A Study of the Pharmacology of G protein-coupled Potassium Channels in Rat

Atrial Myocytes and Guinea-pig Submucous Plexus Neurones.

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Abstract

This study has sought to expand the rather limited pharmacological knowledge of the G protein-coupled potassium channel of the rat atrial myocyte and guinea-pig submucous plexus neurone. These tissues were enzymatically dissociated and the cells kept in short term culture. Initially they were studied with microelectrodes using agonists to induce G protein-coupled conductances; noradrenaline and somatostatin in the case of the neurone, adenosine in the myocyte.

A number of agents known to block other K⁺ channels were tested, of which the three most studied were cetiedil, which was developed as an antisickling agent, clotrimazole, an antifungal drug and propafenone, an antidysrhythmic. Using these and other drugs, the pharmacological profiles of the two tissues were found to be similar, but not identical. In the submucous plexus the order of potency was clotrimazole>propafenone>cetiedil, in the atrial myocyte clotrimazole>cetiedil>propafenone. The three drugs also depolarised the two tissues and reduced the resting membrane conductance, suggesting that they also blocked a tonically-active potassium conductance. There was also some suggestion from intracellular experiments that clotrimazole may have been use-dependent in its mode of action; the level of block seemed to increase after the application of agonist. Active drugs were also tested on voltage-clamped cells in whole cell configuration in which the potassium conductance had been permanently activated with GTP-γ-S to confirm that the drugs were not simply acting as receptor antagonists, and to rule out the possibility of membrane rectification distorting the results obtained from voltage recording experiments.

Other currents of the submucous plexus neurone were studied; the delayed rectifier was sensitive to all three blockers (clotrimazole>cetiedil>propafenone), whereas the A-current was insensitive to clotrimazole and cetiedil. The tetrodotoxin-sensitive sodium current of these neurones was blocked by all three compounds at very similar concentrations.

Cetiedil, clotrimazole and propafenone turned out to be fairly non-selective in their actions; their relative activities did, however, suggest that there may be a difference between the G protein-linked channels of rat atrial myocyte and guinea-pig submucous plexus neurones.
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CHAPTER 1

INTRODUCTION
1.1 G proteins; structure and function.

1.1.1 Introduction; the discovery of G proteins.

The elucidation of cellular signal transduction mechanisms began in the late 1950s when Sutherland and co-workers became interested in how hormones like glucagon and adrenaline initiate glycogen breakdown in hepatocytes. When hepatocyte membranes were treated with hormone, a small heat-stable molecule was generated. This molecