding preimmune serum did not produce any immunofluorescent signal when incubated with detergent-permeabilised DRGs (Figure 3.15C).

3.2.2.2 Immunostaining associated with the α2-subunit antiserum was abolished following preadsorption of the antisera with its immunising peptide
Immunoreactivity associated with the anti-α2-subunit antiserum in permeabilised (Figure 3.16A) and non-permeabilised (Figure 3.16C) cells was abolished following preadsorption of the anti-α2-subunit antiserum with its immunising peptide (20 μg/ml, 1h at 37°C) in both permeabilised (Figure 3.16B) and intact DRG neurones (Figure 3.16D). The degree of VDCC α2-subunit immunoreactivity associated with permeabilised DRG neurones was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the fluorescent region associated with each cell, and using the corresponding phase contrast image to differentiate the boundary of the cell, an average grey value intensity (GVI) value was ascribed to each DRG. GVI values were obtained for permeabilised cells which had been incubated with the antipeptide anti-α2 subunit antiserum (Figure 3.17), either in the presence or absence of the immunising peptide (20 μg/ml, 1h at 37°C). Preadsorption of the anti-α2-subunit antiserum with its immunising peptide reduced α2-subunit immunoreactivity to the level of immunofluorescence observed following treatment of the cells with control preimmune serum (Figure 3.17).

3.2.2.3 Permeabilisation enhances the degree of α2 subunit immunostaining
Although α2 subunit immunostaining was found to occur around the plasma membrane and neurites under both permeabilised and nonpermeabilised conditions, a greater degree of immunostaining at the soma was observed in permeabilised cells (Figure 3.17). This may be explained by the α2 subunit having partial membrane association, which is revealed to the antiserum following permeabilisation.
Figure 3.15  Immunolocalisation of the VDCC $\alpha$2 subunit in DRG neurones

A, DRGs exhibited intense immunostaining associated with the plasma membrane and neurites, following permeabilisation with 0.02$\%$ tritonX and incubation with the antisera raised against aminio acid residues 469-483 of the $\alpha$2 subunit polypeptide (1:1000 dilution). B, In the absence of permeabilisation agents, $\alpha$2 immunostaining was associated with the exofacial surface of the plasma membrane. C, No immunofluorescence was observed following incubation of the DRGs with the corresponding preimmune serum (1:1000 dilution).

All cells were permeabilised with 0.02$\%$ triton prior to incubation with the antisera, except for the cells in image B. Images are 2 $\mu$m confocal sections, taken 10 $\mu$m up from the attachment plaque. Scale bar = 25 $\mu$m.
Figure 3.16 Immunolocalisation of the putative intracellular loop of the VDCC \(\alpha_2\) subunit

A, DRG neurones were permeabilised with 0.02% triton and incubated with the \(\alpha_2\) subunit antiserum (1:1000), raised against amino acid residues 469-483 of the \(\alpha_2\) subunit polypeptide. Intense immunostaining was observed around the plasma membrane of the soma and neurites. Preadsorption of the anti-\(\alpha_2\) subunit antiserum with its immunising peptide (20 \(\mu\)g/ml, 1h at 37°C) (B) abolished \(\alpha_2\) immunostaining in permeabilised cells.

C, In the absence of permeabilisation agents, \(\alpha_2\) immunostaining was observed around the exofacial surface of the plasma membrane of the soma and neurites. Preadsorption of the anti-\(\alpha_2\) subunit antiserum with its immunising peptide (20 \(\mu\)g/ml, 1h at 37°C) (D) abolished \(\alpha_2\) immunostaining in non-permeabilised cells.

Fluorescent images on the left are 2 \(\mu\)m confocal sections, taken 10 \(\mu\)m up from the attachment plaque. The respective phase images are on the right.

Scale bar = 10 \(\mu\)m.
In order to measure the level of α2-subunit immunoreactivity associated with each cell, the image analysis facility supplied with the confocal microscope was used. A cursor outlined the region of immunofluorescence associated with each cell, and using the corresponding phase contrast image to mark the cell boundary, an average grey value intensity (GVI) value was ascribed to each cell.

The GVI value for cells incubated with the anti-α2 subunit antiserum (aa 469-483) either in the presence (■) or absence (□) of permeabilisation was obtained. Results are expressed as mean GVI (arbitrary units) ± sem.*p< 0.05 compared to the GVI value for non-permeabilised cells incubated with the anti-α2 subunit antiserum, n=8.

The GVI value for permeabilised cells incubated with the corresponding preimmune serum (□□□) or anti-α2 subunit antiserum which had been preincubated with its immunising peptide (20 μg/ml, 1h at 37°C) (□□□□□) is also shown, +p< 0.05 compared to the GVI for cells incubated with the anti-α2 subunit antiserum in the absence of immunising peptide (■■) n=7.

Figure 3.17 Permeabilisation enhances α2-subunit immunoreactivity
3.2.2.4 Topography of the N-termini of the α2 and δ components of the α2/δ complex

In order to address the membrane topography of the rest of the α2/δ complex antipeptide antisera raised against the N-termini of the α2 (Figure 3.18 A, B) and δ components (Figure 3.18 C, D) of the VDCC were used to label DRGs for immunocytochemical studies. The N-terminal α2-subunit antiserum corresponded to amino acid residues 1-15 of the α2 polypeptide and the N-terminal δ subunit antiserum was raised against amino acid residues 1-17 of the δ polypeptide. Both antisera were found to immunolocalise at the plasma membrane and neurites in detergent-permeabilised (Figure 3.18 A, C) and intact cells (Figure 3.18 B, D). The finding that both the α2 and δ subunit polypeptide N-termini were immunolocalised to the plasma membrane in the absence of permeabilisation, suggests that these regions are also located on the extracellular surface of the plasma membrane.

The immunostaining associated with the N-terminal α2-subunit polypeptide antiserum in permeabilised (Figure 3.19A) and non-permeabilised cells (Figure 3.19C) was abolished following preincubation of the antisera with its immunising peptide (20 μg/ml, 1h at 37°C) in both permeabilised (Figure 3.19B) and non-permeabilised (Figure 3.19D) neurones. Similarly, immunostaining associated with the N-terminal δ-component polypeptide antiserum in permeabilised (Figure 3.19E) and non-permeabilised cells (Figure 3.19G) was abolished following preincubation of the antisera with its immunising peptide (20 μg/ml, 1h at 37°C) in permeabilised neurones (Figure 3.19F) and intact neurones (Figure 3.19H).

3.2.2.5 Localisation to two intracellular proteins; the VDCC β-subunit and Goα

Since the α2-subunit was raised against a region in the putative intracellular loop in the topography model of Ellis et al (1988), it was surprising that α2 immunostaining could be detected in intact cells, as well as in detergent-permeabilised cells. In order to test this phenomenon the localisation of two well characterised intracellular proteins was examined in intact and detergent-permeabilised cells. Immunostaining associated with the α subunit of the intracellular G-protein G0 was only observed following permeabilisation of the cells (Figure 3.16A). Similarly, immunostaining associated with the VDCC intracellular β-subunit, was observed only following detergent-permeabilisation of the cells (Figure 3.16B). The lack of VDCC β-subunit and Gα0 staining observed at the cytoplasmic surface following fixation confirms the integrity of the plasma membrane barrier if fixed cells. These results suggest that antibodies designed to recognise intracellular domains are unable to interact with these domains unless pores have been formed in the cell membrane during permeabilisation. The finding that the α2 antiserum recognises its binding domain, in a peptide specific manner, in the absence of permeabilisation, suggests that the amino acid residues 469-483 are in fact located on the exofacial surface of the plasma membrane.
Figure 3.18 Immunolocalisation of the N-termini of the α2 and δ components of the VDCC

A, DRGs were permeabilised with 0.02 % triton, and incubated with an α2 antiserum, raised against amino acids 1-15 of the α2 subunit (N-terminus) (1:1000). Immunostaining associated with this antiserum was found to occur around the plasma membrane.

B, in the absence of permeabilisation agents, immunostaining associated with the N-terminal α2 antiserum was still observed around the membrane.

C, DRGs exhibited plasma membrane immunostaining, following permeabilisation with 0.02% triton and incubation with an antiserum (1:1000) raised against the N-terminus of the δ component, corresponding to amino acid residues 1-17 of the δ polypeptide.

D, in non-permeabilisation cells immunostaining associated with the N-terminal δ- subunit antiserum was observed around the plasma membrane.

Fluorescent confocal images are 2 μm sections taken midway through the cell.

Scale bar=10 μm.
Figure 3.19 N-terminal α2 and δ reactivity is abolished following preincubation of the antisera with their respective immunising peptides.

Immunostaining associated with the α2 subunit N-terminal antiserum (1:1000) (corresponding to amino acids 1-15 of the α2 polypeptide) was found to occur around the plasma membrane in the presence (A) and absence (C) of the permeabilisation agent 0.02 % triton. Preincubation of the antiserum with its immnunising peptide (20 µg/ml 1h at 37°C) abolished the N-terminal α2 subunit staining under permeabilised (B) and nonpermeabilised conditions (D).

Immunostaining associated with the δ component N-terminal antiserum (1:1000) (corresponding to amino acid residues 1-17 of the δ polypeptide) was found to occur around the plasma membrane in permeabilised (E) and nonpermeabilised (G) DRGs. Preincubation of the antiserum with its corresponding immunising peptide (20 µg/ml, 1h at 37°C) abolished the N-terminal δ subunit immunostaining in both permeabilised (F) and nonpermeabilised (H) conditions.

Images are 1 µm confocal sections, taken approximately 10 µm up from the attachment plaque. Scale bar = 10 µm.
Figure 3.20  Immunolocalisation of two intracellular proteins, the VDCC β-subunit and \(G_{\alpha_0}\) in DRG neurones

a, DRGs exhibited intense immunostaining associated with the inner plasma membrane, following permeabilisation with 0.02% triton and incubation with an affinity purified anti \(G_{\alpha_0}\) antibody (50 µg/ml).

In the absence of permeabilisation, no \(G_{\alpha_0}\) immunostaining was observed.

b, immunostaining associated with the VDCC intracellular β-subunit was found to occur around the inner plasma membrane in DRGs which had been permeabilised with 0.02% triton, prior to incubation with the antipeptide anti-VDCC β-subunit antiserum (1:1000).

In the absence of permeabilisation, no VDCC β-subunit immunoreactivity was observed.

Fluorescent images on the left are 2 µm confocal sections taken midway through the cell. The respective phase contrast images are on the right. Scale bar = 25 µm.
3.2.3 Discussion of VDCC α2-subunit localisation and topography

The original model for the membrane topography of the calcium channel α2/δ complex polypeptide, proposed by Ellis et al (1988), suggested that the amino terminus of the α2 subunit is extracellular and that the α2 component has two transmembrane domains which are linked by a large intracellular loop. This model also proposes that the α2 subunit is connected to the δ peptide via disulphide interaction, and that the δ peptide has a further transmembrane region resulting in an intracellular carboxy-terminus. The second model for the membrane topography of the α2/δ complex proposed that the entire α2 component is extracellular, and is anchored to the membrane via disulphide interaction with the δ component, which has a single transmembrane domain (Jay et al 1991).

In this study antipeptide anti-α2/δ subunit antisera have been utilised for immunocytochemical studies on DRG neurones, in order to gain further insight into the orientation of the α2/δ subunit within the membrane. An antipeptide antiserum, raised against amino acid residues 469-483 of the α2 polypeptide was used as a probe to investigate where this region resides in the membrane. The recognition domain for this antiserum is located in the putative intracellular loop in the topography model proposed by Ellis et al (1991), and contains a consensus sequence for cAMP-dependent phosphorylation at threonine. Since calcium channels have been shown in several systems to be modulated by processes involving cAMP-dependent phosphorylation (Dolphin 1995), the identification of this threonine residue may have important implications concerning calcium channel modulation. In the second topography model (Jay et al 1991), where the entire α2 component is proposed to exofacial, the antiserum raised against amino acids 469-483 would be expected to interact with an extracellular domain.

When DRG neurones were incubated with the anti-α2 subunit antiserum, immunostaining was observed primarily around the plasma membrane and neurites. This distribution of immunostaining is characteristic of the staining patterns observed with the anti-α1 subunit and anti-β-subunit antisera (Sections 3.1 and 3.3). α2 subunit immunostaining associated with the plasma membrane and neurites was found to occur in the presence and absence of the permeabilisation agent 0.02% Triton X-100. Triton X-100 is a mild nonionic detergent with a hydrophobic region which disrupts the lipid bilayer by displacing the lipid molecules. The other end of the detergent is polar and therefore acts to bring the membrane proteins into solution as detergent-protein complexes, although in cells pretreated with paraformaldehyde the free amino groups of adjacent protein molecules have been cross-linked via covalent interaction and immobilised. The ability of the detergent to form pores in the cell membrane allows extracellularly applied antibodies access to the intracellular milieu. The permeabilisation procedure was found to have no adverse effect on the integrity of the membrane at the concentration used in this study, since phase contrast microscopy of...
permeabilised cells revealed an intact plasma membrane which was comparable to that of nonpermeabilised cells.

The finding that α2 subunit immunofluorescence was observed in nonpermeabilised intact cells suggests that the domain corresponding to amino acids 469-483, is located on the exofacial surface of the membrane. This result disagrees with the model of Ellis et al (1988), and dismisses the functional role of threonine477 in the modulation of calcium channels by phosphorylation. The finding that membrane permeabilisation enhanced immunostaining of the α2 subunit by anti-α2 (469-483) serum in DRG neurones suggests that the antigenic domain is partially occluded by membrane association.

In order to examine the orientation of other regions of the α2/δ complex within the membrane, DRGs were immunolabelled with antipeptide antisera raised against the N-terminus of the VDCC α2 (amino acids 1-15) and δ (amino acids 1-17) components. The antiserum raised against the N-terminus of the α2 subunit revealed immunostaining around the plasma membrane and neurites in intact and detergent-permeabilised cells. Similarly, the antiserum raised against the N-terminus of the δ peptide component, also showed membrane associated immunostaining under permeabilised and nonpermeabilised conditions. The intensity of fluorescence for each of the antisera was unaffected by permeabilisation procedures. This result suggests that the N-termini of both the α2 and δ component are located on the extracellular surface of the cell membrane. Taken together, the immunocytochemical results using the anti-α2 (480-503), α2 N-termini (1-15) and δ N-termini (1-17) antisera provide direct evidence for the arrangement of the α2/δ complex within the membrane of intact cells. Based on these findings the entire α2 subunit is proposed to be exofacial (Figure 3.14 B). This is in agreement with the study of Jay et al (1991). Those authors suggested that the α2 component is wholly extracellular, based on the finding that α2 underwent a large mobility shift on SDS-PAGE gel under deglycosylation conditions in a microsomal membrane preparation. In the original topography model proposed by Ellis et al (1988), only five out of the fifteen putative glycosylation sites would be available to the deglycosylation reaction. The large mobility shift following deglycosylation, observed in the study of Jay et al (1991) is predicted to be possible only if all fifteen consensus glycosylation sites are extracellular. Furthermore, the technique of alkali extraction has also been used to verify the latter topography model. Alkali extraction has been reported to solubilise cytoskeletal and peripheral proteins leaving integral proteins intact in the bilayer of erythrocytes (Steck et al 1973). In the absence of reducing agents the entire α2/δ complex was found to pellet with the membrane under alkali extraction and centrifugation. In contrast, the inclusion of reducing agents promoted solubilization of the larger α2 fragment but not the δ fragment (Jay et al 1991). Those authors therefore proposed that the larger α2 component is a peripheral membrane associated protein, linked via disulfide bonds to the δ an integral membrane protein with a single transmembrane
region. Although the specific amino acids involved in the disulphide linkage have not yet
been identified, 11 cysteine residues exist in the N-terminal peptide, and 9 exist in the
carboxy (δ)-terminal peptide, clearly providing candidate sites for disulphide linkages. The
model for the membrane orientation of the α2/δ complex within the membrane of intact
cells is depicted in Figure 3.14 B.

The α2/δ subunit has been shown to modulate the biophysical properties of calcium channel
α1 subunits in a variety of expression systems. Coexpression of skeletal muscle α2/δ with
the cardiac α1-subunit (α1C), produced an enhanced amplitude and increased rate of
activation of the expressed α1C current in *Xenopus* oocytes, these parameters were further
enhanced following coexpression of the β-subunit (Singer *et al* 1991). Expression of the rat
brain α1B Ca²⁺ channel clone with the β1b subunit in oocytes resulted in expression of a
slowly activating high threshold current (Stea *et al* 1993). Coexpression with the α2 subunit
with α1B alone had no effect on the biophysical properties of the expressed current.
However, when α2 was expressed with α1B + β1b an increase in the level of whole cell
currents was observed (Stea *et al* 1993). Coexpression of α2/δ with the rat brain α1-subunit
(α1A) enhanced the expressed current amplitude in *Xenopus* oocytes (Mori *et al* 1991).
Stable transfection of α2/δ with the smooth muscle α1 subunit, together with the β-subunit,
in Chinese hamster ovary cells, was found to accelerate the inactivation kinetics of the
channel without having a major effect on the other parameters (Welling *et al* 1993).

It has been demonstrated that the VDCC α1 and α2 subunits colocalise in clusters
associated with the membranes of developing t-tubules in normal mice myotubes, and that
the absence of the VDCC α1 subunit in dysgenic myotubes caused an aberrant distribution
of the VDCC α2 subunit (Flucher *et al* 1991). The study of Flucher *et al* (1991) provides
evidence that an interaction exists between the VDCC α1 and α2 subunits of the DHP
receptor in order to facilitate their normal organisation within t-tubule membranes. During
development the VDCC α2 subunit is abundant in muscle of newborn rats while expression
levels of the VDCC α1 subunit are initially low but rise dramatically two weeks postnatally
(Morton & Froehner 1989). Given the high glycosylation of the VDCC α2-subunit which
probably aids in its direction to the exofacial surface of the plasma membrane, and the
finding that the VDCC α2 subunit is expressed prior to the VDCC α1 subunit, α2 may have
a role in targeting and stabilising the α1 subunit to the membrane. This may represent the
mechanism by which the VDCC α2 subunit modulates the functional properties of
expressed calcium channel α1-subunits.
3.3 Calcium Channel β-subunit

3.3.1 Introduction

The cloning of the skeletal muscle β-subunit by Ruth et al. (1989), revealed a protein with a molecular weight of 58 kDa. The deduced amino acid sequence of the skeletal muscle β-subunit indicates that this protein contains four α-helical domains three of which contain a heptad repeat structure, similar to that found in cytoskeletal proteins (Fuch & Hanakoglu 1983). The α-helices were found to contain many charged amino acid residues, suggesting that the α-helices were not membrane spanning segments. The absence of glycosylation and hydrophilic nature of the β-subunit promoted Takahashi et al. (1987) to suggest that the β-subunit was located on the intracellular side of the membrane.

In this study an antipeptide anti-β subunit antiserum has been used to localise the β-subunit in cultured DRG neurones using immunocytochemical techniques. The antipeptide antiserum was raised to a sequence common to β1, β2 and β4, and it cross-reacted with the corresponding β3 peptide.
3.3.2 Results

3.3.2.1 Immunolocalisation of the VDCC β-subunit in DRG neurones.

DRGs were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the anti-β-subunit antiserum in order to examine the immunocytochemical distribution of the β-subunit. Confocal microscopy was utilised to reveal β-subunit immunostaining predominantly around the inner plasma membrane of the soma, although some β-immunostaining was also detected in the cytoplasmic region (Figure 3.21 A-L). Immunoreactivity associated with the β-subunit was also detected in the neurites (Figure 3.21 B-G).

3.3.2.2 Effect of preimmune serum and the anti-β subunit antiserum immunising peptide on β-subunit immunostaining

Immunostaining associated with the VDCC β-subunit (Figure 3.22 A) was abolished when the anti-β-subunit antiserum was preincubated with its immunising peptide (50 μg/ml, 1h at 37°C) (Figure 3.22 B). Similarly, when the DRG neurones were incubated with the corresponding preimmune serum, no specific immunostaining was detected (Figure 3.22 C).

3.3.2.3 Analysis of β-subunit immunoreactivity following preadsorption of the anti-β subunit antiserum with its immunising peptide

The degree of VDCC β-subunit immunoreactivity associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the fluorescent region associated with each cell, and using the corresponding phase contrast image to differentiate the boundary of the cell, an average grey value intensity (GVI) value was ascribed to each DRG. GVI values were obtained for cells with had been incubated with the antipeptide anti-β subunit antiserum (Figure 3.23), either in the presence or absence of the immunising peptide (50 μg/ml, 1h at 37°C). Preadsorption of the anti-β subunit antiserum with its immunising peptide reduced β-subunit immunoreactivity to the level of immunofluorescence observed following treatment of the cells with control preimmune serum.

3.3.2.4 β-subunit immunolocalisation in intact DRGs.

β-subunit immunoreactivity was absent in intact DRGs (Figure 3.20A), but could be detected around the inner plasma membrane in detergent-permeabilised DRG neurones (Figure 3.20B).
DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the antipeptide anti-β subunit antiserum (1:1000 dilution).
A series of 2 μm confocal sections (A-L) taken through the z-plane of the cells from the top surface of the cell (A) to the region of cell-attachment (L).
β-subunit immunoreactivity was revealed primarily around the inner plasma membrane (ipm) and neurites (n), although some β-subunit immunoreactivity was also detected in cytoplasmic compartments (c). Scale bar = 25 μm.
Figure 3.22 Immunolocalisation of the VDCC β-subunit

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with either anti-β subunit antiserum (1:1000 dilution) or preimmune serum (1:1000 dilution). A, immunostaining associated with the VDCC β-subunit was observed around the inner plasma membrane.

B, incubation of the DRGs with preimmune serum produced no detectable immunoreactivity.

C, preadsorption of the anti-β subunit antiserum with its immunising peptide (50 μg/ml 1h at 37°C) abolished β-subunit immunostaining.

The fluorescent images on the left are 2 μm confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 25 μm.
Figure 3.23 Analysis of the reduction in β-subunit immunoreactivity following preadsorption of the anti-β subunit antiserum with its immunising peptide

The level of β-subunit immunoreactivity associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the region of immunofluorescence associated with each cell, using the corresponding phase contrast image to mark the boundary of the cell. An average grey value intensity (GVI-arbitrary units) was then ascribed to each cell.

Cells were incubated in either the anti-β subunit antiserum (■■) or anti-β subunit antiserum which had been preincubated with the corresponding immunising peptide (50 μg/ml, 1h at 37°C) (■■). The average GVI value for cells incubated with the corresponding preimmune serum is also shown (□□□□). Results are expressed as mean ± sem. *p<0.05, n=6.
3.3.3 Discussion of VDCC β-subunit localisation

The antipeptide anti-β subunit antiserum used in this study has been characterised previously (Berrow et al 1995). The antiserum was raised against a sequence common to β₁, β₂ and β₄ and cross-reacted with the corresponding β₃ peptide sequence. The antiserum recognised a protein of approximate molecular mass 52 kDa in rat skeletal muscle t-tubules, and a protein of 55 kDa in a purified DHP receptor from rabbit skeletal muscle. Two protein bands were recognised in rat brain membranes by the β-subunit antiserum, with molecular masses of approximately 52 and 65 kDa, similar to the molecular mass of the rat brain β₃- (54.6 kDa) and β₂- (68.2 kDa) or β₁β (65.7 kDa) subunits, respectively (Pragnell et al 1991, Perez-Reyes et al 1992, Castellano et al 1993b). The anti-β-subunit antiserum also recognised β₁β, β₃ and β₄ overexpressed in COS cells (Campbell et al 1995).

In DRG neurones incubation with the anti-β subunit antiserum revealed localisation of the β-subunit at the inner surface of the plasma membrane, consistent with its association with VDCCs. Immunoreactivity was also observed at the soma, neurites and cytoplasm, the latter suggesting that β-subunits have other functions in neurones in addition to their roles in VDCCs. No β-subunit immunoreactivity was observed in the absence of detergent-permeabilisation when the DRG membrane was intact. An intact membrane would prevent externally applied antisera access to the intracellular environment. The finding that detergent-permeabilisation was a prerequisite for detection of β-subunit immunoreactivity therefore indicates that the β-subunit is wholly intracellular. Preincubation of the β-antiserum with its immunising peptide abolished β-subunit immunoreactivity. Similarly, no specific immunostaining was observed following incubation of the DRGs with the corresponding preimmune serum. These results indicate that the anti-β-subunit antiserum interacted with its recognition domain in a peptide-specific manner.

The immunocytochemical localisation of the VDCC β-subunit is consistent with the intracellular localisation of the β-subunit based on the absence of posttranslational glycosylation, the hydrophilic nature of the amino acid residues, and the presence of protein phosphorylation sites (Takahashi et al 1987).

The skeletal muscle β-subunit has been shown to be a substrate for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) (Takahashi et al 1987, Nastainczyk et al 1987). cDNA cloning and sequencing of the VDCC β-subunits has subsequently revealed the presence of multiple consensus sites for phosphorylation by different protein kinases. While the skeletal
muscle β-subunit has been found to contain potential consensus sequences for phosphorylation by PKA and PKC (Ruth et al 1989), the rat brain β2-subunit has been reported to contain consensus sequences for phosphorylation by casein kinase II, as well as PKA and PKC (Perez-Reyes et al 1992). In contrast the β3- and β4-subunits only contain consensus sequences for phosphorylation by PKC or casein kinase II (Castellano et al 1993a, Castellano 1993b). Although calcium channel currents have been shown to be modulated by phosphorylation events occurring on the calcium channel α1-subunit (Dolphin 1995), it has also been shown that expression of the β-subunit is required for the cAMP-dependent increase of cloned cardiac and vascular Ca\(^{2+}\) channel currents (Klockner et al 1992). Furthermore, β-adrenergic modulation of Ca\(^{2+}\) channel currents in canine myocytes has been reported to be due to cAMP-dependent phosphorylation of the β-subunit (Haase et al 1993). The β-subunit has been shown to exert a modulatory effect on Ca\(^{2+}\) channel α1-subunits. Coexpression of the cardiac or smooth muscle forms of the class C L-type α1-subunit with the β1, β2 or β3 subunits in Xenopus oocytes increased the peak current, accelerated activation kinetics and shifted the voltage-dependence of activation to more negative potentials (Singer et al 1991, Wei et al 1991, Itagaki et al 1992, Perez-Reyes et al 1992, Hullin et al 1992, Castellano 1993a, 1993b). The amplitudes of the α1A, α1B, α1D and α1E classes of Ca\(^{2+}\) channels have all been shown to be increased by coexpression with β subunits (Mori et al 1991, Williams et al 1992a, 1992b, Sather et al 1993, Ellinor et al 1993, Stea et al 1993). The increase in expressed current amplitude, increase in inactivation kinetics and hyperpolarising shift in the activation threshold, observed upon coexpression of the β-subunit with the calcium channel α1-subunit may be due in part to protein phosphorylation mechanisms.

An intracellular localisation of the β-subunit has been verified by the finding that the β-subunit interacts with a region on the intracellular loop linking transmembrane motifs I and II for skeletal, cardiac, and neuronal calcium channel α1 subunits (Pragnell et al 1994). A conserved sequence motif was identified in all six Ca\(^{2+}\) channel α1 subunits as a consensus sequence for 'binding' of the β-subunit. Mutations within this sequence reduced the stimulation of the peak current density by the β-subunit and altered the inactivation currents and voltage-dependence of activation. A 30 amino acid domain located at the N-terminus of the β-subunit, a region conserved in all β-subunits, was found to be of crucial importance in the α1-β interaction (De Waard et al 1994). Modifications of this region, by truncation or single-point mutation, was found to alter the stimulation of Ca\(^{2+}\) currents by the β-subunit. This domain was also found to be a prerequisite for β-subunit-induced kinetic changes and the voltage-dependence of steady state inactivation (De Waard et al 1994).

In summary, the finding that the VDCC β-subunit is localised to the inner surface of the plasma membrane in DRG neurones is therefore consistent with the high level of potential consensus...
sequences for protein phosphorylation on β-subunits, and the finding that the β-subunit is
coupled to the VDCC α1-subunit via interaction with the intracellular loop linking
transmembrane motifs I and II of the α1-subunit.
3.4 Localisation of the G-protein $G_q$ and $G_i \alpha$ subunits

3.4.1 Introduction

The interaction of a ligand with its membrane-bound receptor leads to a change in the intracellular concentration of a second messenger, such as cyclic nucleotide or $Ca^{2+}$, which then activates other intracellular processes. The GTP-binding proteins (G proteins) are heterotrimers, composed of an $\alpha$, $\beta$ and $\gamma$ subunit, which couple receptors to their respective effectors (Simon et al 1991). Activation of a receptor results in the $\alpha$ subunit of the G protein interacting with an intracellular domain of the receptor molecule and GDP is exchanged for GTP. In the GTP-bound form the $\alpha$ subunit is in the activated configuration, it dissociates from the $\beta\gamma$ complex and proceeds to interact and activate various other molecules involved in the signal transduction pathway. This process produces an amplification of the receptor-mediated signal and a variety of subsequent second messenger activities.

The G protein $G_{q1}$ has been detected in the brain, where it is heterogenously distributed (Largent et al 1991) and occurs in three isoforms, $G_{q11}$, $G_{q12}$ and $G_{q13}$, (Itoh et al 1986; Jones & Reed 1987; Yatani et al 1988; Carty et al 1990), where $G_{q11}$ is reported to be the most prevalent isoform in the brain (Neer & Clapham 1988). $G_{q1}$ is involved in the modulation of membrane-bound enzymes such as phospholipases and adenylate cyclase (Bimbaumer et al 1990). $G_{q11}$ has also been shown to interact with the GABA$_B$ receptor in bovine brain (Morishita et al 1990). In the rat pituitary cells $G_{q1}$ has been reported to mediate the dopaminergic stimulation of $K^+$ currents (Lledo et al 1992) and LHRH-mediated activation of calcium channel currents.

$G_q$ exists in two isoforms, $G_{q1}$ and $G_{q2}$ (Hui et al 1990), and is highly abundant in brain tissue (Bockaert 1990). Negative coupling between receptors and voltage-dependent calcium channels occurs via $G_q$. Injection of antipeptide anti-$G_{q}$ antibodies into snail neurones revealed the role of $G_{q}$ in the dopaminergic inhibition of calcium channel currents (Harris-Warrick et al 1988). Similarly, dialysis of antipeptide anti-$G_{q}$ antibodies into NG108-15 cells, attenuated the adrenaline-induced inhibition of calcium channel currents (McFadzean et al 1989) and microinjection of antisense oligonucleotides with complementarity to the mRNA of $G_{q1}$ or $G_{q2}$ selectively abolished the muscarinic and somatostatin-induced inhibition of calcium channel currents in GH3 cells (Kleuss et al 1991).

In this study an antipeptide anti $G_{q}$ antiserum and an affinity purified anti-$G_{q}$ antibody were used to immunolocalise $G_{q}$ in DRG neurones. An antipeptide affinity purified anti-$G_{q}$ antibody was also used to investigate the localisation of $G_{q1}$ in these cells. The primary reason
for examining the distribution of these proteins in DRGs was to enable the expression levels of the G-proteins to be monitored following treatment of the cells with antisense oligonucleotides complementary to the mRNA of either $G\alpha_\text{o}$ or $G\alpha_\text{i}$. The use of antisense oligonucleotides to deplete DRG neurones of $G\alpha_\text{o}$ and $G\alpha_\text{i}$ is described in Chapter 4.
3.4.2 Results

3.4.2.1 Immunolocalisation of Goα

DRGs were fixed, detergent permeabilised, incubated with the antipeptide anti-Goα antiserum (OC2) and prepared for immunocytochemistry. Goα immunostaining was observed around the inner plasma membrane and neurites (Figure 3.24 C-K). Goα immunoreactivity was also detected in the perinuclear region (Figure 3.24 D-K). The corresponding preimmune serum produced no detectable immunostaining (Figure 3.24 M).

A similar Goα distribution was observed when the DRGs were prepared for immunocytochemistry utilising the affinity purified antipeptide anti-Goα antibody. The DRG neurones exhibited Goα immunoreactivity around the inner plasma membrane and neurites (Figure 3.25 B-G). Staining associated with Goα was also observed at the attachment plaque (Figure 3.25 I-J) and at regions of cell-to-cell contact (Figure 3.26 A). Preadsorption of the anti-Goα antibody with its immunising peptide (50 µg/ml 1h at 37°C) abolished the specific Goα staining (Figure 3.26 A). Incubation of the DRGs with rabbit IgG (50 µg/ml) produced no detectable immunostaining (Figure 3.26 C).

3.4.2.2 Immunolocalisation of Gαi

DRGs were fixed, detergent-permeabilised, incubated with the affinity purified antipeptide anti-Gαi antibody and prepared for immunocytochemistry. Gαi immunostaining was associated with the inner plasma membrane (Figure 3.27 C-J), weak immunoreactivity was also observed in the neurites (Figure 3.27 A-G) and cytoplasm (Figure 3.28 A). Preincubation of the anti-Gαi antibody with its immunising peptide (50 µg/ml 1h at 37°C) abolished specific Gαi immunoreactivity (Figure 3.28 B). The control rabbit IgG (50 µg/ml) produced no discernible immunostaining (Figure 3.28 C).

3.4.2.3 Analysis of the loss of Goα and Gαi immunoreactivity following preadsorption of the anti-Goα and anti-Gαi antibodies with their respective immunising peptides.

The degree of Goα and Gαi immunoreactivity associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the fluorescent region associated with each cell, and using the corresponding phase contrast image to differentiate the boundary of the cell, an average grey value intensity (GVI) value was ascribed to each DRG. GVI values were obtained for cells with had been incubated with the affinity purified anti-Goα (Figure 3.29 A) and anti-Gαi (Figure 3.29 B) antibodies, either in the presence or absence of their respective immunising peptides (50 µg/ml).
1h at 37°C. Preadsorption of anti-\( \Gamma_0 \) antibody with its immunising peptide reduced \( \Gamma_0 \) immunoreactivity to the level of immunofluorescence observed following treatment of the cells with control rabbit IgG (50 \( \mu \)g/ml) (Figure 3.29 A). Similarly, preadsorption of anti-\( \Gamma_1 \) antibody with its immunising peptide, reduced \( \Gamma_1 \) immunoreactivity to the level of immunofluorescence observed following treatment of the cells with control rabbit IgG (50 \( \mu \)g/ml) (Figure 3.29 B)
Figure 3.24 Immunolocalisation of G\textsubscript{\alpha_0} throughout the z-plane of DRG neurones

DRG neurones were fixed with 4\% paraformaldehyde, detergent-permeabilised with 0.2\% Triton-X100, incubated with anti-G\textsubscript{\alpha_0} antiserum (OC2-Provided by Professor Milligan, Glasgow University) and prepared for immunocytochemistry.

A series of 2 \(\mu\)m confocal sections (A-L) taken through the z-plane of the cell from the uppermost surface of the cell (A) to the region of cell-attachment (L). G\textsubscript{\alpha_0} immunoreactivity was prevalent at the inner surface of the plasma membrane (pm), in the neurites (n) and within cytoplasmic regions (c).

A 2 \(\mu\)m section taken 10 \(\mu\)m up from the attachment plaque in a cell incubated with the corresponding preimmune serum (M) failed to produce any immunoreactivity. Scale bar = 25 \(\mu\)m.
Figure 3.25 Immunolocalisation of $\mathrm{G\alpha}_o$ throughout the z-plane of DRG neurones

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised with 0.02% Triton-X100, incubated with the affinity purified anti-$\mathrm{G\alpha}_o$ antibody and prepared for immunocytochemistry.

A series of 2 μm confocal sections (A-J) taken through the z-plane of the cell from the uppermost surface of the cell (A) to the region of cell-attachment (J).

$\mathrm{G\alpha}_o$ immunoreactivity was prevalent at the inner surface of the plasma membrane (pm), in the neurites (n) and at the attachment plaque (ap).

The corresponding phase contrast image (K) is also shown. Scale bar = 25 μm.
Figure 3.26 Immunolocalisation of $\alpha_0$

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised with 0.02% Triton-X100 and incubated with the affinity-purified anti-$\alpha_0$ antibody (50 $\mu$g/ml).

A, immunostaining associated with $\alpha_0$ was observed around the inner plasma membrane and at regions of cell-to-cell contact.

B, preadsorption of the anti-$\alpha_0$ antibody with its immunising peptide (50 $\mu$g/ml, 1h at 30°C) abolished $\alpha_0$ immunoreactivity.

C, control rabbit IgG (50 $\mu$g/ml) failed to produce any immunofluorescent signal.

Fluorescent images are 2 $\mu$m sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 20 $\mu$m.
Figure 3.27 Immunolocalisation of Gα₂ throughout the z-plane of DRG neurones

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised with 0.02% Triton-X100 and incubated with the affinity-purified anti-Gα₂ antibody (50 µg/ml).

A series of 2 µm confocal sections (A-K) taken through the z-plane of the cells from the top surface of the cell (A) to the region of cell-attachment (K). The corresponding phase contrast image (L) is also shown.

Gα₂ immunoreactivity was revealed primarily around the inner plasma membrane (ipm), although some Gα₂ immunoreactivity was also detected in neurites (n). Weak Gα₂ immunoreactivity was also observed in the cytoplasm (c). Scale bar = 25 µm.
Figure 3.28  \( \Gamma \alpha_3 \) immunoreactivity was abolished by preadsorption of the affinity-purified anti-\( \Gamma \alpha_3 \) antibody with its immunising peptide.

DRG neurones were fixed with 4% paraformaldehyde, detergent-permeabilised and incubated with the affinity-purified anti-\( \Gamma \alpha_3 \) antibody (50 \( \mu \)g/ml).

A, immunostaining associated with \( \Gamma \alpha_3 \) was observed around the inner plasma membrane. B, preadsorption of the anti-\( \Gamma \alpha_3 \) antibody with its immunising peptide (50 \( \mu \)g/ml 1h at 37\(^\circ\)C) abolished \( \Gamma \alpha_3 \) immunoreactivity. C, incubation of the cells with control rabbit IgG (50 \( \mu \)g/ml) produced no immunofluorescent signal.

Fluorescent images are 2 \( \mu \)m confocal sections taken midway through the cell. The corresponding phase contrast images are on the right. Scale bar = 20 \( \mu \)m.
Figure 3.29 Analysis of the reduction in $\alpha_0$ and $\alpha_i$ immunoreactivity following preadsorption of the antibodies with their respective immunising peptides

The level of $\alpha_0$ and $\alpha_i$ immunoreactivity associated with each DRG neurone was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the region of immunofluorescence associated with each cell, using the corresponding phase contrast image to mark the boundary of the cell. An average grey value intensity (GVI-arbitrary units) was then ascribed to each cell.

A, cells were incubated in either the anti-$\alpha_0$ antiserum (■■) or anti-$\alpha_0$ antiserum which had been preincubated with the corresponding immunising peptide (50 µg/ml, 1h at 37°C) (□). The average GVI value for cells incubated with control rabbit IgG (50 µg/ml) is also shown (□□□□). Results are expressed as mean ± sem. *p<0.05 compared to the GVI of cells incubated with anti-$\alpha_0$ antiserum in the absence of immunising peptide, n=8.

B, cells were incubated in either the anti-$\alpha_i$ antiserum (■■) or anti-$\alpha_i$ antiserum which had been preincubated with the corresponding immunising peptide (50 µg/ml, 1h at 37°C) (□). The average GVI value for cells incubated with control rabbit IgG (50 µg/ml) is also shown (□□□□). Results are expressed as mean ± sem. *p<0.05 compared to the GVI of cells incubated with anti-$\alpha_i$ antiserum in the absence of immunising peptide, n=5.
3.4.3 Discussion of G-protein localisation

An antipeptide anti-Go antiserum (OC2) and affinity purified anti-Go antibody were used in this study to examine the immunolocalisation of the G-protein Gq in DRG neurones. The antipeptide Go antiserum was raised against the C-terminal decapeptide of Go1. Antisera raised to this peptide are reported to be specific for G proteins of the Gq subtype (Goldsmith et al. 1987). The polyclonal antiserum (OC2) was found to label two distinct proteins of approximately 39 kDa corresponding to Go1 and Go2, in a rat cortical membrane immunoblot (Menon-Johansson et al. 1993). Furthermore, the OC2 antiserum has been shown to immunoprecipitate a 39 kDa polypeptide in solubilized rat brain cortical membranes, which was identified as Go by immunoblotting with anti-Go antiserum (OC1) (Georgoussi et al. 1995). The ON1 antiserum has previously demonstrated a high specificity and selectivity to recognise a 39 kDa protein corresponding to Go while not cross reacting with Gi (Goldsmith et al. 1987). The affinity purified anti-Go antibody was also raised against the same C-terminal decapeptide sequence on Go1, as the OC2 antiserum, and was found to label two 39 kDa proteins, following immunoblotting on a rat cortical membrane preparation (N Berrow, unpublished observations).

Immunocytochemical confocal microscopy, utilising the anti-Go antiserum (OC2), revealed Go immunostaining primarily associated with the inner plasma membrane, cytoplasm, nuclear region and neurites in prefixed and permeabilised DRG neurones. The preimmune serum corresponding to the anti-Go antiserum (OC2) produced no immunostaining of the DRG neurones. The anti-Go antiserum labelling of two distinct 39 kDa proteins on rat brain immunoblots has also previously been shown to be reduced following pretreatment of the antiserum with its immunising peptide sequence (Menon-Johansson et al. 1993).

The distribution at the inner plasma membrane is consistent with the role of Go in coupling membrane-bound receptor activation to signal transduction pathways. Ultrastructural studies using anti-Go antibodies have identified Go labelling at the inner plasma membrane as well as in the cytoplasmic matrix at the regions of the endoplasmic reticulum and Golgi (Gabrion et al. 1989). Subcellular localisation of Go has also revealed Go immunoreactivity at the plasma membrane and neurites in mouse striatal and cerebellar granule neurones (Brabet et al. 1988).

The affinity purified anti-Go antibody also revealed Go immunostaining at the inner plasma membrane and neurites. The immunoreactivity associated with Go was abolished following pretreatment of the affinity purified anti-Go protein antibody with its immunising peptide thereby indicating that the antibody was interacting with its recognition site in a peptide specific manner. Control rabbit IgG produced no immunoreactivity. Unlike the immunostaining...
observed using the anti-Gαo antiserum, immunoreactivity associated with the affinity-purified anti-Gαo antibody was extremely weak in the cellular cytoplasmic and nuclear regions. The concentration of Triton-X100 used to permeabilise the cells prior to treatment with the anti-Gαo antiserum (OC2) (0.2 %) was 10-fold higher than the concentration used to permeabilise cells prior to treatment with the affinity-purified anti-Gαo antibody (0.02%). It has been reported that 0.02 % Triton permeabilises the plasma membrane while leaving the nuclear membrane intact, and that higher concentrations of Triton permeabilise the nuclear membrane as well as the plasma membrane (Peebles 1988).

Discrete cytoplasmic Gαo immunostaining between the endoplasmic reticulum and Golgi while nuclei remained unstained, has previously been observed in neuronal cells (Brabet et al. 1988, Brabet et al. 1989). Gαo and has also been found attached to the membranes of synaptic vesicles in chromaffin cells and hypothalamic neurones (Ahner-Hilger et al. 1994), and at secretory granules in chromaffin cells (Toutant et al. 1987). Its attachment to secretory granules and the finding that Ca2+-dependent exocytosis was enhanced by PTX and anti-Gαo antibodies (Ohara-Imaizumi et al. 1992) indicates that Gαo may be involved in the maturation of secretory granules or their fusion with the cell membrane. The localisation of Gαo at the Golgi of the adrenal medulla (Maier et al. 1995) indicates that Gαo may additionally be involved in the formation of immature secretory granules at the trans-Golgi network.

Intense Gαo immunofluorescence was also detected at regions of cell-to-cell contact and at the attachment plaque of DRG neurones. Gαo expression at regions of cell-to-cell contact has also been reported in mouse striatal and cerebellar granule neurones (Brabet et al. 1988) and in mouse brain slices (Gabrion et al. 1989). In differentiated NIE-115 cells a similar Gαo immunostaining pattern was observed, but in the undifferentiated state, only a positive reaction was seen in the cytoplasmic matrix, indicating a relocalisation of Gαo during differentiation (Brabet et al. 1990). The intense Gαo immunoreactivity at the cell attachment plaque and at regions of cell-to-cell contact could possibly represent additional roles of Gαo in signal transduction associated with cell surface recognition molecules. The morphoregulatory activities of neural cell adhesion molecule (NCAM) and N-cadherin have been shown to be mediated via a pertussis toxin sensitive G-protein-dependent activation of L- and N-type Ca2+ channels in PC12 cells (Doherty et al. 1991). Activation of tyrosine kinase has more recently been shown to be an important step upstream of G-protein activation in the CAM pathway leading to neurite outgrowth (Williams et al. 1994).

Although Gαo was found to be expressed in the neurites of DRG neurones, Gαo was not detected at the growth cone regions. In contrast, a strong Gαo immunofluorescent signal was observed at neuritic processes and growth cones in cultured neuroectodermal cells (Pituello et al. 1991) and at the distal tips of the cellular processes of differentiated PC12 cells (Strittmatter et al. 1991).
et al 1990). In the latter study, $\Gamma_\alpha_0$ was found to be regulated by the intracellular growth cone protein GAP-43, whose expression is tightly correlated with axonal growth (Skene 1989). Activation of $\Gamma_\alpha_0$ has more recently been reported to increase neurite outgrowth in neuroblastoma cells (Strittmatter et al 1994a), this has been shown to be due to activation of $\Gamma_\alpha_0$ by the N-terminal region of GAP-43 (Strittmatter et al 1994b).

In ciliated ependymocytes and outer hair cells $\Gamma_\alpha_0$ has been localised along the axonemes of kinocilia (Peraldi 1987), and at the base of the cell and intraflagellar network (Canlon et al 1991) respectively. These localisations of $\Gamma_\alpha_0$ are similar to the distribution of actin filaments, and suggest a possible interaction of $\Gamma_\alpha_0$ with some cytoskeletal components. In rat peritoneal mast cells, heterotrimeric G-proteins have been reported to participate in affecting the changes in the actin cytoskeleton observed following activation of the mast cells (Norman et al 1994).

Immunofluorescent studies have been employed to reveal the cellular distribution of G-protein subunits in comparison with that of the voltage-dependent calcium channel (Toutant et al 1990). $\Gamma_\alpha_0$ colocalised with the Ca$^{2+}$ channel immunofluorescence, indicating that both proteins were located in very close subcellular structures. The immunocytochemical results presented in this section, reveal $\Gamma_\alpha_0$ to have a similar distribution as the subunit components of the various calcium channel subtypes in DRGs (Sections 3.1-3.3). Since various neurotransmitters have been shown to modulate calcium channel activity via $\Gamma_\alpha_0$ in a variety of preparations (Dolphin 1995), such an anatomical distribution of $\Gamma_\alpha_0$ with respect to the calcium channel would allow close coupling of G-proteins to the Ca$^{2+}$ channel effector.

The affinity purified anti-$\Gamma_\alpha_i$ antibody used in this study was raised against a C-terminal decapeptide of $\Gamma_\alpha_{i1}$ and $\Gamma_\alpha_{i2}$ (Campbell et al 1993). The specificity of the anti-$\Gamma_\alpha_i$ antibody preparation for the $\Gamma_i$ protein classes was confirmed by immunoblotting against a rat brain membrane preparation (N Berrow, unpublished observations). The anti-$\Gamma_\alpha_i$ antibody was found to label a set of proteins of molecular mass 40-43 kDa.

Immunocytochemical confocal microscopy utilising the anti-$\Gamma_\alpha_i$ antibody revealed $\Gamma_\alpha_i$ immunoreactivity primarily around the inner plasma membrane of paraformaldehyde-fixed and detergent-permeabilised DRG neurones. The neurites and cytoplasm exhibited weak $\Gamma_\alpha_i$ immunofluorescence. The immunoreactivity associated with $\Gamma_\alpha_i$ was abolished following pretreatment of the affinity purified anti-$\Gamma_\alpha_i$ antibody with its immunising peptide, thereby indicating that the antibody was interacting with its recognition site in a peptide specific manner. Control rabbit IgG produced no immunoreactivity.

The anti-$\Gamma_\alpha_i$ antibody failed to stain regions of cell-to-cell contact. This is in contrast to the immunostaining observed with the anti-$\Gamma_\alpha_0$ antibody, which showed marked immunofluorescence associated with the neurites and zones of cell-to-cell contact. In ra
pituitary cells, G\(\alpha_i\) localisation at the cell periphery (Wilson et al. 1994) and membrane
(Degtyarev et al. 1994) has been reported. The \(\alpha\) subunit of G\(_i\) also localised at the Golg
membrane in rat pituitary cells (Wilson et al. 1994) and liver and adrenal medulla tissue (Maiei
et al. 1995). In the neostriatum, G\(\alpha_i\) immunoreactivity has been observed in the axon terminals,
plasma membranes and synaptic vesicles (Aronin & DiFiglia 1992). The localisation of G\(\alpha_i\) in
intracellular compartments, particularly at the Golgi, indicates that G\(\alpha_i\) may have a role in
regulating vesicle formation and intracellular membrane trafficking for certain constitutive
signalling pathways. The Rab superfamily of small GTP-binding proteins also appear to be
transiently associated with secretory vesicles during trafficking and maturation (Fischer et al.
1991, Matteoli et al. 1991). Activation of G proteins by GTP\(_\gamma\)S has been reported to disrupt
endocytosis and trans-Golgi recycling of endosomal vesicles (Mayorga et al. 1989). G\(\alpha_{i2}\) and G
\(\alpha_{i3}\) have since been immunolocalised in endosomal membranes where they have been reported
to stimulate H\(^+\)-ATPase activity to regulate endosomal acidification processes (Gurich &
DuBose, 1993). G\(\alpha_i\) therefore appears to have a role in regulating the maturation of both
endocytotic and exocytotic vesicles.
Chapter 4

Use of Antisense Oligodeoxynucleotides to Deplete Dorsal Root Ganglia of the G-proteins $G_0/G_i$ and the Voltage-dependent Calcium Channel $\beta$-Subunit.
4.1 The use of biological and molecular tools to investigate the G-protein modulation of ion channels.

In order to establish which subtype of G-protein mediates the neurotransmitter modulation of ion channels, various approaches have been used. Pertussis toxin (PTX) is an exotoxin produced by the bacterium Bordetella pertussis. PTX catalyzes the ADP-ribosylation of the α-subunit of the G-protein subtypes Gi and Go (see Section 1.5, Chapter 1). This covalent modification prevents Gi and Go from coupling with their receptors and consequently these G-proteins remain in the inactive GDP-bound configuration (Ui et al., 1984). PTX has been shown to block the GABAergic (Dolphin & Scott., 1987), muscarinic (Toselli., 1989) and opioid (Gross et al., 1990) inhibition of voltage-dependent calcium channels (VDCCs) in neuronal cells. The activation of neuronal K^+ currents upon GABA_B receptor activation is also linked to a PTX-sensitive G-protein (Andrade et al., 1986). The results from such studies would suggest that a Gi or Go subtype is involved in the neurotransmitter modulation of ion channels. However, the inability of PTX to discriminate between Gi and Go is the major limitation of this approach. Anti-G protein antibodies, specific to either Gi or Go, were subsequently developed in order to determine which subtype of PTX-sensitive G-protein coupled neurotransmitter receptor activation to ion channel modulation. An antipeptide antibody raised against a conserved sequence on the α subunit of the Go subtype of G-protein suppressed the adrenergic inhibition of Ca^{2+} currents when it was dialyzed into NG108-15 cells (McFadzean et al., 1989); an anti Gi antibody had no effect on this noradrenaline-mediated inhibition of Ca^{2+} current. In rat dorsal root ganglion neurones an antiserum specific for Go, attenuated opioid receptor-mediated inhibition of Ca^{2+} channel current while a Gi antiserum was without effect (Moises et al., 1994). Similarly, when dorsal root ganglion neurones were replated in the presence of an antipeptide anti G_{α_0}, but not G_{α_0} antiserum, the GABAergic inhibition of N-type calcium channels was suppressed (Menon Johansson et al., 1993). In rat pituitary cells anti G_{α_0}-antibodies were found to reduce the inhibition of Ca^{2+} currents, but not the stimulation of K^+ currents, via dopamine D2 receptors (Lledo et al., 1992). In that study, antibodies raised against the α subunits of Gi1, Gi2 or Gi3 were found to be inactive with regard to Ca^{2+} current inhibition, however, the anti Gi3 antibody blocked the dopamine-induced activation of the K^+ current. The use of specific Gi or Go antibodies therefore provides a useful tool with which to identify the PTX-sensitive G-protein subtype involved in ion channel modulation. There are, however, problems inherent in the use of antibodies, including the difficulty of introducing them into cells, the low affinity of many antipeptide antibodies, and uncertainty as to their specificity in preventing function because of their large size.
The development of antisense oligonucleotide technology provides a novel technique which offers the potential to block the expression of specific genes within cells. In GH3 pituitary cells, the expression of the G-protein G\textsubscript{0}, G\textsubscript{i} or G\textsubscript{s} \(\alpha\)-subunits were transiently down-regulated following intracellular injection of antisense oligonucleotides complementary to unique motifs on each of the G-protein subtype \(\alpha\) subunit mRNA sequences (Kleuss et al., 1991). The study by these authors revealed that the muscarinic and somatostatin induced inhibition of L-type \(\text{Ca}^{2+}\) channel currents was abolished following exclusive depletion of \(G_{\alpha 0}\) expression levels. The antisense strategy has also revealed that the muscarinic and somatostatin receptors are coupled to different splice variants of G-protein \(\alpha, \beta\) and \(\gamma\) subunits in order to mediate the observed inhibition of calcium channel currents in GH3 cells (Kleuss et al., 1992). Furthermore, in GH3 cells, the stimulation of L-type \(\text{Ca}^{2+}\) channel current by thyrotrophin-releasing hormone (TRH) has been shown to be specifically abolished following treatment with antisense oligonucleotides complementary to the mRNA of the G-protein subtype G\textsubscript{i2} (Gollasch et al., 1993). The antisense strategy can therefore be successfully employed to produce a selective depletion of G-protein subunits in intact cells. This method has subsequently identified the G-protein subtypes involved in mediating ion channel modulation by certain neurotransmitters and hormones in a variety of cell types (Kleuss et al., 1991; Campbell et al., 1993).

Antisense oligonucleotides have been used in this study in an attempt to selectively deplete cultured dorsal root ganglion neurones of G-protein G\textsubscript{0} and G\textsubscript{i} \(\alpha\) subunits. This approach has also been manipulated to deplete expression levels of the calcium channel \(\beta\)-subunit in these cells (this will be discussed in section 4.8).

In order to appreciate the benefits, and recognise the limitations of this technique it is necessary to discuss: 1) the mode of action of antisense oligonucleotides 2) the choice of delivery route 3) the choice of oligonucleotide sequence 4) essential controls.

These aspects of the antisense strategy are addressed in the following sections.

### 4.2 Mode of Action of Antisense Oligodeoxynucleotides

Gene expression requires an information flow from gene to protein. Each stage of this process requires specific base pairing between complementary nucleic acids in order to ensure the accurate transmission and interpretation of this information; this forms the basis of the antisense strategy. The nature of the double helix, proposed by Watson and Crick (1953), describes a complementary interaction between the purine adenine and guanine residues with the pyrimidine thymine, uracil and cytosine nucleic acid residues. Adenine forms complementary base pairs via hydrogen bonding with thymine or uracil, while guanine forms complementary base pairs with cytosine. The description of this interaction gives an
Figure 4.1 Site of antisense oligonucleotide action

1. Synthesis of single stranded mRNA from a double stranded DNA template.  
2. Nucelocytoplasmic transport of newly synthesised mRNA.  
3. Activation of RNaseH cleaves the RNA primer, DNA elongation ensues  
4. Translation of mRNA into amino acids and thus protein
appreciation that single-stranded DNA and RNA can form homo- and heterodimers of DNA:DNA, RNA:DNA, and RNA:RNA hybrids; such complementary interactions are of intrinsic importance to transcription, RNA processing, and translation.

The fundamental importance of specific base pairing in the function of nucleic acids offers the possibility of interfering with the expression of target genes in a highly selective manner by using a complementary antisense sequence. Antisense DNA deoxyoligonucleotides (ODNs) are complementary in sequence to the mRNA strand. The ODN can therefore form complementary base-pairs with the mRNA and may block expression by several possible means. The mechanisms by which may exert their effects are described below and summarised in Figure 4.1

• 4.2.1 Transcriptional arrest:

A nuclear target for antisense oligonucleotide action has been proposed based on the findings that 32p-labeled oligomers, associated with the target mRNA, have been extracted from the nucleus of rat PC12 cells following incubation with antisense oligonucleotides (Teichman-Weinberg et al 1981). It has also been demonstrated that fluorescence accumulated in the nucleus of Swiss 3T3 cells, following microinjection of oligonucleotides covalently conjugated to the fluorochrome fluorescein (Leonetti et al 1991). Given the nuclear association of oligonucleotides, it seems apparent that, in intact cells, antisense oligonucleotides can bind to their complementary sequence on pre-mRNA and even DNA. By doing so they have the potential to interfere with nuclear events such as transcription or the nucleocytoplasmic transfer of mature transcripts.

Under normal transcription conditions, a coding strand of DNA forms complementary base-pairs with the non-coding strand of DNA. An RNA polymerase II enzyme moves along the non-coding DNA template to catalyze the synthesis of a messenger RNA molecule (mRNA), (Figure 4.2). In order to accommodate RNA polymerase II during RNA chain elongation, the DNA double helix is opened locally to form a transcription 'bubble'. These sites may be potentially accessible to complementary oligonucleotides, at least transiently.

The single stranded region of the transcription initiation complex has been shown to be targeted by oligonucleotides. Oligonucleotides complementary to the bacterial T7 promoter A2 have been used to ascertain the size of the open loop created by binding of the polymerase (Grachev et al 1984). Those authors describe the bacterial open loop to be 17 base pairs, although the size of the eukaryotic open loop has not yet been ascertained. It has also been reported that the initiation of transcription by E.Coli RNA polymerase on the bla gene was inhibited by an oligonucleotide, complementary to the open region in the RNA polymerase-promoter complex. (Helene et al 1985). The effect was found to be sequence specific and the anti-bla oligonucleotide had no effect on transcriptional initiation of the lac promoter.
Normal Transcription

Transcription in the presence of antisense oligonucleotide

Figure 4.2 Effect of antisense oligonucleotide on transcription
It has been shown that triple-stranded structures can form between pyrimidine oligonucleotides and DNA homopurine regions via Hoogsteen hydrogen bonding. This interaction forms the basis of the anti-gene strategy. In the triple helix motif pyrimidine-purine pairing of the oligonucleotide with the DNA of the duplex occurs in the major groove of the Watson Crick double helix. It is possible for thymidine to form hydrogen bonds with adenine, while the adenine residue is simultaneously involved in Watson Crick interaction with another thymidine residue. Similarly, protonated cytosine can bind to the guanine of a G.C base pair (Riordan & Martin 1991). The occurrence of such triple helix structures has been demonstrated using oligonucleotides covalently linked to crosslinking or cleaving groups; the association of the oligomer with a DNA duplex brought the crosslinking or cleaving reagent into the vicinity of the DNA bases, this introduced irreversible damage to the target DNA strand (Praseuth et al 1988 Francois et al 1989). The current major limitations of the triple helix model include the pH-dependence of binding. In order for cytosine to interact with a G.C base pair, the cytosine must be protonated and the Hoogsteen binding dissociates with increasing pH (Povsic & Dervan 1989). Another limitation of the triple helix approach is the available code, since it is restricted to the recognition of homopurine tracts by pyrimidine oligonucleotides, although Cooney et al (1988) reported inhibition of c-myc gene transcription via triplex formation with an oligonucleotide containing both purines (guanine and adenine) and pyrimidines (thymine). It would clearly be desirable to extend the triple helical recognition code to a general solution in which all four base pairs of Watson-Crick double strands could be recognised under physiological conditions. Perhaps the development of new derivatives of oligonucleotide will help to solve the problems associated with the anti-gene approach in the future.

ODNs can therefore be targeted to DNA in order to achieve transcriptional arrest and thus a direct inhibition of gene function. The ODN strand is accommodated in the major groove of the DNA duplex, and may form complementary base pairs with the coding DNA strand via Hoogsteen base pairing (Moffat 1991). The resulting nucleotide triplex may displace the RNA polymerase enzyme from its catalytic binding site and consequently a truncated version of mRNA is produced, (Figure 4.2).

**4.2.2 Inhibition of mRNA transport into cytoplasm:**

RNA II polymerase transcripts, premessenger RNAs, associate in the nucleus with specific proteins that bind premessenger mRNA, heterogeneous ribonucleoproteins (hnRNPs) and with smaller nuclear ribonucleoprotein particles (snRNPs). The premessenger RNA-hnRNP-snRNP complexes assemble on nascent transcripts where the premessenger RNA is processed to mRNA by snRNP-induced splicing. The mRNA molecules are then transferred from the nucleus to the cytoplasm via nuclear pore complexes which occur on the surface of the nucleus. The

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nucleocytoplasmic transport of mRNA is an energy-driven process and is dependent on the facilitatory hnRNP-RNA complexes which shuttle between the nucleus and cytoplasm (Pinol-Roma & Dreyfus 1992). It has been postulated that single-stranded entities are preferential for nucleocytoplasmic transport (Kim & Wold 1985). In that study thymidine kinase (TK) activity was found to be reduced in cells expressing high levels of TK antisense RNA. In the TK antisense-expressing cells, sense:antisense duplexes were detected primarily in the nucleus, whereas in control cells sense TK transcripts were predominantly located in the cytoplasm. Those authors proposed that nuclear antisense inhibition involves the blockade of sense:antisense duplex export from the nucleus, possibly by disrupting the interaction between the sense RNA strand and the facilitatory hnRNP protein, and that consequently this reduces the amount of transcribed RNA found in the cytoplasmic region.

**4.2.3 Attenuation of translation:**

In normal translation the transcribed mRNA signal is converted to an amino acid sequence and thus a protein. This event occurs at the ribosome. The mRNA interacts with the ribosome and with the aid of activating enzymes, protein factors and transfer RNA molecules (tRNA) the mRNA strand is translated into the protein for which it encodes. (Figure 4.3). Antisense DNA inhibition of translation was first demonstrated by Wickstrom et al (1988). Those authors reported that an antisense oligonucleotide against the predicted hairpin loop containing the initiation codon of mRNA of the protooncogene c-myc mRNA, caused a sequence-selective inhibition of c-myc gene expression and proliferation in human leukemic HL-60 cells.

ODNs are complementary in sequence to the mRNA strand. The ODN can therefore form complementary base-pairs with the mRNA. The resulting duplex may act through steric hindrance to prevent the ribosome from binding to or scanning the message, and thus result in a truncated protein or no translation at all (Figure 4.3).

**4.2.4 Activation of RNaseH:**

Another possible mode of action of ODNs is the activation of RNaseH. This enzyme specifically degrades the hybridized RNA of an RNA-DNA duplex (Stein & Hausen 1969. The RNA-DNA duplex resulting from the interaction of the antisense ODN with the mRNA therefore provides a substrate for RNaseH.

Reliable hybrid arrest of tobacco mosic virus (TMV) translation using complementary DNA (cDNA) was found to occur in reticulocyte cell-free systems, following digestion of the hybrid with RNaseH, before assaying TMV translation (Minshull & Hunt 1986). Those authors have
more recently shown that the use of antisense oligonucleotides, complementary to a sequence common to the coding region of rabbit α and β-globin, inhibited translation of globin mRNA in reticulocyte lysate cell free systems, provided RNaseH was added before globin translation was assayed (Minshull and Hunt 1992). RNaseH-induced cleavage of mRNA has been shown to mediate the antisense elimination of endogenous calmodulin mRNA in Xenopus oocytes (Dash et al 1987) and to decrease levels of protooncogenic c-mos mRNA in mouse oocytes (O'Keefe et al., 1989). The cleaving of the mRNA strand of a mRNA/antisense DNA hybrid, by this enzyme may thus contribute to the attenuation of protein expression levels following antisense ODN treatment.

Antisense deoxynucleotides may therefore act to deplete expression levels of the target protein using several modes of action. Nuclear inhibition of protein expression may arise from the inhibition of mRNA transcription due to displacement of the RNA polymerase enzyme from its catalytic site on the non-coding DNA strand. Mature transcripts, when associated with the antisense strand, may be prevented from leaving the nucleus and will consequently be unavailable for translation. Cytoplasmic inhibition may occur as a result of RNaseH activation which will degrade the RNA strand of an RNA:DNA antisense duplex. Antisense ODNs are complementary to the mRNA target, the antisense molecule may form complementary base pairs any remaining target mRNA. This interaction may displace the ribosome through steric hindrance to block translation and hence, inhibit protein synthesis.
1. Normal Translation:

mRNA 5' UUUAAAUUUAAUUUAAAAUUUAAUUUAAA 3'

2HN-Phe-Lys-Phe-Lys-Phe-Lys

Ribosome

Result; Normal Protein

2. Translation in presence of antisense oligonucleotide:

Oligo 3' AAATTTAAATT 5'

mRNA 5' UUUAAAUUUAAUUUAAAAUUUAAUUUAAA 3'

2HN-Phe-Lys-Phe-Lys-Phe-Lys

Ribosome

Result; Displaced Ribosome

Truncated Protein

Figure 4.3 Effect of Antisense Oligonucleotides on Translation
4.3 Chemical Modifications of Oligonucleotides

Various chemical modifications have been introduced into the phosphodiester backbone of antisense oligonucleotides in order to render them uncharged and nuclease resistant (Figure 4.4). ODNs containing the phosphodiester linkage are rapidly degraded in many cells, with a half life of approximately 20 min (Fisher et al., 1993). With the phosphorothioate ODN analogue, in which one of the non-bridging equivalent internucleotide oxygen molecules is replaced by sulphur (Figure 4.4), the half life is greatly increased. Phosphorothioate modified ODNs can remain intact for several hours (Lledo et al., 1993 Campbell et al., 1993) or even days (Kirsch et al., 1993 Berrow et al., 1993).

In methylphosphonate analogues of ODNs, -CH$_3$ replaces the internucleotide oxygen (Figure 4.4). This modification also provides greater extra- and intracellular longevity because of increased nuclease resistance (Tidd, 1992). The elimination of the negative residue on the internucleotide phosphate bridge has been suggested to enhance cellular uptake, probably by improving lipid solubility (Miller et al., 1981). In contrast, phosphorothioate ODNs do not penetrate the cell membrane efficiently, requiring their use at relatively high concentrations.

As discussed in Section 4.2, the main mechanism of action of phosphodiester and phosphorothioate antisense ODNs is activation of RNaseH. Methylphosphonate ODN analogs do not activate RNaseH-mediated cleavage of RNA (Furdon et al., 1989), and yet sequence-specific inhibition of protein synthesis has been reported for ras (Brown et al., 1989) and acquired immunodeficiency syndrome virus (Sarin et al., 1988) by methylphosphonate ODNs.

The formation of methylphosphonate/phosphodiester oligonucleotide chimeras is a recent development which offers several advantages. With methylphosphonates occupying terminal positions and phosphodiesters occupying the central section, the chimera is exonuclease-resistant but retains the capacity to activate RNaseH-cleavage of mRNA. Such chimeras have proven effective in mediating antisense inhibition of the protooncogene c-myc in HL-60 cells (Tidd, 1992).
Figure 4.4 Structures and modifications of oligonucleotide internucleotide linkages
4.4 Delivery routes of oligonucleotides

• 4.4.1 External application:  
External application of antisense ODNs relies on cellular uptake of the ODNs from the culture medium. This method has had several achievements using antisense oligonucleotides. Listurud et al (1991) have investigated the contribution of the nicotinic acetylcholine receptor (nAChR) subunits to nAChR-operated channels of embryonic chick sympathetic neurones. These neurones express four nAChR channel subtypes and six nAChR subunit genes (α3, α4, α5, α7, β2 and β4). Treatment of the neuronal cultures with α7 antisense greatly reduced the number and altered the properties of all nAChR subtypes with the α7 subunit apparently making a significant functional contribution to the remaining nAChR channels. The use of antisense oligonucleotides should permit the microdissection of the functional contribution of individual subunits to the function of various ion channels, within their native environment.

The antisense technique has also been manipulated to demonstrate that the GABA_B receptor, which inhibits adenylyl cyclase in cerebellar granule neurones, belongs to the superfamily of receptors having seven transmembrane domains (Holopainen & Wojcik 1993). Since the GABA_B receptor has not yet been cloned, characterisation of this receptor has only been by pharmacological means. Antisense methods may therefore prove useful in the investigation of the secondary structure of this, and other non-cloned receptors. The study by these authors reported an optimal uptake of ODN from the external medium after 24h, although the maximal reduction in muscarinic and GABA_B activity occurred at least 3 days later. Phosphorothioate modified ODNs have also been introduced into primary cultures of rat anterior pituitary cells to reduce levels of expression of the dopamine D2 receptor (Valerio et al., 1994). In that case the ODNs began to accumulate in the cell cytoplasm just 1h after application, and a maximal uptake of ODN was observed after 24h. However, in order to substantially reduce dopamine receptor expression it was necessary for the cells to be incubated with the ODNs for several days. The long incubation times required for the ODN to exert an antisense effect was probably due to the slow turnover rates of these proteins.

The mechanism by which ODNs enter the cell is not yet fully understood. It has been reported that an 80kD protein may be a putative receptor for phosphodiester and phosphorothioate ODNs. This cell surface protein is thought to mediate phosphodiester and phosphorothioate ODN uptake in a manner compatible with receptor-mediated endocytosis in HL-60 leukaemic cells (Loke et al., 1989). However, it has also been reported that uptake of phosphodiester and phosphorothioate ODNs into HL-60 cells proceeds via a pinocytotic mechanism which is
dependent on intact protein kinase C activity (Stein et al., 1993). That study showed that internalisation of fluorescently labelled ODNs promoted exocytosis of fluid-phase markers via ODN-induced inhibition of protein kinase C, thereby suggesting that ODNs may have a blocking effect on their own uptake. In uptake studies with $^{35}$S-labeled phosphorothioate ODNs, endocytosis, mediated by a receptor-like mechanism, predominated at ODN concentrations < 1 μM, whereas fluid-phase endocytosis prevailed at higher concentrations, in K562 human leukemia cells (Beltinger et al. 1995).

In L929 and Krebs ascite carcinoma cells, cell surface receptors (of molecular masses 79 and 90 kDa) were found to bind ODNs and promote phosphodiester and phosphorothioate ODN uptake through endocytosis (Yakubov et al., 1989), using a saturable, energy-dependent mechanism. In primary neuronal cultures unmodified ODN uptake is saturable and is also reminiscent of receptor-mediated endocytosis (Yu et al., 1993).

Methylphosphonate modified ODNs however are thought to enter cells using a separate mechanism. Fluorescent labelling studies have revealed a co-localisation of methylphosphonate ODNs and endosomal/lysosomal markers in Chinese hamster ovary cells (Shoji et al. 1992). The uptake of labelled methylphosphonate ODNs was not blocked by competition for uptake by nonlabelled phosphodiester ODN or methylphosphonate ODNs, nor was ODN uptake affected by ATP levels. Following extracytoplasmic acidification and trypsinisation, receptor-mediated endocytosis is inhibited but pinocytosis is unaffected (Shoji et al. 1991). The lowered pH was found to inhibit phosphorothioate modified ODN uptake but the cellular uptake of the methylphosphonate ODNs remained intact. Since methylphosphonate ODNs have been shown to bind to the outer cell membrane (Akhtar et al. 1991), pinocytosis may play the key role in cellular uptake without involving specific oligonucleotide cell surface receptors. The described results suggest that cellular uptake of the methylphosphonate modified ODNs proceeds via the non specific pinocytotic mechanism.

The punctate distribution of fluorescently labelled ODNs within cells indicates that externally applied ODNs may accumulate in endosomal/lysosomal compartments (Loke et al. 1989). This distribution is characteristic of endocytosis, in which receptors cluster in coated pits, enter the cell via clathrin-coated vesicles, and pass through an acidified endosomal compartment before either fusing with lysosomes and being degraded or recycling to the cell surface. In order to reach targets in the nucleus and cytoplasm, ODNs need to escape from the endosomal vesicles. Since ODNs themselves are unable to destabilise membranes (Akhtar et al. 1991), an alternative, as yet unknown, mechanism must exist in order to allow ODNs access to the cytoplasmic and nuclear compartments.

Cellular uptake of phosphodiester ODNs was found to be promoted upon treatment of mouse kidney cell lines with the pore-forming agent, streptolysin O (Barry 1993), thereby providing a
means for simple and rapid introduction of ODNs into eukaryotic cells. The pore-forming tendency of streptolysin O is dependent on the cholesterol composition of the plasma membrane (Bhakdi et al., 1985), but since the plasma membrane cholesterol organisation may vary between cell types, sensitivity to streptolysin O is also likely to be variable.

In order to evaluate which antisense ODN analogue is the most advantageous, it is necessary to establish the stability of the different analogues in the cell culture milieu. Akhtar et al. (1991) found that the phosphodiester ODN was the least stable to serum nuclease degradation; although this unmodified analog had increased stability in nuclear and cytoplasmic HeLa cell extracts, where serum nuclease activity was absent. The phosphorothioate and methylphosphonate ODNs were found to have a greater stability than unmodified ODNs, particularly following heat inactivation of the serum. The presence of serum-supplemented growth media in the culture media of L929 cells was also found to promote degradation of unmodified ODNs by serum 3' exonucleases, thereby limiting the efficacy of antisense ODNs (Degols et al., 1992). It is therefore of benefit to use heat-inactivated serum or to omit serum completely during the ODN incubation in order to maximise antisense ODN longevity. This may pose a problem in many culture systems where the survival of certain cell types may be heavily dependent on serum factors.

- **4.4.2 Cationic Liposomes:** Another approach to in vitro targeting of antisense oligonucleotides consists of ODNs, of the appropriate specificity, being covalently attached to poly-L-lysine (Lemaitre et al., 1987) or encapsulated into antibody-targeted liposomes (Leonetti et al., 1990). The latter method ensures specific interaction with cells that carry the corresponding surface antigen, into which they are taken up by receptor-mediated endocytosis. Both of these approaches protect the ODNs from degradation by serum nucleases.

The efficacy of antisense ODNs complementary to the c-myc protooncogene mRNA or vesicular stomatitis virus nucleocapsid mRNA, to reduce levels of these proteins was greatly enhanced, in L929 cells, following covalent conjugation of these ODNs to poly-L-lysine (Degols et al., 1992). The mechanism by which poly-L-lysine conjugation potentiates the biological activity of antisense ODNs if not fully understood. Poly-L-lysine has been shown to act as a transmembrane carrier of antisense ODNs (Lemaitre et al., 1987); the conjugate is taken up by a nonspecific receptor-mediated endocytotic pathway, and the conjugated material is eventually released somewhere within the cell through proteolytic degradation of the polypeptide moiety. In addition, the antiviral activity of the antisense ODN complementary to the mRNA of the vesicular stomatitis virus, was enhanced following incorporation of the ODN into a liposome bearing an antibody recognition molecule to the major histocompatibility complex immunological cell surface determinant on L929 cells (Degols et al., 1992)
• **4.4.3 Microinjection:** Microinjection of the ODN directly into the cell cytoplasm avoids problems associated with uptake from the extracellular medium and degradation by serum nucleases. This technique also avoids ODN compartmentalisation within the cell, since the endocytotic pathway is bypassed. Microinjection of fluorescently-labelled ODNs into the cytoplasm showed a rapid translocation to the nucleus (Leonetti et al. 1992, Fisher et al. 1993, Wagner 1994). The nuclear accumulation of ODNs was found to be independent of intracellular ATP levels (Leonetti et al. 1992), thereby suggesting that nuclear uptake is a passive process. However, nuclear localisation was also found to occur within seconds of microinjection indicating a rapid nuclear uptake mechanism. The reason for oligonucleotides concentrating in the nucleus was proposed to be the binding of the compounds to nuclear proteins (Chin et al. 1990), subsequently, oligonucleotides conjugated to bromodeoxyuridine were found to affinity label a set of nuclear proteins, of molecular masses 36-50 kDa in HeLa cells (Leonetti et al. 1991). The rapid accumulation of unmodified and phosphorothioate ODNs predominantly in the nucleus suggests that the antisense effects of ODNs are mediated via an interaction with nascent RNA rather than arresting mRNA translation, although these ODNs may undergo nucleocytoplasmic translocation, by an as yet unknown mechanism, in order to exert an effect on cytoplasmic targets. Methylphosphonate ODN analogs however, have been shown to exhibit a strong cytoplasmic location as well as nuclear accumulation following microinjection into HeLa cells (Clarenc et al. 1993). The study of Fisher et al. (1993) demonstrated that the incorporation of a 3' phosphoroamidate linkage moiety to a phosphodiester ODN promoted ODN accumulation in cytoplasmic compartments such as the endoplasmic reticulum, in rat2 fibroblast cells. Modifications of ODNs allowing a cytoplasmic location may therefore prove useful in the development of more efficient molecules. Unmodified phosphorothioate ODNs were found to be rapidly degraded following microinjection by 3' exo- and endonuclease activity in the nucleus, in contrast, phosphorothioate ODNs were found to have greater nuclease resistance (Fisher et al. 1993). The microinjection technique has been applied to successfully deplete the GH3 pituitary cell line and cultured rat sensory neurones of G-protein α-subunits (Kleuss et al., 1991 Campbell et al., 1993).
4.5 Nonspecific effects of ODNs

Antisense ODNs can cause accidental side-effects through a number of non-sequence specific mechanisms. In *xenopus* oocytes antisense ODN have been shown to cause cleavage of imperfectly matched target sequences (Woolf *et al.*, 1992). It has also been reported that ODNs bind in a sequence specific manner to protein complexes such as thrombin (Bock *et al.*, 1992) and to small organic molecules (Ellington & Szostak 1990). ODNs containing a C-G motif, which was flanked by two 5' purines and two 3' pyrimidines, induced murine B-cell proliferation and promoted immunoglobulin secretion in a sequence-specific manner (Krieg *et al.* 1995). Intracellular breakdown of ODNs by endonucleases (Fisher *et al.*, 1993) and extracellular degradation of ODNs by serum exonucleases has been described (Shaw *et al.*, 1991), the resulting nucleoside and nucleotide degradation products have been reported to stimulate cell proliferation in a variety of cell types (Rathbone *et al.*, 1992) and inhibit differentiation of leukaemic cells (Kamano *et al.*, 1992).

It is therefore crucial to perform a range of control experiments in order to verify that any biological effects observed following antisense ODN treatment is due to a selective depletion of the target protein and not as a result of a nonspecific mechanism.

4.6 Controls for measuring antisense effects

Direct measurement of the target RNA or protein levels is required in order to evaluate the efficacy of the antisense ODN. In a cell culture system this may sometimes be problematic. For example, in primary cells, ODN uptake may be heterogeneous and quite poor (Krieg *et al.*, 1991), target protein levels may be altered in a subset of cells but overall target protein levels may not change significantly.

An advantage of the microinjection technique is that the cells injected with ODNs can be localised on gridded coverslips and the depletion of the target protein can be monitored using immunocytochemical confocal microscopy (Campbell *et al.*, 1993); however this method of measuring protein levels relies on the availability of specific target protein antibodies.

There are several control ODNs which should be considered in order to assess the specificity of the antisense ODN. This choice of control sequence is a critical element in the design of any antisense experiment.

Sense control ODNs have a sequence complementary to the antisense ODN and should not therefore interact with the target mRNA. These control ODNs are good for maintaining structural features such as palindromes and hairpin loops but do not maintain base composition.
They are useful as a control for non-sequence specific effects. However, they may interact with the non-coding strand of DNA to produce a steric hindrance of RNA transcription. Scrambled control sequences are ideal for the maintenance of base composition but do not support the structural features.

A mismatched control ODN has essentially the same sequence as the antisense ODN except for one or two mismatches in the central region of the oligonucleotide. This is an adequate control for demonstrating target hybridization selectivity and for maintaining a certain degree of base composition. However, the maintenance of structural features depends on where the mismatches are made.

Telomeres are portions of linear DNA located at the ends of eukaryotic chromosomes. Telomeres are composed of short guanine-rich repeats (G-quartet). The G-quartet domain is recognized by a telomerase enzyme which promotes its elongation in the 5’ to 3’ direction. The complementary lagging strand is then completed by DNA polymerase enzyme, allowing chromosome replication, and thus mitosis to ensue (Blackburn 1991). It has been proposed that an antisense oligonucleotide containing a G-quartet sequence may compete with the telomerase for the telomerase enzyme, thereby disrupting chromosome replication and mitosis (Stein & Krieg 1994).

Antisense oligonucleotides have been reported to inhibit leukaemic cell proliferation (Skorski 1994) and tumour growth in mice (Kitajima 1992). The basis for these antisense effects was a decrease in the observed cell proliferation; there was no quantitative data on protein or mRNA reduction in the targeted cells. Since G-quartet structures may interfere with the final stages of mitosis, and thus proliferation, it is imperative to 1) show an inhibition of target protein levels or 2) avoid antisense sequences which contain a G-quartet, when performing experiments which aim to show the anti-proliferic actions of certain antisense sequences.

4.7 Choice of antisense sequence

The choice of antisense sequence is dependent on several factors, in order to ensure efficient depletion of the target protein.

Sequences which form secondary structures should be avoided. The secondary structure may lead to intramolecular hybridization competing with the antisense-sense interaction (Rhodes & James 1990). The stem and loop structures that oligonucleotides may adopt can be predicted using thermodynamic parameters (Freier et al 1986) and computational analysis of the sequence on a variety of programs (Verma et al 1994).

Palindromic sequence arrangements and sequences with regions of high 3’ and 5’ complementation should be avoided in order to prevent self-folding of the oligonucleotide.
since folded antisense ODN molecules would have a decreased affinity for the target mRNA. The 5'-CG-3' palindromic sequence has also been reported to induce interferon and enhance natural killer cell activity (Kuramoto et al 1992), such an effect may be misinterpreted as antiproliferic action of antisense ODNs.

Complications arising from a folded conformation are more likely to occur in longer oligonucleotide molecules. Longer ODN molecules may also have an increased probability of having significant complementation with more than one target mRNA. Effective inhibition of protein expression has been observed when the antisense oligonucleotide is directed to the initiation codon or to a region at the 5' end of the mRNA molecule (Degols et al 1992, Murray & Crockett 1992). Kim & Wold (1985) found that inhibition of thymidine kinase expression was caused by a failure to export duplex RNA into the cytoplasm. There was no difference in the inhibition of nucleocytoplasmic translocation of the duplex RNA, between constructs that contained sequences homologous only to the 3', or to both the 5' and 3' regions of the target. However, for antisense RNA that escapes from the nucleus and causes inhibition of translation, it is probable that the 5' end of the mRNA is more likely to be an effective target, since inhibition of initiation seems more effective than inhibition of elongation. Targeting ODNs to the 3' region of the mRNA may result in expression of a protein with only a truncated C-terminus, although antisense ODNs interacting with a 3' target have shown successful depletion of Xenopus oocyte ribosomal proteins (Wormington et al 1986). Although evidence exists to support a 3' target site as being effective in producing an antisense effect, the antisense ODNs used in this study were complementary to a 5' region of the target mRNA.

As mentioned in Section 4.6, ODN sequences containing a G-quartet motif, have been shown to interfere with the final stages of mitosis and thus affect cellular proliferation. Studies involved in the use of antisense oligonucleotides to reduce expression levels of protooncogenes use the inhibition of proliferation as an indication of successful antisense depletion of the target protein. The presence of a G-quartet may, however, cause a non-target sequence selective inhibition of proliferation. The selection process for a suitable antisense oligonucleotide sequence should therefore avoid sequences which contain a quartet of guanine bases, particularly in studies investigating the anti-proliferic action of antisense oligonucleotides.

In order to ensure that the selected antisense ODN sequence has a highly selective complementarity unique to the target mRNA, it is necessary to screen the antisense ODN sequence through a genome database such as EMBL/Genbank. A suitable anisense sequence should only recognise the complementary mRNA sequence of the target protein, if the ODN has any >60% homology with any other protein then it should be disregarded. The control nonsense and scrambled oligonucleotides should also be screened on the EMBL/Genbank databases in order to ensure that these oligonucleotides have no target sequence of significant homology.
antisense oligonucleotides used in this study were screened through the EMBL/Genbank databases, and were found to have no other targets of significant homology. Similarly, the control nonsense and scrambled ODNs had no homology with any oligonucleotide sequence stored in the EMBL/Genbank databases.
4.8 Use of antisense ODNs to deplete cultured rat dorsal root ganglion neurones of the G-proteins $G_\text{q}$ and $G_\text{i}$

The aim of this study was to selectively deplete intact DRG neurones of the G-protein $G_\text{q}$ and $G_\text{i}$ $\alpha$ subunits, using the antisense strategy. After consideration of the possible antisense ODN delivery routes, which have been described in section 4.3 of this chapter, we elected to use the microinjection technique, thereby allowing direct intracellular application of the antisense ODN. In order to prolong the antisense ODN halflife and retain RNaseH activity, phosphorothioate-modified ODN were used. The choice of phosphorothioate antisense ODN and control nonsense ODN sequences are described in the Methods section.

4.8.1 Results

4.8.1.1 Efficiency of microinjection procedure

In order to assess the efficiency of the microinjection system the cells were injected with Dextran (1mg/ml) conjugated to the fluorescent marker fluorescein-isothiocyanate (FITC). The success of the injection was then verified by observing the injected cell under fluorescent illumination at 450 nm, through a filter selective for the fluorescein fluorochrome. Figure 4.5 shows two cells 30 min after microinjection with Dextran-FITC. Intense fluorescence was observed throughout the cell thus indicating the presence of injected dextran-FITC within the cell cytoplasm. It is unlikely that the dextran-FITC was binding non specifically to the outer cell membrane since no other cell in the immediate region emitted a fluorescent signal when excited at 450nm.

4.8.1.2 Viability of cells following microinjection

In order to assess the survival of the DRGs following microinjection, the cells were grown on Cellolocate coverslips (Eppendorf, Germany). These coverslips have a 3 mm diameter labelled grid marked on them, thereby allowing the localisation of cells following microinjection. Table 4.1 shows the proportion of viable cells remaining 1h and 24h after microinjection with 20 fl of distilled H$_2$O. 60-100% of injected cells were found to survive the injection procedure. Taken together, these results indicate that the DRG neurones were efficiently microinjected and were largely able to withstand the microinjection procedure.
Dorsal root ganglion neurones were microinjected with 20 fl of Dextran (Mr 38,000; 1 mg/ml) conjugated to the fluorochrome fluorescein-isothiocyanate (FITC). 30 minutes later the cells were viewed under fluorescent illumination at 450 nm, using a filter selective for FITC, in order to verify the success of the microinjection procedure. The injected cells are indicated by the arrows. Scale bar = 50 μm.
Table 4.1 Viability of DRG neurones 1h and 24h following microinjection

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Number of viable cells remaining after microinjection 1h</th>
<th>Number of viable cells remaining after microinjection 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7/12 (58%)</td>
<td>13/15 (87%)</td>
</tr>
<tr>
<td>2</td>
<td>6/10 (60%)</td>
<td>12/15 (80%)</td>
</tr>
<tr>
<td>3</td>
<td>11/11 (100%)</td>
<td>16/20 (80%)</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>9/11 (81%)</td>
</tr>
<tr>
<td>Mean percentage of viable cells</td>
<td>73 ± 13 %</td>
<td>82 ± 2 %</td>
</tr>
</tbody>
</table>

DRG neurones were plated out on gridded coverslips. After 5 days in culture the cell cytoplasm was microinjected with 20μl of distilled H₂O. Following microinjection the cells were returned to the incubator for either 1h or 24h in separate experiments. In order to assess cell viability, the coverslips were examined under phase contrast optics after 1h or 24h and the number of phase bright cells were counted. Cells which had not survived the injection procedure were granular, flat in morphology or had become detached from the coverslip.

The number of viable cells remaining after microinjection are expressed as a proportion of the total number of cells originally injected on each coverlip, and the percentages of viable cells remaining after injection are in parentheses.
4.8.1.3 Depletion of Go following microinjection of the Go antisense oligonucleotide

Immunocytochemical confocal microscopy, using an antipeptide anti-Gαo antiserum (OC2), revealed Go immunofluorescence associated with the inner plasma membrane and cytoplasm in control non-injected DRG neurones (Figure 4.6A). Following microinjection of the control nonsense ODN, 24h prior to fixation of the cells, Gαo immunofluorescence remained intact (Figure 4.6B). Similarly, when the cells were microinjected with antisense ODNs complementary to the mRNA of Gi there was no effect on the level of expression of Gαo (Figure 4.6C). However, 24h after microinjection of the Gαo antisense ODN the immunofluorescence associated with Go was diminished (Figure 4.6D). A similar result was obtained when the depletion of Gαo was monitored using an affinity purified anti-Gαo antibody. Figure 4.7 shows DRGs 24h after microinjection with control nonsense oligonucleotide (A) or Gαo antisense oligonucleotide (B). The cells were prepared for immunocytochemical confocal microscopy utilising an affinity purified anti-Gαo antibody. Gαo immunostaining was found primarily around the inner plasma membrane. Immunoreactivity associated with the inner plasma membrane was depleted following microinjection of Gαo antisense oligonucleotide (Figure 4.7B).

The affinity purified anti-Gαo antibody was also used to monitor the timecourse of Gαo antisense depletion of Gαo expression levels. Figure 4.8A shows the time course of Gαo antisense oligonucleotide depletion of the Go α-subunit. Following microinjection with the ODNs, the cells were fixed at various time points, incubated with the affinity purified anti-Gαo antibody and prepared for immunocytochemical confocal microscopy. An image analysis facility on the confocal microscope allows measurement of the fluorescence intensity associated with each cell, this value is expressed as grey value intensity. A maximal 76 ± 12 %, n=6 to 95 ± 9%, n=7 depletion of Gαo immunofluorescence was observed 24-36h after microinjection of the Gαo antisense oligonucleotide (Table 4.2). This gives an estimated half life of approximately 16h for Gαo. There was no effect on Gαo expression levels 24h after microinjection of control nonsense oligonucleotides or antisense ODNs complementary to the mRNA of Gi.
Figure 4.6 The effect of injection of nonsense, and $\Gamma_{\alpha_1}$ or $\Gamma_{\alpha_0}$ antisense DNA on the immunocytochemical localization of $\Gamma_{\alpha_0}$.

DRG neurones were cytoplasmically injected with 20 fl of control nonsense, and $\Gamma_{\alpha_1}$ or $\Gamma_{\alpha_0}$ antisense DNA. 24 hours after microinjection the DRGs were fixed with 4% paraformaldehyde, stained for $\Gamma_{\alpha_0}$ immunoreactivity using an anti peptide anti-$\Gamma_{\alpha_0}$ antiserum (OC2) and viewed with a confocal laser scanning microscope.

A, $\Gamma_{\alpha_0}$ localization in a control non-injected cell, showing prominent $\Gamma_{\alpha_0}$ immunostaining at the cell membrane. Some staining was also observed in the nucleus and perinuclear cytoplasm. B, $\Gamma_{\alpha_0}$ localization in a cell 24 h after injection with a nonsense DNA oligonucleotide. C, $\Gamma_{\alpha_0}$ localization in a cell 24 h after injection with the $\Gamma_{\alpha_1}$ antisense DNA oligonucleotide. D, $\Gamma_{\alpha_0}$ localization in a cell 24 h after injection with $\Gamma_{\alpha_0}$ antisense DNA oligonucleotide. Note lack of staining at cell membrane and cytoplasm.

In each case the fluorescence image is on the left, and is a 1 µm confocal section taken through the cell about 10 µm up from the attachment plaque. On the right is shown the respective phase image. Scale bar = 10 µm.
Figure 4.7  \( G_{\alpha_0} \) antisense oligonucleotide depletion of \( G_{\alpha_0} \) immunostaining

DRGs were microinjected with \( G_{\alpha_0} \) antisense oligonucleotide, fixed after 24h and prepared for immunocytochemical confocal microscopy utilising an affinity purified anti-\( G_{\alpha_0} \) antibody.

A. Cells microinjected with control nonsense oligonucleotide exhibit intense \( G_{\alpha_0} \) immunostaining associated with the inner plasma membrane.

B. 24h after microinjection with \( G_{\alpha_0} \) antisense oligonucleotide, \( G_{\alpha_0} \) immunoreactivity is lost from the inner plasma membrane.

Images are 2 \( \mu m \) confocal sections taken 10 \( \mu m \) up from the attachment plaque.

Scale bar = 25 \( \mu m \)
Figure 4.8 Time course of antisense DNA oligonucleotide depletion of $G\alpha_0$ and $G\alpha_1$ immunoreactivity

DRG neurones were microinjected with 20 fl of $G\alpha_0$ or $G\alpha_1$ antisense DNA oligonucleotides. Cells were fixed at various intervals following injection, stained for $G\alpha_0$ or $G\alpha_1$ immunoreactivity and viewed with the confocal laser scanning microscope. The image analysis facility on the confocal microscope ascribed a fluorescence intensity value to each individual cell, expressed as grey value intensity (GVI) (arbitrary units; mean± s.e.m).

A, time course of $G\alpha_0$ antisense DNA oligonucleotide depletion of $G\alpha_0$ immunoreactivity. A maximal loss of $G\alpha_0$ immunoreactivity was observed 24 h after injection of $G\alpha_0$ antisense. The control nonsense DNA oligonucleotide had no effect on $G\alpha_0$ expression levels at the 24h timepoint. The $G\alpha_0$ depletion was transient since $G\alpha_0$ levels began to recover after a further 120 h.

B, time course of $G\alpha_1$ antisense DNA oligonucleotide depletion of $G\alpha_1$ immunoreactivity. In this case a maximal depletion in $G\alpha_1$ expression levels was also observed 24 h after injection of the $G\alpha_1$ antisense oligonucleotide. The control nonsense oligonucleotide had no effect on $G\alpha_1$ levels at 24 h. $G\alpha_1$ levels were found to recover after 90 h.

n=7 for $G_0$, n=4 for $G_1$, **p<0.01, Student's t-test compared to cells microinjected with control nonsense oligonucleotide.
Table 4.2 Range of inhibition of Gα<sub>Q</sub> immunoreactivity following microinjection of Gα<sub>Q</sub> antisense oligonucleotides.

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>% Depletion of Gα&lt;sub&gt;Q&lt;/sub&gt; immunoreactivity following microinjection with Gα&lt;sub&gt;Q&lt;/sub&gt; antisense oligonucleotide (mean ± s.e.m. number of cells in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76 ± 12 % (n=6)</td>
</tr>
<tr>
<td>2</td>
<td>79 ± 21 % (n=6)</td>
</tr>
<tr>
<td>3</td>
<td>84 ± 9 % (n=11)</td>
</tr>
<tr>
<td>4</td>
<td>89 ± 16 % (n=9)</td>
</tr>
<tr>
<td>5</td>
<td>95 ± 9 % (n=7)</td>
</tr>
</tbody>
</table>

The % inhibition of Gα<sub>Q</sub> immunoreactivity 24h after microinjection of Gα<sub>Q</sub> antisense oligonucleotide was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the area of immunofluorescence associated with a 2 μm section taken midway through the cell and, using the corresponding phase contrast image to outline the boundary of the cell, an average grey value intensity (GVI) was ascribed to each cell. The % depletion of Gα<sub>Q</sub> immunoreactivity following treatment with Gα<sub>Q</sub> antisense was calculated by comparing the GVI value of Gα<sub>Q</sub> antisense injected cells with the GVI of nonsense injected cells at 24h.

4.8.1.4 Depletion of Gα<sub>Q</sub> following microinjection with Gα<sub>Q</sub> antisense oligonucleotide

Immunocytochemical confocal microscopy, utilising an affinity purified anti peptide Gα<sub>Q</sub> antibody, revealed Gα<sub>Q</sub> immunostaining primarily around the inner plasma membrane in control non-injected DRGs (Figure 4.9A). This Gα<sub>Q</sub> immunostaining was unaffected by microinjection of control nonsense oligonucleotides (Figure 4.9B) or antisense ODNs complementary to the mRNA of Gα<sub>Q</sub> (Figure 4.9C). In comparison, Gα<sub>Q</sub> immunostaining associated with the inner plasma membrane was completely abolished following microinjection of the antisense ODN complementary to the mRNA of Gα<sub>Q</sub> (Figure 4.9D).

The timecourse of Gα<sub>Q</sub> antisense oligonucleotide depletion of Gα<sub>Q</sub> expression levels was also monitored using the affinity purified anti-Gα<sub>Q</sub> antibody. The DRG neurones were injected with either Gα<sub>Q</sub> antisense or control nonsense oligonucleotides and fixed at various timepoints. The cells were then incubated with the affinity purified anti-Gα<sub>Q</sub> antibody and prepared for immunocytochemistry. Figure 4.8B shows the time course of Gα<sub>Q</sub> antisense ODN depletion of G<sub>I</sub> α subunit levels. The Gα<sub>Q</sub> antisense ODN also produced a maximal 68 ± 11 %, n=5 to 89 ± 8 %, n=7 depletion of its target protein 24-32 h after microinjection (Table 4.3), giving a half life of approximately 16 h for Gα<sub>Q</sub>. There was no effect on levels of Gα<sub>Q</sub> expression 24h after microinjection of a control nonsense oligonucleotide or antisense ODNs complementary to the mRNA of Gα<sub>Q</sub>.
Figure 4.9 The effect of injection of nonsense, and GαQ or Gαi antisense DNA on the immunocytochemical localization of Gαi.

DRG neurones were cytoplasmically injected with 20 fl of control nonsense, and GαQ, or Gαi antisense DNA. 24 hours after microinjection the DRGs were fixed with 4% paraformaldehyde, stained for Gαi immunoreactivity using an affinity purified anti-peptide anti-Gαi antiseraum and viewed with a confocal laser scanning microscope.

A, Gαi localization in a control non-injected cell, showing prominent Gαi immunostaining at the cell membrane and in some parts of the cytoplasm. No nuclear localization was observed.

B, Gαi localization in a cell 24 h after injection with a nonsense DNA oligonucleotide.

C, Gαi localization in a cell 24 h after injection with the GαQ antisense DNA oligonucleotide.

D, Gαi localization in a cell 24 h after injection with Gαi antisense DNA oligonucleotide. Note lack of staining at cell membrane.

In each case the fluorescence image is on the left, and is a 1 μm confocal section taken through the cell about 10 μm up from the attachment plaque. On the right is shown the respective phase image. Scale bar = 10 μm.
Table 4.3 Range of inhibition of Gα1 immunoreactivity following microinjection of
Gα1 antisense oligonucleotides.

The % inhibition of Gα1 immunoreactivity 24h after microinjection of Gα1 antisense oligonucleotide was measured using the image analysis facility supplied with the confocal microscope. A cursor was used to outline the area of immunofluorescence associated with a 2 μm section taken midway through the cell and, using the corresponding phase contrast image to outline the boundary of the cell, an average grey value intensity (GVI) was ascribed to each cell. The % depletion of Gα1 immunoreactivity following treatment with Gα1 antisense was calculated by comparing the GVI value of Gα1 antisense injected cells with the GVI of nonsense injected cells at 24h.

4.8.2 Discussion

The microinjection technique has proved to be a useful method by which to apply ODNs directly into non-transformed cells. The injection procedure is semi-automated which, although technically demanding, allows the injection of a large population of cells. The DRG neurone is ideally suited for microinjection. The large cell diameter (15-20 μm) and spherical morphology of the DRGs neurone allows easy visualisation of the microinjection procedure using phase contrast optics. These cells are also particularly robust and can readily withstand the injection. The DRG cell cytoplasm was the preferential injection target since ODNs have been found to be rapidly taken up into nucleus following cytoplasmic injection (Leonetti et al., 1991). The nucleus of these cells was quite prominent and could be injected, albeit with greater difficulty than cytoplasmic injections, but a cytoplasmic injection site was preferred in order to avoid nuclear damage occurring as a result of microinjection.
The ability to microinject antisense ODNs directly into DRG neurones avoids complications associated with cellular ODN uptake, and degradation of the ODN by serum exonucleases. It is also possible using this technique to record exactly which neurones were successfully microinjected, this avoids problems associated with differential ODN uptake by subpopulations of cells, which may be the case when applying ODNs via the extracellular medium.

Microinjection was found to be a successful route of ODN delivery into the DRG neurones. The expression levels of the target G-proteins, as measured by immunocytochemical confocal microscopy, were successfully depleted following microinjection of the G-protein antisense ODNs. Furthermore, this depletion was found to be specific to the target protein; $\alpha_0$ levels were exclusively reduced by antisense ODNs complementary to the mRNA of $\alpha_1$ and $\alpha_2$, furthermore, $\alpha_i$ levels were selectively depleted by antisense ODNs complementary to the mRNA of $\alpha_{11}$, $\alpha_{12}$ and $\alpha_{13}$. The expression levels of $\alpha_0$ were unaffected following microinjection of a control nonsense oligonucleotide, which had no target of significant homology, nor were $\alpha_0$ levels affected by microinjection of an antisense DNA oligonucleotide which was highly complementary to the mRNA of $\alpha_{11}$, $\alpha_{12}$ and $\alpha_{13}$. Similarly, $\alpha_i$ expression levels were unaffected following microinjection of the control nonsense oligonucleotide or the antisense DNA oligonucleotide which had high complementarity to the mRNA of $\alpha_0$ and $\alpha_0$.

The selective depletion of either $\alpha_0$ or $\alpha_i$ from DRGs using this antisense strategy has been manipulated in this laboratory in order to address which subtype of G protein mediates the GABAergic inhibition of Ca$^{2+}$ channel currents (Campbell et al., 1993). The inhibition of the Ca$^{2+}$ channel current by the GABA agonist (-)-baclofen was measured 24h after microinjection of either $\alpha_0$ or $\alpha_i$ antisense ODNs. The $\alpha_i$ antisense ODN had no effect on the GABAergic inhibition of the Ca$^{2+}$ current. In contrast, the $\alpha_0$ antisense ODN reduced the GABAergic inhibition of Ca$^{2+}$ current by approximately 60%. The failure to produce a complete inhibition of $\alpha_0$ expression in some batches of DRG cultures, or to completely abolish the GABAergic modulation of the Ca$^{2+}$ current by $\alpha_0$ antisense ODNs may be explained by the excess of $\alpha_0$ levels normally present in these cells or to the ability of the coupling process to utilise $\alpha_i$ when $\alpha_0$ is absent. The result from the antisense study of Campbell et al (1993) suggests that the GABAergic inhibition of Ca$^{2+}$ channel currents is via the $\alpha_0$ subtype of G-protein. This is agreement with previous reports which showed a selective inhibition of the GABAergic modulation of N-type Ca$^{2+}$ currents following treatment of the cells with anti $\alpha_0$, but not anti $\alpha_i$ antibodies (Menon-Johannson et al., 1993). Furthermore, in GH3 cells, microinjection of antisense oligonucleotides complementary to the sequences of $\alpha_0$ and $\alpha_i$, showed that muscarinic and somatostatin receptors were coupled to $\alpha_0$ and $\alpha_0$ respectively to inhibit L-type calcium channel.
currents, $\mathrm{G}_\alpha_1$ was found to have no role in this modulation (Kleuss et al., 1991). Similarly, using the antisense strategy it was shown that the dopaminergic inhibition of calcium currents in pituitary lactotrophes was exclusively via $\mathrm{G}_\alpha_\mathrm{O}$ (Baertschi et al., 1992). Antisense studies have also been applied to investigate the G-protein modulation of other ion channels; the muscarinic inhibition of K$^+$ channel currents in rat ventromedial hypothalamic neurones was attributed to the $\alpha$-subunit of $\mathrm{G}_{11}$, following depletion of various G-protein $\alpha$-subunits by application of antisense oligonucleotides to the cell culture medium (Buckley et al., 1995).

The finding that loss of $\mathrm{G}_\alpha_0$ and $\mathrm{G}_\iota$ immunoreactivity was most extensive 24-32h after microinjection of the respective antisense oligonucleotides gives an estimated half life of approximately 16$h$ for these proteins. A similar turnover rate was previously observed for $\mathrm{G}_\alpha_\mathrm{O}$ in GH$_3$ cells (Kleuss et al., 1991), although the turnover rate in GH$_4$ pituitary cells, estimated from metabolic labelling experiments, was slightly slower ($t_{1/2} 28 \pm 7$h) (Silbert et al., 1990).

The levels of $\mathrm{G}_\alpha_0$ and $\mathrm{G}_\iota$ expression began to recover after 96$h$, this is presumably indicative of oligonucleotide degradation or export from the cell. The ODNs used in this study had a modified phosphorothioate backbone to improve nuclease resistance, however, it would appear that this modification only allows a temporary stability and eventually the ODN is broken down and rendered inactive. It is unknown to what extent the resulting ODN degradation products affect the cell, although some reports have described an affect on cell proliferation (Rathbone, 1992) and differentiation (Kamano, 1992).

In summary, microinjection of antisense oligonucleotides, complementary to the mRNA of $\mathrm{G}_\alpha_0$ or $\mathrm{G}_\iota$, into the cytoplasm of cultured DRG neurones produces a selective depletion of these target proteins. This has subsequently allowed the functional relevance of these G-proteins on the GABAergic modulation of calcium channel currents to be evaluated.

The relative ease of applying antisense ODNs to DRG neurones through microinjection has wide potential in the investigation into the functional significance of various proteins in these cells.

The next section is concerned with manipulating the antisense strategy to deplete DRGs of the voltage-dependent calcium channel $\beta$-subunit.
4.9 Use of antisense oligonucleotides to deplete DRG neurones of the voltage-dependent calcium channel β-subunit

The aim of this part of the study was to use antisense oligonucleotides to produce a selective
depletion of the VDCC β-subunit from DRG neurones.

Using an identical protocol to the G-protein antisense study described in section 4.8, the
delivery route for the ODNs was via microinjection directly into the cell cytoplasm. The
antisense oligonucleotide had a modified phosphorothioate backbone in order enhance nuclease
resistance and retain RNaseH activity. The antisense, control nonsense and scrambled
sequences are described in the Methods section.

4.9.1 Introduction

The voltage-dependent calcium channel (VDCC) is a multi subunit complex consisting of a
core forming α1 subunit and ancillary β, α2/δ and, in skeletal muscle, γ subunits (see General
Introduction). Evidence from co-expression studies in oocytes and mammalian cells suggests
that the VDCC β subunit modifies the biophysical and pharmacological characteristics of the
current observed on expression of the α1-subunits alone (Singlet et al., 1991 Itagaki et al., 1992
Zhang et al., 1993). A spectrum of effects has been attributed to the β-subunits, including an
increase in the expressed current amplitude (Singlet et al., 1991 Lory et al., 1993 Stea et al., 1993)
increase in the activation and inactivation kinetics (Varadi et al., 1991) and a hyperpolarising
shift in the voltage dependence of activation (Perez-Reyes et al., 1992 Stea et al., 1993).

In order to investigate further the role of the VDCC β subunit in calcium channel function and
GABAergic modulation, the antisense strategy was applied. The DRGs were microinjected
with antisense oligonucleotides complementary to the mRNA of the four known β subunits.
The microinjection procedure was essentially the same as described for the G-protein antisense
oligonucleotide study (Section 4.7). Immunocytochemical confocal microscopy, utilising an
anti peptide VDCC β-subunit antiserum, was then employed in order to examine the efficiency
of the β subunit antisense ODN in depleting expression levels of the VDCC β subunit.

The antipeptide anti-β subunit antiserum was raised against a penta-decapeptide corresponding
to amino acid residues 65-79 inclusive of the deduced sequence of the rat brain β1β-subunit
(Pragnell et al., 1991). This peptide sequence is common to all current rat brain β-subunit clones
with the exception of β3, which has a single leucine insertion (Castellano et al., 1993). The β
subunit antisense DNA oligonucleotide sequence is described in the Methods section. The
oligonucleotide sequence has 96% complementarity to nucleotides 487-511 of the β1β mRNA
(Pragnell et al 1991), and 92% complementarity to nucleotides 676-701 of the β2 mRNA (Perez-Reyes et al 1992), 394-419 of the β3 mRNA (Castellano et al 1993b) and 397-422 of the β4 mRNA (Castellano et al 1993a). The antisense ODN interaction site is therefore downstream to the anti-β subunit antiserum recognition site.

4.9.2 Results

4.9.2.1 Time course of β-subunit antisense ODN depletion of VDCC β-subunit levels
Figure 4.10 shows the time course for depletion of VDCC β-subunit levels by the β-subunit antisense ODN. DRG neurones were fixed with 4% paraformaldehyde at various time intervals following antisense ODN microinjection and prepared for immunocytochemical microscopy. The degree of immunofluorescence associated with each cell was measured using the image analysis facility supplied with the confocal microscope. A maximal depletion (93 ± 9%, n=9, p<0.01, t-test) of VDCC β-subunit immunoreactivity was observed 110h after microinjection of the antisense sequence, suggesting a half-life for the turnover of the β-subunit greater than 50h. At 110h there was no effect on β-subunit immunofluorescence by the scrambled control ODN. The β subunit immunoreactivity began to rise after 130h, probably as a result of ODN degradation.

4.9.2.2 Immunocytochemical evidence for β-subunit antisense ODN depletion of VDCC β-subunit expression levels
Figure 4.11A shows β subunit immunostaining around the inner plasma membrane and cytoplasm in a control non-injected DRG neurone. 110h after microinjection with a control nonsense ODN the cells were fixed and prepared for immunocytochemistry (Figure 4.11B), the nonsense ODN was found to have no effect on β-subunit immunoreactivity. However, 110h after microinjection of the β subunit antisense sequence, VDCC β subunit immunofluorescence was substantially reduced (Figure 4.11C), thereby indicating that the β-subunit antisense ODN was effective in reducing expression levels of its target protein.
Figure 4.12A shows a group of DRGs 110h after microinjection with control scrambled oligonucleotide. The cells exhibited β-subunit immunoreactivity around the inner plasma membrane, with a lesser degree of immunofluorescence observed in the cytoplasm. 110 h after microinjection of β-subunit antisense oligonucleotide (Figure 4.12B), β-subunit immunolocalisation around the plasma membrane was depleted.
VDCC β-subunit Depletion

![Graph showing time course of antisense DNA oligonucleotide depletion of VDCC β-subunit immunoreactivity](image)

**Figure 4.10 Time course of antisense DNA oligonucleotide depletion of VDCC β-subunit immunoreactivity**

DRG neurones were microinjected with 20 fl of VDCC β-subunit antisense DNA oligonucleotides. Cells were fixed at various intervals following injection, stained for VDCC β-subunit immunoreactivity and viewed with the confocal laser scanning microscope. The image analysis facility on the confocal microscope ascribed a fluorescence intensity value to each individual cell, expressed as grey value intensity (GVI) (arbitrary units; mean± s.e.m). The time course of β-subunit antisense DNA oligonucleotide depletion of VDCC β-subunit immunoreactivity showed a maximal loss of β-subunit immunoreactivity 110 h after injection of the β-subunit antisense oligonucleotide. The control nonsense DNA oligonucleotide had no effect β-subunit expression levels at the 110 h timepoint. The β-subunit depletion was transient since G\(\alpha\) levels began to recover at 144 h.

\(n=9\), **\(p<0.01\) Student's t-test. compared to the GVI value ascribed to cells injected with nonsense oligonucleotide.
Figure 4.11 The effect of injection of nonsense and VDCC β-subunit antisense DNA on the immunocytochemical localization of the VDCC β-subunit.

DRG neurones were cytoplasmically injected with 20 fl of control nonsense and VDCC β-subunit antisense DNA. 110 hours after microinjection the DRGs were fixed with 4% paraformaldehyde, stained for VDCC β-subunit immunoreactivity using an anti-VDCC β-subunit antisera and viewed with a confocal laser scanning microscope. A, VDCC β-subunit localization in a control non-injected cell, showing prominent β-subunit immunostaining at the cell membrane with a lesser degree of immunostaining observed in the cytoplasm. B, VDCC β-subunit localization in a cell 110 h after injection with a control nonsense DNA oligonucleotide. C, VDCC β-subunit localization in a cell 110 h after injection with the VDCC β-subunit antisense oligonucleotide. Note lack of staining at cell membrane. In each case the fluorescence image is on the left, and is a 2 μm confocal section taken through the cell about 10 μm up from the attachment plaque. On the right is shown the respective phase image. Scale bar = 10 μm.
Figure 4.12  Antisense oligonucleotide depletion of the VDCC β-subunit from a group of DRG neurones

A, immunostaining for the VDCC β-subunit. The staining is largely localized to the inner plasma membrane with a lesser degree of immunostaining observed in the cytoplasm, 110 h after injection of a control scrambled DNA oligonucleotide. B, reduction in VDCC β-subunit staining 110 h after injection with a VDCC β-subunit antisense DNA oligonucleotide. The fluorescent image is on the left, and is a 2 μm confocal section taken through the cell approximately 10 μm up from the attachment plaque. On the right is shown the respective phase image. Scale bar = 20 μm.
4.9.2.3 Effect of the β subunit antisense ODN on levels of expression of other VDCC associated proteins

To examine whether the β subunit antisense ODN caused a non-specific reduction of the synthesis of other proteins involved in the VDCC complex or in signal transduction involving the GABAB receptor, the levels of the VDCC α2 subunit and G-protein Gαo were observed following β subunit antisense microinjection. Depletion of β subunit immunoreactivity was found to have no effect on expression levels of the VDCC α2 subunit (Figure 4.13A). Furthermore, depletion of β-subunit immunoreactivity had no effect on Gαo expression levels at 110h (Figure 4.13B) or at 24h (Figure 4.14).

4.9.2.4 Effect of β subunit antisense ODN on levels of expression of VDCC α1-subunits

In order to investigate whether depletion of the β-subunit disrupted the association of the VDCC main pore-forming α1 subunits with the membrane, or caused a non-sequence specific reduction in the synthesis of α1 subunits, the expression levels of α1-subunits were examined immunocytochemically 110h after microinjection of the β-subunit antisense ODN. Since VDCC β subunits can interact with a number of different clones of the VDCC α1 subunit (for review see Perez-Reyes & Schnieder) it was necessary to examine the effect of the β subunit antisense sequence on levels of expression of the A, B, C and D-type calcium channel clones. The β subunit antisense sequence was found to have no effect on expression levels of the α1 subunit of the ω-Aga IVA-toxin sensitive A-type calcium channel clone (Figure 4.15A) or on the α1 subunit of the ω-conotoxin GVIA-sensitive B-type calcium channel clone (Figure 4.15B). Similarly, expression levels of the α1 subunits of the dihydropyridine-sensitive C- and D-type calcium channel clones were unaltered following β subunit depletion (Figure 4.15 C,D).
Figure 4.13 The lack of effect of the VDCC β-subunit antisense oligonucleotide injection on immunostaining for Gα_{q} or VDCC α2-subunit.

DRG neurones were microinjected with 20 fl of VDCC β-subunit antisense DNA oligonucleotide. 110 h after microinjection of the β-subunit antisense, the cells were fixed with 4% paraformaldehyde and stained for Gα_{q} or VDCC α2-subunit immunoreactivity utilizing an affinity purified anti Gα_{q} antibody or anti VDCC α2-subunit antiserum respectively.

A, immunostaining for Gα_{q} was observed around the inner plasma membrane before (left) and 110 h after (right) microinjection of VDCC β-subunit antisense oligonucleotides. No reduction in staining for Gα_{q} was observed.

B, membrane associated immunostaining was observed for the VDCC α2-subunit No reduction in staining for VDCC α2-subunit was observed 110 h following β-subunit antisense oligonucleotide microinjection (right) compared with control (left).

Images are 2 μm confocal sections taken midway through the cell. Scale bar = 5 μm.
DRGs were microinjected with VDCC β-subunit antisense oligonucleotide and fixed after 24h. Cells were prepared for immunocytochemical confocal microscopy utilising an affinity purified anti-$G_{\alpha\text{O}}$ antibody.

A, $G_{\alpha\text{O}}$ immunolocalisation was associated with the inner plasma membrane in control non-injected cells.

B, 24h after microinjection with β-subunit antisense, $G_{\alpha\text{O}}$ immunostaining was still found to occur around the inner plasma membrane.

All images are 2 μm confocal sections taken midway through the cell.

Scale bar = 25 μm
Figure 4.15 The lack of effect of the VDCC β-subunit antisense oligonucleotide injection on immunostaining of the VDCC clone α1-subunits.

DRG neurones were microinjected with 20 fl of VDCC β-subunit antisense DNA oligonucleotide. 110 h after microinjection of the β-subunit antisense, the cells were fixed with 4% paraformaldehyde and stained for VDCC α1-subunit immunoreactivity utilizing a range of anti peptide antiserum specific to the α1-subunit of each of the calcium channel A, B, C and D-clones.

A, immunostaining for the o-Aga IVA toxin-sensitive calcium channel A-clone was observed around the plasma membrane before (left) and 110 h after (right) microinjection of VDCC β-subunit antisense oligonucleotides. No reduction in staining for the calcium channel A-clone was observed.

B, plasma membrane and neurite associated immunostaining was observed for the o-conotoxin-sensitive calcium channel B-clone No reduction in staining for calcium channel B-clone was observed 110 h following B-subunit antisense oligonucleotide microinjection (right) compared with control (left).

C, no reduction in the plasma membrane and slight neurite immunostaining of the dihydropyridine-sensitive calcium channel C-clone was observed before (left) and 110 h after (right) microinjection of the VDCC β-subunit antisense oligonucleotide.

D, no reduction in the plasma membrane and neurite immunostaining of the dihydropyridine-sensitive calcium channel D-clone was observed before (left) and 110 h after (right) microinjection of the VDCC β-subunit antisense oligonucleotide.

Images are 2 μm confocal sections taken midway through the cell. Scale bar = 5 μm.
4.9.3 Discussion

The results described in this section indicate that it is possible to deplete DRGs of VDCC β subunit immunoreactivity, by injection of an antisense oligonucleotide with a high complementarity to all four VDCC β subunit mRNAs. VDCC β-subunit immunolocalisation was unaffected following microinjection of either control nonsense oligonucleotide, which had no target sequence of significant homology or control scrambled oligonucleotide, which had the same base composition as the β-subunit antisense oligonucleotide but had no target sequence of significant homology. It is unlikely that a truncated version of the β-subunit protein was produced following antisense ODN treatment. The β subunit antisense ODN interaction site is downstream (3') from the region of the β-subunit mRNA that encodes for the anti-β subunit antisem recognition site. If antisense treatment resulted in a truncated β-subunit, the antisem would still have the ability to bind to its recognition domain on the β-subunit, and consequently, β-subunit immunostaining would be unaffected following microinjection of antisense ODN.

Depletion of the VDCC β subunit had no effect on the expression levels or localisation within the cell of other calcium channel subunits, such as the α1 or α2 subunits.

The effects of β subunit depletion on calcium channel function were examined by co-workers from this laboratory. The peak calcium channel amplitude was attenuated, a depolarising shift in the voltage dependence of activation was observed and the ability of the dihydropyridine agonist BayK 8644 to enhance calcium channel currents was reduced following β subunit depletion (Berrow et al., 1995). Since the β-antisense ODN had no effect on expression levels of other components of the VDCC complex (α1, α2 subunits), the alteration in the biophysical and pharmacological properties of the calcium channel current may be solely attributed to loss of the β subunit. The functional effects observed in the absence of the VDCC β subunit are the converse from results obtained by co-expression of the β-subunit with α1 subunits of skeletal muscle, cardiac and neuronal calcium channels (Varadi et al., 1991 Hullin et al., 1991 Castellano, et al 1993). Taken together, these findings suggest that the VDCC β subunit exerted a considerable influence on the biophysical properties of functional calcium channels.

A particularly intriguing result, concerning GABAergic modulation of calcium channel current, was discovered following β subunit depletion. The inhibition of the residual Ca2+-current by the GABAβ agonist (-)-baclofen was markedly enhanced following loss of β subunit immunoreactivity (Campbell et al., 1995). The GABAβ-ergic modulation of calcium channel currents has previously been shown to be mediated via Gαo (Campbell et al., 1993). Since Gαo expression levels and Gαo localisation were unaffected following β-subunit antisense.
treatment, the observed alteration in GABAergic modulation of calcium channel currents may solely be attributed to loss of the β-subunit. The hypothesis resulting from the study of Campbell et al. (1995) described a competition between the VDCC β subunit and Gαq for binding to the VDCC α1 subunit, and that the β subunit, when complexed with the VDCC α1 subunit has a GTPase activating effect to promote the hydrolysis of Gαq. This would limit the time for which activated Gαq interacts with the VDCC α1 subunit, thereby limiting the efficacy of the Gα-mediated inhibitory modulation. This hypothesis will be further described in Chapter 5.

A maximal inhibition of β subunit immunoreactivity was observed 110h after microinjection with β antisense ODNs. This gives an estimated half-life for β subunit turnover of at least 50h. The VDCC β-subunit therefore has a slower turnover rate than that of the α subunit of the G-protein Gαq, also a membrane-associated protein, which was found to have a half-life of just 16h. Antisense oligonucleotides have demonstrated a turnover of 20 h for the α1-subunit of the L-type VDCC in mouse kidney cell line (Barry et al. 1993). The VDCC α1-subunit polypeptide is much larger than the β-subunit polypeptide. The higher turnover rate reported for the α1-subunit in kidney cell lines by Barry et al. (1993) compared to the β-subunit turnover in DRG neurones may reflect differences in the properties of protein synthesis between cell lines and primary neuronal cultures. The expression levels of the β-subunit began to recover at 144 h after oligonucleotide microinjection, this indicates a limit of the effectiveness of a single phosphorothioate injection in this system.

4.9.5 Summary

In summary, the antisense strategy has been successfully applied in this study to produce a selective depletion of the target proteins within intact cells. Microinjection of antisense sequences, complementary to the mRNA of the G-proteins Gαo/Gαi and the calcium channel β-subunit, produced a selective depletion of these target proteins in cultured dorsal root ganglion neurones, as measured by immunocytochemical confocal microscopy. The G-proteins Gαo and Gαi were both maximally depleted 24 h after injection of antisense DNA oligonucleotides, which have high complementarity to each of the respective Gαo and Gαi mRNA sequences. This gives a half-life of approximately 16 h for these two intracellular proteins. In contrast the calcium channel β-subunit, which is also an intracellular protein associated with the plasma membrane, was maximally depleted 110 h after injection of an antisense DNA oligonucleotide which is highly complementary to the mRNA of all four
cloned VDCC β-subunits. This gives an estimated halflife of approximately 55h for this protein.

The successful depletion of these proteins from DRG neurones allowed their influence on calcium channel function and modulation to be examined. The GABAergic inhibition of calcium channel currents was abolished following antisense oligonucleotide depletion of GαQ, but not Gαi. This result indicated that the GABAβ receptor is coupled to the inhibition of calcium channel currents via GαQ in DRG neurones.

Antisense depletion of the calcium channel β-subunit had profound effects on the biophysical and pharmacological characteristics of the calcium channel current. The peak inward current was attenuated, a depolarising shift in the kinetics of activation was observed and the ability of the dihydropyridine agonist BAYK 8644 to enhance calcium channel currents was reduced. The GABAergic inhibition of the calcium channel current was potentiated following depletion of the β-subunit immunoreactivity.

The development of antisense technology therefore provides a strategy with widespread implications into further understanding of the proteins and mechanisms underlying a broad spectrum of events occurring in the field of cell biology.
Chapter 5

Voltage-dependent calcium channel β-subunits, in combination with α1-subunits, have a GTPase-activating effect to promote hydrolysis of GTP by Gαo in rat frontal cortex membranes.
5.1 Introduction

Neuronal voltage-dependent Ca$^{2+}$ channels (VDCCs) have been shown to be modulated by a variety of neurotransmitters via an interaction with pertussis toxin (PTX)-sensitive GTP-binding proteins (G proteins) (for review see Dolphin 1995). Evidence from studies using anti-G protein antibodies (McFadzean et al. 1989), and more recently, antisense oligonucleotides complementary to the mRNA of G protein α-subunits, suggest that the Go subtype of G proteins may be pivotal in mediating the neurotransmitter inhibition of Ca$^{2+}$ channel currents (Kleuss et al. 1991, Campbell et al. 1993). In many neuronal systems, neurotransmitters inhibit N and P/Q-type VDCCs (for review see Dolphin 1995) with a smaller effect on L-type Ca$^{2+}$ channels (Kleuss et al. 1991). L-type VDCCs are the primary target for modulation in neurosecretory (Kleuss et al. 1991) and non-neuronal secretory cells (Schmidt et al. 1991).

G proteins have also been found to modulate the interaction of dihydropyridine (DHP) agonists with L-type Ca$^{2+}$ channels. The agonist effects of DHPs on dorsal root ganglion neuron Ca$^{2+}$ channel currents is promoted upon activation of a PTX-sensitive G protein (Scott & Dolphin 1987) and GTP analogues have been shown to enhance DHP agonist binding to cortical synaptic membranes (Bergamaschi et al. 1988).

Purification of the DHP receptor from skeletal muscle has revealed a subunit composition for the L-type VDCC of four non-covalently linked heterologous polypeptides (α1, α2/δ, β and γ) (see General Introduction Chapter 1). Expression studies have indicated that the α1 subunit is capable of forming a functional ion pore that binds DHPs (Perez-Reyes et al. 1989) and can be modulated by binding of the VDCC β subunit to increase the expressed current amplitude and influence its kinetics (Wei et al. 1991, Lory et al. 1993, Stea et al. 1993). The use of antisense oligonucleotides complementary to all four cloned VDCC β-subunits has also revealed the importance of endogenous β-subunits in the amplitude, activation kinetics, DHP modulation (Berrow et al. 1995) and G protein modulation (Campbell et al. 1995) of Ca$^{2+}$ currents in dorsal root ganglion neurones.

G protein α subunits have an intrinsic GTPase activity which hydrolyses GTP, bound to the activated form of the G protein α subunit, to GDP, thereby inactivating an recycling the G protein (Bourne et al. 1990). Activation of a G protein linked neurotransmitter receptor results in an increase in GTPase activity. Such an enhancement of GTPase has been observed for example following activation of the G protein linked dopamine D2 receptor in the rat striatum (Onali & Olianas 1988) or muscarinic M2 receptors in the rat forebrain (Hoss et al. 1990), and this results from an increased exchange of GTP for GDP. However, GTPase can also be potentiated by an effector protein acting as a GTPase-activating-protein (GAP) to stimulate the intrinsic GTPase activity of the activated G protein. For example, cGMP phosphodiesterase has...
been shown to stimulate the GTPase activity of the photoreceptor G protein transducin in amphibian (Arshavsky & Bownds 1992) and bovine (Arshavsky et al 1994) rod outer segments. Moreover, the GTPase activity of the G protein Gq/11 is stimulated by its effector phospholipase-Cβ1 (Bernstein et al 1992).

It has been shown previously that the GTPase activity of the G protein Go can be stimulated by a number of DHP agonists (Sweeney & Dolphin 1992). Using an antipeptide anti-VDCC β subunit antiserum and a peptide, which mimics the β-subunit binding site on the VDCC α1 subunit (Pragnell et al 1994), the data presented in this Chapter provides evidence that the VDCC β subunit is the principal component of the L-type Ca^{2+} channel involved in linking DHP agonist binding with enhanced GTPase activity of the L-type VDCC associated G protein.
5.2 Results

5.2.1 Stimulation of GTPase by the DHP agonist (-)-BayK 8644
The L-type calcium channel activator (-)-BayK 8644 stimulates the GTPase activity of rat frontal cortical membranes in a dose-dependent manner, a maximal 21.5 ± 4.7 % stimulation above basal GTPase is found to occur with a 10 nM concentration of (-)-BayK 8644 (with 0.5 μM GTP as a substrate) (Figure 5.1). In contrast the L-type calcium channel antagonist (+)-BayK 8644 is found to produce only a modest 3.72 ± 0.63 % stimulation above basal GTPase activity (Figure 5.1).

5.2.2 Stimulation of GTPase by the GABA agonist (-)-baclofen
The GABA agonist (-)-baclofen has been observed to increase the GTPase activity of rat frontal cortex membranes in a dose-dependent manner. A maximal 30-35 % stimulation of GTPase above basal GTPase (46 ± 8.0 pmol/mg/min) is found to occur at 10-100 μM (-) baclofen, with 0.5 μM GTP as substrate (Figure 5.2).

5.2.3 Stimulation of GTPase by the DHP agonist (-)-BayK 8644 and GABA agonist (-)-baclofen is reduced by affinity purified anti-GαO and anti-GαO/Gαi antibodies respectively.
Treatment of the membranes with the affinity purified anti-GαO antibody caused an 84 ± 5 % reduction in the stimulation of GTPase by the DHP agonist (-)-BayK 8644 (10nM), with 0.5 μM GTP as a substrate (Figure 5.3A). The affinity purified anti-Gαi antibody had no effect on the DHP agonist-induced stimulation of GTPase (Figure 5.3) at 0.5 μM GTP. Preadsorption of the anti-GαO antibody with its immunising peptide (500 μg/ml, 1h at 30°C) prevented the inhibition of DHP agonist-stimulation of GTPase produced by the anti-GαO antibody, the % stimulation of GTPase by (-)-BayK 8644 being 13.1 ± 2.0 % in control membranes and 15 ± 1.8 % following preadsorption of the anti-GαO antibody (Table 5.1).
In contrast, the stimulation of GTPase by the GABA agonist (-)-baclofen (10 μM) was reduced by 89 ± 4 % and 41 ± 5 % following treatment of the membranes with the affinity purified anti-Gαi and anti-GαO antibodies respectively (Figure 5.3B). The attenuation of GABA-stimulated GTPase by the anti-Gαi and anti-GαO antibodies was prevented following preadsorption of the anti-G protein antibodies with their respective immunising peptides (500 μg/ml, 1h at 30°C). The % stimulation of GTPase above basal by 10 μM (-) baclofen being 21.7 ± 3.0 % in membranes incubated with control rabbit IgG, and 19.1 ± 2.1 %, and 21.7 ± 3.0 % following preadsorption of the anti-Gαi and anti-GαO antibodies with their respective immunising peptides (Table 5.1).

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A concentration-response curve for the stimulation of GTPase by the L-type calcium channel activator (-)-BayK 8644 and L-type calcium channel antagonist (+)-BayK 8644. GTPase is 33 ± 6 pmol/mg/min (n=9) and stimulation by (-)-BayK 8644 (●) or (+)-BayK at 0.5 μM GTP, is expressed as stimulation of GTPase above basal activity. Results nted as mean ± sem and the number of experiments is given in parentheses.
Figure 5.2  A concentration-response curve for the stimulation of GTPase by the GABA<sub>B</sub> agonist (-)-baclofen

Basal GTPase is 46 ± 8 pmol/mg/min (n=4) and stimulation by (-)-baclofen, at 0.5 μM GTP, is expressed as % stimulation of GTPase above basal activity. Results are expressed as mean ± sem., n=4.
Figure 5.3   Effect of anti G-protein antibodies on (-)-BayK 8644 and (-)-Baclofen stimulation of GTPase.

A:-- An affinity purified anti-Gi antibody (50 μg/ml) (SSSS) had no effect on the ability of (-)-BayK 8644 to stimulate GTPase. Stimulation of GTPase by (-)-BayK 8644 was reduced by 84 ± 5% when the membranes were pretreated with an affinity purified anti-Go antibody (50 μg/ml) (ΩΩ), (n=6, **p<0.01, paired t-test compared to membranes treated with control rabbit IgG (■)).

B:--Baclofen-stimulated GTPase was attenuated by 89 ± 4% and 41 ± 5% when the membranes were treated with affinity purified anti-Go (ΩΩ) and anti-Gi (SSSS) antibodies respectively (n=8, **2p<0.01, paired t-test compared to membranes treated with control rabbit IgG)
### % Stimulation of GTPase above basal by 10 nM (-)-BayK 8644

<table>
<thead>
<tr>
<th>Control IgG</th>
<th>Anti-Gα&lt;sub&gt;q&lt;/sub&gt; antibody</th>
<th>Anti-Gα&lt;sub&gt;i&lt;/sub&gt; antibody</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>18.9 ± 4.2 (6)</td>
<td>13.1 ± 2.0 (6)</td>
</tr>
<tr>
<td>Immunising peptides (500 µg/ml, 1h at 30°C)</td>
<td>*2.4 ± 1.0 (6)</td>
<td>15.3 ± 4.6 (6)</td>
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<tr>
<td>Gα&lt;sub&gt;q&lt;/sub&gt; antibody + Gα&lt;sub&gt;i&lt;/sub&gt; immunising peptide (500 µg/ml, 1h at 30°C)</td>
<td>15.1 ± 1.8 (6)</td>
<td>17.0 ± 3.1 (6)</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;i&lt;/sub&gt; antibody + Gα&lt;sub&gt;q&lt;/sub&gt; immunising peptide (500 µg/ml, 1h at 30°C)</td>
<td>*1.85 ± 1.3 (6)</td>
<td></td>
</tr>
</tbody>
</table>

### % Stimulation of GTPase above basal by 10 µM (-)-baclofen

<table>
<thead>
<tr>
<th>Control IgG</th>
<th>Anti-Gα&lt;sub&gt;q&lt;/sub&gt; antibody</th>
<th>Anti-Gα&lt;sub&gt;i&lt;/sub&gt; antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.7 ± 3.0 (6)</td>
<td>*12.2 ± 2.5 (6)</td>
</tr>
<tr>
<td>Immunising peptides (500 µg/ml, 1h at 30°C)</td>
<td>22.0 ± 3.0 (4)</td>
<td>*7.5 ± 3.0 (4)</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;q&lt;/sub&gt; antibody + Gα&lt;sub&gt;i&lt;/sub&gt; immunising peptide (500 µg/ml, 1h at 30°C)</td>
<td>21.7 ± 3.0 (6)</td>
<td>19.1 ± 2.1 (4)</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;i&lt;/sub&gt; antibody + Gα&lt;sub&gt;q&lt;/sub&gt; immunising peptide (500 µg/ml, 1h at 30°C)</td>
<td>*7.2 ± 4 (6)</td>
<td>*11.0 ± 3.5 (4)</td>
</tr>
</tbody>
</table>

Table 5.1 Effect on anti-G protein antibody immunising peptides on stimulation of GTPase by (-)-BayK 8644 and (-)-baclofen.

Anti-G protein antibodies (50 µg/ml) were incubated with the immunising peptides (500 µg/ml) for 1h at 30°C. In control conditions the antibodies were also incubated for 1h at 30°C, before being incubated with the membranes for a further 1h at 37°C. *p<0.05. Students t-test, compared to the stimulation of GTPase in the presence of control rabbit IgG (50 µg/ml). All samples taken from a single experiment are presented in the same colour. Number of experiments are in parentheses.
5.2.4 An antipeptide anti-VDCC β subunit antiserum, but not an antipeptide anti-VDCC α2 subunit antiserum, abolishes (-)-BayK 8644-stimulated GTPase.

Incubation of the cortical membranes with an antipeptide antiserum raised against the VDCC β subunit (1:50) abolished the (-)-BayK 8644-stimulation of GTPase. In the presence of control preimmune serum Eadie-Hostee analysis shows a 31 ± 6% increase in GTPase Vmax (basal Vmax was increased from 91 ± 5 pmol/mg/min to 120 ± 8 pmol/mg/min by 10 nM (-)-BayK 8644) with no change in Km for GTP (basal Km = 0.24 ± 0.01 μM, Km in the presence of 10 nM (-)-BayK 8644 = 0.23 ± 0.02 μM).

Following incubation of the membranes with the antipeptide anti-β subunit antiserum no increase in GTPase Vmax or change in Km for GTP is observed (basal Vmax = 88 ± 9 pmol/mg/min, basal Km = 0.24 ± 0.01 μM, and Vmax and Km in the presence of 10 nM (-)-BayK 8644 = 78 ± 10 pmol/mg/min and 0.23 ± 0.007 μM, respectively) (Figure 5.4A). Eadie-Hofstee transformation also shows that the stimulation of Vmax by 10 nM (-)-BayK 8644 is unaffected by incubation of the membranes with antiserum raised against the VDCC α2 subunit (Figure 5.4B). Following incubation of the membranes with control preimmune serum basal Vmax is increased from 62 ± 12 pmol/mg/min to 82 ± 9 pmol/mg/min by (-)-BayK 8644 (10nM). Basal Km for GTP = 0.16 ± 0.08 mM, and Km for GTP in the presence of 10 nM (-)-BayK 8644 = 0.18 ± 0.03 μM. Following incubation of the membranes with anti-VDCC α2 subunit antiserum Vmax is still increased from a basal value of 63 ± 8 pmol/mg/min to 79 ± 7.0 pmol/mg/min by (-)-BayK 8644 (10nM) with no effect on Km for GTP (basal Km = 0.17 ± 0.05 μM, Km in the presence of (-)-BayK 8644 = 0.19 ± 0.08 μM).

5.2.5 An antipeptide anti-VDCC β subunit antiserum attenuates (-)-BayK 8644-induced stimulation of GTPase, but not (-)-baclofen-stimulated GTPase

Incubation of the membranes with the anti-β subunit antiserum abolishes the stimulation of GTPase induced by (-)-BayK 8644 (10nM), with 0.5 μM GTP as substrate (Figure 5.5A). Preadsorption of the anti-β subunit antiserum with its immunising peptide (100 μg/ml, 1h at 37°C) prevents the inhibition of (-)-BayK 8644-stimulated GTPase by the anti-β subunit antiserum, the % stimulation of GTPase by (-)-BayK 8644 being 18.0 ± 1.7% in control membranes, and 14.4 ± 2.15% in membranes incubated with the anti-β subunit antiserum that had been preadsorbed with the immunising peptide (n=5).

In contrast, the % stimulation of GTPase by the GABAB3 agonist (-)-baclofen (10 μM) (24.02 ± 3.6% in membranes incubated with control preimmune serum) was unaffected by pretreatment of the membrane with the anti-β subunit antiserum (24.02 ± 5.42%).
Figure 5.4 Eadie-Hofstee transformation for the effect of (-)-BayK 8644 on GTPase $V_{\text{max}}$ and $K_m$ after incubation of the membrane with anti-VDCC $\beta$-subunit and VDCC $\alpha_2$-subunit antisera.

In A, after incubation with preimmune serum basal $V_{\text{max}}$ (○) was increased from 91 ± 5 pmol/mg/min to 120 ± 8 pmol/mg/min by 10 nM (-)-BayK 8644 (●). There was no effect of (-)-BayK 8644 on $K_m$ (basal $K_m=0.24 \pm 0.01$ μM, $K_m$ in (-)-BayK 8644-stimulated membranes is 0.23 ± 0.02 μM). In the presence of anti VDCC β-subunit antiserum basal $V_{\text{max}}$ was 88 ± 9 pmol/mg/min (basal $K_m=0.24 \pm 0.01$ μM), the subsequent addition of 10 nM (-)-BayK 8644 had no effect on $V_{\text{max}}$ (78 ± 10 pmol/mg/min) or on $K_m$ (0.23 ± 0.007 μM), (n=9).

In B, following incubation of the membrane with preimmune serum basal $V_{\text{max}}$ (○) was increased from 62 ± 12 pmol/mg/min to 82 ± 9 pmol/mg/min by 10nM (-)-BayK 8644 (●). Basal $K_m=0.16 \pm 0.08$ μM and $K_m$ in the presence of 10 nM (-)-BayK 8644 = 0.18 ± 0.03 μM. Following treatment of the membranes with anti VDCC $\alpha_2$-subunit antiserum $V_{\text{max}}$ was still increased from a basal value of 63 ± 8 pmol/mg/min (○) to 79.0 ± 7.0 pmol/mg/min by 10 nM (-)-BayK 8644 (●) with no affect on $K_m$ (basal $K_m = 0.17 \pm 0.05$ mM, $K_m$ in the presence of (-)-BayK 8644 = 0.19 ± 0.08, n=7)
Figure 5.5  Effect of β-subunit antiserum on (-)-BayK 8644 and (-)-baclofen-stimulated GTPase

Stimulation of GTPase (at 0.5μM GTP) by 10nM (-)-BayK 8644 was abolished by pretreatment of the membrane with anti-VDCC β-subunit antiserum (dilution 1:50) (■) (n=20 **2p<0.01, paired t-test compared to GTPase activity in membranes treated with control preimmune serum (□). In contrast, stimulation of GTPase by the GABAB agonist (-)-baclofen (10μM) (24 ± 3.6 %) was unaffected by pretreatment of the membrane with anti VDCC β-subunit antiserum (24.02 ± 5.42 %) (■), n=14.

Preadsorption of the anti-β subunit antiserum with its immunising peptide (□), (100 μg/ml, 1h, 30°C) retained the ability of (-)-BayK 8644 (10 nM) to stimulate GTPase (14.4 ± 2.15 %, n=5) and had no effect on the stimulation of GTPase by (-)-Baclofen (10μM), (20.4 ± 4.17%, n=5). There was no difference in the ability of the anti-β subunit antiserum to reduce (-)-BayK 8644-stimulation of GTPase following incubation at 30°C for 1h (-1.3 ± 3.6 % stimulation of GTPase, n=20) or 2h (1.4 ± 0.8% stimulation of GTPase, n=5)
5.2.6 A VDCC β-subunit binding site peptide abolishes (-)-BayK 8644-stimulation of GTPase, but has no effect on the GABAergic-stimulation of GTPase.

The VDCC β-subunit binding site peptide used in this Study has identical sequence homology to the β-subunit interaction site on the VDCC α1-subunit (Pragnell et al 1994). The synthetic peptide may act to prevent the β-subunit interaction with the Ca2+ channel by directly competing with the α1-motif for its association with the β-subunit.

The % stimulation of GTPase by (-)-BayK 8644 (10nM) was abolished following preincubation of the membranes with the β-subunit binding site peptide (20 μg/ml, 1h at 30°C) (Figure 5.6A). There was no effect of a control scrambled peptide on (-)-BayK 8644-mediated stimulation of GTPase (% stimulation of GTPase by (-)-BayK 8644 in non-treated membranes; 11%, 17%, and 21% stimulation of GTPase by (-)-BayK 8644 in scrambled peptide-treated membranes; 12.95 ± 2.5 %).

The % stimulation of GTPase by the GABAB agonist (-)-baclofen (10 μM), is 30.08 ± 3.02 % following treatment of the membranes with the control scrambled peptide and is unaffected by the β-subunit binding site peptide (24 ± 2.5 %).

5.2.7 Effect of antipeptide anti-VDCC β subunit and anti-α2 subunit antisera on [3H]PN200 110 binding to cortical membranes

In coexpression study systems the VDCC β-subunit has been reported to increase the DHP binding capacity of the expressed α1-subunit without having any discernible effect on the Kd for DHPs (Lacerda et al., 1991) we therefore investigated the effect of the VDCC β-subunit antiserum on specific binding of the DHP antagonist [3H]PN200 110 to frontal cortex membranes. In the presence of control preimmune serum Bmax =346 ± 29 fmol/mg protein, Kd = 2.40 ± 0.35 nM (n=8). Incubation with the anti-VDCC β-subunit antiserum results in a 30 ± 6% reduction in Bmax (237 ± 47 fmol/mg protein) with no change in the apparent Kd (2.0 ± 0.38 nM), n=8 (Figure 5.7A).

The effect of the antipeptide anti-VDCC α2 subunit antiserum on the [3H]PN200 110 binding capacity of the cortical membranes was also examined. In the presence of control preimmune serum Bmax =255 ± 61 fmol/mg protein, Kd= 2.36 ± 0.25 nM, n=4. Following treatment of the membranes with anti VDCC α2-subunit antiserum no change in Bmax (220 ± 49 fmol/mg protein) or Kd (2.1 ± 0.36 nM, n=4) is observed.
A: Pretreatment of the membrane for 1h with the VDCC β-subunit binding site peptide (■) (20μg/ml) abolished the ability of (-)-BayK 8644 (10nM) to stimulate GTPase (at 0.5μM GTP) (n=10, **p<0.01, paired t-test compared to membranes treated with a control scrambled peptide sequence (□) (20μg/ml). There was no difference in (-)-BayK 8644 stimulation of GTPase between control non-treated membranes (△) (11 %, 17 % stimulation of GTPase, n=2.) and scrambled peptide treated membranes (12.95 ± 5.4 % stimulation of GTPase, n=10).

B: Stimulation of GTPase by (-)-baclofen (10μM) is 30.08 ± 3.02 % following treatment with the control scrambled peptide and is unaffected by the β-site peptide (24 ± 2.5 % stimulation of GTPase, n=10). There was no difference in (-)-baclofen (10μM) stimulation of GTPase between control non-treated membranes (25 %, 33 % stimulation of GTPase, n=2) and scrambled peptide treated membranes.

Figure 5.6 Effect of a VDCC β-subunit binding site peptide on (-)-BayK 8644 and (-)-baclofen-stimulation of GTPase.
Figure 5.7 Effect of anti-VDCC β-subunit and anti-VDCC α2-subunit antisera on binding of $[^{3}H]$-PN200 110 to frontal cortex membranes

Graph A shows a Scatchard transformation of $[^{3}H]$-PN200 110 binding to frontal cortex membranes following pretreatment of the membrane with preimmune serum (dilution 1:50) (□) and anti VDCC β-subunit antiserum (dilution 1:50) (■). In the presence of preimmune serum $B_{\text{max}} = 346 \pm 29$ fmol/mg protein, $K_{d} = 2.40 \pm 0.35$ nM (n=8). Incubation with anti-VDCC β-subunit antiserum results in a 30 ± 6% reduction in $B_{\text{max}} (237 \pm 47$ fmol/mg protein) with no change in the apparent $K_{d} (2.0 \pm 0.38$ nM), n=8. Inset:- Competition binding curve for $[^{3}H]$-PN200 110 binding to cortex membrane in the presence of preimmune serum (□) and anti VDCC β-subunit antiserum (■).

Graph B shows a Scatchard transformation of $[^{3}H]$-PN200 110 binding to cortex membranes. In the presence of preimmune serum (dilution 1:50) (Δ) $B_{\text{max}} = 255 \pm 61$ fmol/mg protein, $K_{d} = 2.36 \pm 0.25$ nM, n=4, following treatment of the membranes with anti VDCC α2-subunit antiserum (dilution 1:50) (▼) no change in $B_{\text{max}} (220 \pm 49$ fmol/mg protein) or $K_{d} (2.1 \pm 0.36$ nM, n=4) is observed. Inset:- Competition binding curve for $[^{3}H]$-PN200 110 binding to frontal cortex membranes in the presence of preimmune serum (Δ) and anti VDCC α2-subunit antiserum (▼).
5.3 Discussion

This study has demonstrated that activation of the L-type Ca^{2+} channel promotes an increase in high affinity GTPase activity in the rat frontal cortex. The pure agonist isomer of the dihydropyridine (-)-BayK 8644 (Wei et al. 1986) binds to the calcium channel α1-subunit (Mikami et al., 1989) to cause a significant enhancement of GTPase activity. The maximal effective concentration was 10-100 nM which produced approximately 20% stimulation of GTPase. This concentration is some 10-100 fold less than that used by many workers (Grove et al., 1991, Jassar et al., 1993, Byron et al., 1993), however the compound used in many such studies is the racemic isomer of BayK 8644, a partial agonist of L-type calcium channels. The DHP antagonist isomer, (+)-BayK 8644 (Wei et al. 1986), was unable to induce any stimulation of GTP hydrolysis. It may thus be concluded that the GTPase stimulation induced by DHP may only occur when the L-type Ca^{2+} channel is in the agonist bound conformation. This result supports the finding of Sweeney and Dolphin, (1992), which also showed an enhancement of GTPase by racemic BayK 8644.

Several lines of experimental evidence suggest that the GABA_B receptor is also coupled to a G protein: Guanine nucleotides modulate GABA_B receptor binding in the brain (Hill et al 1984) and in cerebellar granule neurones the GABA_B-ergic inhibition of adenylate cyclase is PTX sensitive (Xu & Wojcik 1986). The GABA_B receptor interacts with a PTX sensitive G-protein in the hippocampus (Andrade et al., 1986) and spinal cord (Scott and Dolphin., 1987) to increase K^+ conductance and decrease Ca^{2+} conductance respectively. The antisense strategy has since revealed that the GABA_Bergic inhibition of Ca^{2+} currents in sensory neurones is via Go (Campbell et al 1993). Activation of GABA_B receptors also attenuates neurotransmitter release in cultured cerebellar granule neurones in a PTX-sensitive manner (Huston et al 1990).

In this study activation of the GABA_B receptor by the agonist (-)-baclofen (Kerberle et al. 1972) promotes a dose-dependent increase in GTP hydrolysis, consistent with the coupling of the GABA_B receptor to a G protein. Results from this Laboratory have shown previously that stimulation of GTP hydrolysis by (-)-baclofen is via an increased rate of guanine nucleotide exchange since activation of the GABA_B receptor, promotes an increase in binding of the non-hydrolysable analogue of GTP,GTP\gamma S, to cortical membranes (Sweeney & Dolphin 1992).

Several binding studies have shown that a physical association exists between adenosine A1 receptors and G proteins (Lohse et al 1984, Klotz et al 1986), which have now been identified as the PTX-sensitive G proteins Go and Gi (Munshi & Linden 1989, Freissmuth et al 1991). In frontal cortex membranes, activation of A1 receptors also causes an increase in GTP hydrolysis and promotes binding of GTP\gamma S (Sweeney & Dolphin 1995), indicating that, in a similar manner to the GABA_B receptor, the adenosine A1 receptor promotes GTPase activity by
increasing the rate of guanine nucleotide exchange. In contrast, the stimulation of GTPase activity of the activated G protein, since DHP agonists do not promote enhanced binding of GTPγS, and do not therefore advocate increased guanine nucleotide exchange (Sweeney & Dolphin 1992). The GABAergic effect on K⁺ and Ca²⁺ conductances in hippocampal and sensory neurones are PTX-sensitive (Andrade et al 1986, Dolphin & Scott 1987). The DHP agonist-mediated stimulation of GTPase has also been shown to be PTX sensitive in membranes prepared from cultured cerebellar granule neurones (Silver and Sweeney, unpublished observations). Since PTX can use either G₁ or G₀ as a substrate for ADP-ribosylation, affinity purified anti G protein antibodies, specific to the α subunit of either G₁ or G₀, were used to reveal to which subtype of PTX-sensitive G-protein the L-type Ca²⁺ channel and the GABA_B receptor are linked in cortical membranes.

The specificity of the G-protein antibodies used in this study has been confirmed previously (Campbell et al., 1993, Berrow et al 1995). Immunostaining associated with the anti-Gα₀ antibody in sensory neurones was abolished by antisense oligonucleotides complementary to the mRNA of all published Gα₁ sequences. Similarly anti-Gα₀ immunostaining was abolished by antisense oligonucleotides complementary to the mRNA of all published Gα₀ sequences, with no cross-reactivity between Gα₀ antisense oligonucleotides and Gα₁ antibodies. The use of these affinity purified anti-G protein antibodies revealed that the L-type calcium channel is exclusively linked to Gα₀. Treatment of the cortical membrane with the anti-Gα₀ antibody completely abolished stimulation of GTPase by the DHP agonist, while the anti-Gα₁ antibody had no effect on this stimulation of GTPase. GABA_B receptor-stimulation of high affinity GTPase was substantially reduced by the anti-Gα₁ antibody and partially reduced by anti-Gα₀, indicating that the GABA_B receptor can interact with both Gα₀ and Gα₁ in this preparation. GABA_B receptors have also been reported to couple to both G₀ and G₁₁ in bovine brain (Morishita et al., 1990). However, evidence from the use of G protein antibodies and antisense oligonucleotides suggests that only the coupling of GABA_B receptors via Gα₀ is involved in the inhibition of Ca²⁺ channel currents (Menon-Johansson et al., 1993, Campbell et al., 1993). Ca²⁺ channel currents are inhibited by PTX-sensitive G-protein linked transmitters; e.g. activation of adrenergic and GABA_Bergic receptors reduces Ca²⁺ currents in chick dorsal root ganglia (Holz et al., 1989), dopamine attenuates Ca²⁺ currents in chick sympathetic neurones (Marchetti et al 1986) and somatostatin reduces Ca²⁺ channel currents in rat sympathetic neurones (Shapiro & Hille 1993). In studies investigating the subtype of PTX-sensitive G protein involved in mediating the neurotransmitter inhibition of L-type Ca²⁺ channel currents, Gα₀ appears to be pivotal (Kleuss et al., 1991, Moises et al., 1994). This study, and that of
Sweeney and Dolphin (1992), has shown that DHP agonist interaction with the L-type Ca2+ channel promotes the GTPase activity exclusively of GoO. It is possible that in intact polarised systems a significant proportion of L-type Ca2+ channels are normally in this conformation. Since GoO is the principal G-protein involved in mediating the neurotransmitter modulation of the Ca2+ channel current, this enhanced GTPase activity may represent a mechanism by which the channel can deactivate such a modulatory signal. The kinetics of G protein-mediated signaling in cells strongly predicts the existence of some mechanism to accelerate GTP hydrolysis on trimeric G proteins. Although the rate constant for hydrolysis of bound GTP by purified G proteins usually varies from 0.8 min\(^{-1}\) to 4 min\(^{-1}\) in vitro (Gilman 1987), termination of cellular signalling is almost always much faster. The photoreceptor G protein transducin hydrolyzes bound GTP with a \(t_{1/2}\) of 15 s (Navon & Fung 1984), but the deactivation of transducin-stimulated cGMP phosphodiesterase in rod outer segments takes less than 1 s (Vuong & Chabre 1991). The intrinsic GTPase activity of activated transducin is found to be promoted by its effector, cGMP-phosphodiesterase, by a mechanism independent of guanine nucleotide exchange in order to promote rapid termination of the activated state of the G protein (Arshavsky & Bownds 1992, Arshavsky et al 1994). A similar mechanism for deactivation of the modulatory effects of Gq/11, induced upon activation of muscarinic receptors, has been reported by the study of Berstein et al., (1992). Those authors demonstrated an enhanced GTPase activity of Gq/11 by its effector protein, phospholipase-C. It has also been suggested that K+ channels have a GTPase-activating effect (Breitwieser & Szabo.,1988). Those studies investigated the muscarinic activation of the cardiac inward-rectifying K+ channel (IK,ACh) via the PTX-sensitive G-protein G\(_{i3}\). It was reported that the intrinsic GTP hydrolysis rate of G\(_{i3}\) (\(k_{\text{cat}} \approx 2\) min\(^{-1}\)) was too slow to account for the rapid off rate of the muscarinic effect (\(k_{\text{cat}}\approx 100\) min\(^{-1}\)). The authors therefore proposed that the IK,ACh channel promoted the intrinsic GTPase activity of the activated G\(_{i3}\) to terminate the modulatory muscarinic effect. However, the rapid deactivation of IK,ACh may be explained by an alternative regulatory pathway involving G protein \(\gamma\) subunits (Logothetis et al 1987).

Since the DHP agonist bound L-type Ca2+ channel was found to stimulate the GTPase activity of its associated G-protein it was also of interest to examine which subunit of the VDCC was involved in coupling DHP agonist binding to enhanced GTPase of Go. By using an antipeptide antibody raised against the VDCC \(\beta\)-subunit we have found that the VDCC \(\beta\)-subunit has a preferential role in linking L-type Ca2+ channel DHP agonist binding to GTP hydrolysis. The anti-VDCC \(\beta\) subunit antiserum was raised against a VDCC \(\beta\) subunit peptide as previously described (Berrow et al 1995). It recognised a \(\beta\) subunit (\(\beta_{1a}\)) from purified skeletal muscle VDCCs (Berrow et al 1995) and also recognised \(\beta_{1b}, \beta_{3}\) and \(\beta_{4}\) overexpressed in COS cells (Campbell et al 1995). The stimulation of GTPase by DHP agonists was extensively reduced by
the VDCC β-subunit antiserum yet there was no effect of the VDCC α2-subunit antiserum on this stimulation of GTPase. The anti-VDCC α2 subunit antiserum was raised against a peptide on the VDCC α2 subunit polypeptide, and recognised a 143 kDa protein in immunoblots of rabbit skeletal muscle t-tubule (Brickley et al 1995). The VDCC α2-subunit has an exofacial orientation within the cell membrane (Jay et al., 1991, Brickley et al., 1995) and may therefore be an unlikely candidate for coupling the agonist bound channel to an intracellular G-protein. However, the VDCC β-subunit does have an intracellular localisation (Takahashi et al 1987, Brickley et al., 1995) and would therefore be suitable for such an interaction.

The VDCC β-subunit has profound effects on Ca\(^{2+}\) channel function. Coexpression systems have revealed a role for the β-subunit in the enhancement of the expressed current amplitude (Singer et al 1991, Hullin et al 1992, Stea et al 1993), acceleration of activation and inactivation kinetics (Varadi et al 1991), a hyperpolarising shift in the voltage-dependence of activation of the current (Perez-Reyes et al 1992, Castellano et al 1993, Wei et al., 1991) and an increase in the availability of DHP binding sites (Perez-Reyes et al., 1992). Each β-subunit subtype has the ability to interact with a variety of different VDCC α1-subunit subtypes, and this produces a wide spectrum of modulatory interactions and functional effects (see review Perez-Reyes and Schneider., 1994). From our own studies, the use of antisense oligonucleotides to deplete sensory neurones solely of VDCC β-subunits has also shown the importance of this subunit in normalising the Ca\(^{2+}\) channel current amplitude, activation kinetics and sensitivity to DHPs (Berrow et al., 1995). Furthermore, such studies have implicated the VDCC β-subunit in the G-protein mediated inhibition of the Ca\(^{2+}\) channel current in dorsal root ganglion neurones. The inhibition of the residual Ca\(^{2+}\) channel current by the GABA\(_B\) agonist (-)-baclofen is greatly potentiated following depletion of VDCC β-subunits from these cells (Campbell et al., 1995). A number of hypotheses may be put forward to account for the result that depletion of VDCC β subunits heightens the ability of the residual current to be inhibited by (-)-baclofen. It is possible that agonist activation of Go produces inhibition of Ca\(^{2+}\) current by removal of the bound VDCC β subunit (Figure 5.8; pathway a). However, this hypothesis would predict that following depletion of VDCC β subunits there would be no further effect of G protein activation. Alternatively, activated Go may associate directly with the VDCC α1-subunit (Figure 5.8; pathway b, illustrating direct competition with VDCC β-subunit for a binding site; or pathway c, illustrating an allosteric interaction). Thus, in native cells the VDCC β-subunits may act to limit, possibly by competition for a binding site, the extent of the interaction between the VDCC α1 subunit and Go\(_\alpha\). This may be facilitated by the VDCC α1-β complex stimulating GTP hydrolysis, and thus limiting the temporal effectiveness of the G-protein modulating signal.
The primary binding site for the VDCC β subunit on the α1 subunit is a conserved motif in the I-II cytoplasmic linker of the α1-subunit (Pragnell et al., 1994), termed the α1-subunit interaction domain (AID) (De Waard et al. 1995). The β-site peptide used in this study is homologous to the AID. The synthetic β-site peptide will thus compete with the AID for interaction with the β-subunit thereby disrupting the association between the VDCC α1-subunit and its cytoplasmic β-subunit. This synthetic AID peptide has been shown previously to inhibit binding of the β-subunit to the VDCC α1-subunit in vitro (De Waard et al. 1995). The use of this peptide in the GTPase assay system attenuated the stimulation of GTP hydrolysis by DHP agonists but had no effect on the GABAβ-mediated stimulation of GTPase. This result suggests that the VDCC β-subunit must be associated with the VDCC α1-subunit in order to permit the stimulation of GTPase by DHP agonists. This reinforces the theory that the β-subunit is a prerequisite in coupling DHP agonist binding to GTP hydrolysis.

The VDCC β-subunit is involved in modulating DHP binding to the α1-subunit. Coexpression of the skeletal muscle VDCC β-subunit with the α1-subunit shows enhanced high affinity binding of the DHP antagonist ligand [3H]PN200 110 to the α1-subunit with no discernible effect on the affinity of the α1-subunit for DHPs (Varadi et al., 1991 Lacarda et al., 1991, Mitterdorfer et al. 1994), although the β subunit did not appear to increase the expression levels of VDCC α1 subunit protein (Nishimura et al. 1993, Neely et al. 1993). In the present study a 30% reduction in [3H]PN200 110 binding sites was observed, with no change in the affinity for DHPs, following treatment of the cortical membranes with the anti-VDCC β-subunit antiserum. A reduction in the number DHP binding sites could contribute to the observed decrease in DHP agonist-stimulated GTPase following incubation with the VDCC β-subunit antiserum. However, since the DHP-mediated inhibition of GTP hydrolysis was completely abolished by this antiserum while the number of available DHP binding sites was only partially reduced, we propose that sufficient DHP binding does occur following VDCC β-subunit antiserum treatment and that the decrease in DHP agonist-stimulation of GTPase is downstream of agonist binding.

The involvement of the VDCC α2-subunit in modulating the availability of DHP binding sites is less well defined. Monoclonal antibodies to the α2-subunit have been shown to reduce [3H]PN200 110 binding to T-tubules with a decrease in Ca2+ influx into smooth muscle cells (Kowalski et al., 1990). Furthermore, a heterologous expression system has shown an increased amplitude of the expressed current when the skeletal muscle α2-subunit is co-injected with the cardiac α1-subunit (Mikami et al., 1989). In contrast, the VDCC α2-subunit has very little effect when it is coexpressed with the skeletal muscle α1-subunit in mouse L-cells (Varad et al., 1991), nor does it have any effect on the brain isoform of class C L-type Ca2+ channel expressed in Xenopus oocytes (Tomlinson et al., 1993). One should be cautious however when
interpreting the significance of α2 on calcium channel function in the latter expression system since oocytes possess a high proportion of endogenous α2-subunit (Singer-Lahat et al., 1992). The VDCC α2-subunit antiserum used in this study had no effect on the binding of [3H]PN200 110 which is in agreement with the study of Varadi et al., 1991.

Taken together these findings suggest that the VDCC β-subunit has a role in modulating DHP binding in the rat frontal cortex. The VDCC β-subunit is also the principal subunit of the VDCC complex involved in the coupling of DHP agonist binding to enhanced GTPase of the G-protein Go. The proclivity of the VDCC β subunit to stimulate GTP hydrolysis by Go may represent a mechanism by which the effector Ca2+ channel deactivates the G-protein modulating signal.
Figure 5.8 A scheme for coupling the GABA<sub>B</sub> receptor to VDCCs; interaction between Gα<sub>o</sub> and the VDCC β-subunit
VI. General Discussion

Immunocytochemical studies reveal that cultured rat DRG neurones possess several types of VDCCs. The $\alpha_1$ subunit of the A-type VDCC was localised to the plasma membrane of the soma, while the DHP-sensitive $\alpha_1C$ and $\alpha_1D$ VDCCs were found in the soma and also to a lesser degree in the neurites. The $\alpha_1B$ N-type VDCC was abundant in both the soma and neurites. The different localisations of these VDCCs may reflect their different physiological roles within the cell. For example, the neuritic localisation of $\alpha_1B$ may reflect a role for this subtype of VDCC in the regulation of neurotransmitter release, while the localisation of $\alpha_1A$ exclusively to the somal plasma membrane may indicate that the A-type Ca$^{2+}$ channel is primarily involved in regulating cytosolic Ca$^{2+}$ levels in the cell body. VDCC $\beta$-subunits were localised to the inner surface of the cell body and neurites, and were also found in the cytoplasm. The association of the $\beta$-subunit with the membrane is consistent with the association of the $\beta$-subunit with VDCC $\alpha_1$-subunits and the finding that it is focused on the intracellular surface of the membrane in agreement with the study of Pragnell et al. (1994) which reported that the $\beta$-subunit interacts with the Ca$^{2+}$ channel complex by binding to an intracellular loop on the VDCC $\alpha_1$-subunit. The cytoplasmic localisation of the $\beta$-subunit suggests that $\beta$-subunits may have other functions in addition to their association with Ca$^{2+}$ channels. This study also provides evidence for the membrane topography for the $\alpha_2/\delta$ component of the VDCC complex. The $\alpha_2/\delta$ component was found to be wholly exofacial. The high glycosylation of the $\alpha_2/\delta$ component may aid in directing this subunit to the extracellular surface of the membrane. The $\alpha_1$-subunit of the Ca$^{2+}$ channel is not highly glycosylated, the $\alpha_2/\delta$ component may therefore target the $\alpha_1$-subunit to the membrane and stabilise it within the membrane. In addition to the localisation of Ca$^{2+}$ channel subunits, the localisation of the $\alpha$-subunits of the G proteins $G_o$ and $G_i$ were also investigated. $G_o$ and $G_i$ were found to occur at the cytoplasmic surface of the plasma membrane. Unfortunately the anti-VDCC antisera used in this study have not yet been fully characterised in brain membranes. The low levels of Ca$^{2+}$ channel protein in brain membranes has hampered the characterisation of the antisera by immunoblotting. However, immunocytochemical evidence presented in this Thesis has demonstrated that the anti-VDCC antisera interact with their respective recognition sites in a peptide-specific manner. Furthermore, the anti-$\beta$ subunit antiserum has been shown to recognise neuronal $\beta$-subunits when overexpressed in COS-7 cells. Overexpressing the VDCC $\alpha_1$-subunits in COS-7 cells and subsequent metabolic radiolabelling and immunoprecipitation with the anti-$\alpha_1$ subunit antiserum may provide an alternative procedure with which to characterise the anti-VDCC $\alpha_1$ subunit antisera.
The primary reason for examining the immunolocalisation of the VDCC β-subunit and G-proteins Gαo and Gαi within DRG neurones was to provide a means with which to monitor the levels of expression of these target proteins following treatment of the cells with antisense oligonucleotides. Chapter 4 of this Thesis describes the use of the novel antisense strategy to produce a selective and transient downregulation of expression of a target protein. Antisense oligonucleotides, complementary to the mRNA of either VDCC β-subunits or G-proteins Gαo/Gαi, were microinjected directly into the cytoplasm of DRG neurones and the resulting downregulation of the target proteins was monitored using confocal microscopy.

A maximal depletion of VDCC β-subunits occurred 110 hours after microinjection of the VDCC β-subunit antisense, giving an estimated half-life of 55h for the VDCC β-subunit. In contrast the levels of the α-subunits of Gαi and Gαo were maximally suppressed just 24 hours after injection of the Gαi/Gαo antisense sequences, giving an estimated half life of 12h for these proteins. The VDCC β-subunit and Gαi/Gαo are both intracellular membrane-associated proteins, yet they exhibit marked differences in their turnover rates. The microinjection technique proved to be a successful delivery route for the antisense oligonucleotides. This method avoids complications associated with differential uptake of oligonucleotides by subpopulations of cultured cells, and also limits degradation of the oligonucleotide by extracellular serum nucleases. The antisense strategy provides a useful tool with which to deplete cells of a particular target protein in order to examine the role of the target protein in intact cells. In parallel functional studies the antisense strategy has been used to demonstrate that the GABAergic inhibition of Ca2+ channel currents in DRG neurones is mediated exclusively by Gαo (Campbell et al 1993). Depletion of VDCC β-subunits using antisense oligonucleotides decreased peak inward Ca2+ current, slowed activation kinetics, and caused a hyperpolarising shift in the voltage-dependence of activation in DRG neurones (Berrow et al 1995). Furthermore, loss of VDCC β-subunit immunoreactivity was found to enhance the GABAergic-mediated inhibition of Ca2+ channel current (Campbell et al 1995). The interpretation of ablation experiments should however be made with caution since the resulting physiological responses are occurring in cells devoid of a vital protein component, and under such conditions the physiology of the cell may be altered drastically. It is helpful when results of antisense oligonucleotide studies are the corollary of co-expression studies since extrapolation of the functional properties of an individual protein may be made with greater assurance.

A major disadvantage of using confocal microscopy to measure levels of the target proteins following treatment with antisense oligonucleotides is the fact that the decline in expression levels and the electrophysiological consequences of antisense treatment are monitored in
different batches of cells. Another approach which could be implemented in order to monitor the decrease in target protein mRNA levels following treatment with antisense oligonucleotides is single cell reverse transcriptase polymerase chain reaction (PCR). The PCR technique offers the advantage that the mRNA levels of the target protein following treatment with antisense oligonucleotides, and functional electrophysiological properties could be monitored in the same cell. The cell would be injected with antisense oligonucleotide, and following electrophysiological recording the cell contents would be extracted for PCR. The mRNA of the cell would be amplified and levels of target protein mRNA could be measured by running the PCR product down an agarose gel.

Chapter 5 describes an increase in the intrinsic GTPase activity of activated Goq in rat frontal cortex membranes when the dihydropyridine (DHP) L-type Ca$^{2+}$ channel is in the agonist-bound conformation. Treatment of the membranes with an antipeptide anti-VDCC β-subunit antiseraum, or with a peptide which mimics the β-subunit binding domain on the Ca$^{2+}$ channel α1-subunit, abolished the DHP agonist-induced stimulation of GTPase. The result from this biochemical study suggests that the VDCC β-subunit has a pivotal role in coupling DHP agonist binding to the L-type Ca$^{2+}$ channel to an enhanced hydrolysis of GTP by Goq. It is proposed that the β-subunit may have a GTPase-activating effect which promotes the intrinsic GTPase activity of activated Goq. Goq would subsequently be converted to the inactive GDP-bound form and this may represent a mechanism by which Ca$^{2+}$ channels limit the temporal effectiveness of a G protein modulatory signal. The GTPase-activating effect of VDCC β-subunits may explain the enhanced GABAergic inhibition of Ca$^{2+}$ channel currents observed following treatment of DRG neurones with VDCC β-subunit antisense oligonucleotides (Campbell et al 1995). The ablation of VDCC β-subunits would remove the associated GTPase-activating effect such that Goq may remain in the activated conformation for longer to exert a greater modulatory effect on the Ca$^{2+}$ channel α1-subunit. It is likely that many other G protein-coupled effectors have GTPase-activating effects to inactivate the G protein modulatory signal. Such a mechanism is parsimonious and inherently specific to a defined signalling pathway. Variability may exist among effectors in their ability to stimulate GTP hydrolysis and could allow different effectors under the control of a single G protein to display distinctly temporal patterns of regulation.

The ability of the VDCC β-subunit to act as a GAP has been compared in two different preparations, DRG neurones and frontal cortex membranes. It would be advantageous to investigate the biochemical and electrophysiological aspects of the VDCC β-subunit GAP activity in the same preparation. Cultured cerebellar granule neurones (CBGNs) would provide an ideal system for such a study. CBGNs may be treated with antisense oligonucleotides to
deplete the cells of VDCC β-subunits. Unfortunately the small diameter of the CBGN soma preclude them from the microinjection procedure, and the antisense oligonucleotides would have to be applied to the culture medium and taken up by the cells. Although Holopainen & Wojcik (1992) have reported successful uptake of oligonucleotides by cultured CBGNs this has not been reproduced by workers from this laboratory. Permeabilisation of the cells with streptolysin O may provide a more efficient and convenient method to introduce antisense oligonucleotides into CBGNs. If CBGNs were successfully depleted of VDCC β-subunits by antisense oligonucleotides then measurement of GTPase activity and Ca\(^{2+}\) channel current properties could be monitored in the same cell type. Such experiments would contribute to further understanding of the VDCC β-subunit GTP-ase activating effect.

The combination of techniques utilised in this Study to investigate the structure-function relationship of voltage-dependent Ca\(^{2+}\) channels could equally be applied to the investigation of other types of ion channels and protein systems. Neuroscience research is rapidly tending towards a multi-disciplinary approach involving a range of molecular techniques in conjunction with electrophysiological methods. The advent of molecular cloning and the development of the antisense strategy has allowed further insight into the roles of individual proteins within cells and has also facilitated the identification of critical structural determinants of protein function.
VII. Publications resulting from this Study

The results presented in this Thesis have been published in the following journals:


**Campbell, V., Berrow, B., Brickley, K., Page, K., Wade, R., & Dolphin, A.C. (1995)** Voltage-dependent calcium channel β-subunits, in combination with α1-subunits, have a GTPase-activating effect to promote the hydrolysis of GTP by G\(_{\alpha}\) in rat frontal cortex. *FEBS Lett.* (in press)


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Finally, this Thesis is dedicated to my fabulous husband James, and to my family (present and future!).

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IX Bibliography


Holopainen, I. & Wojcik, W.J. (1993). A specific antisense oligodeoxynucleotide to mRNAs encoding receptors with seven transmembrane spanning regions decreases muscarinic m_2 and y-aminobutyric acid_g receptors in rat cerebellar granule cells. *J.Pharm.Exp. Ther* 264, 423-430.

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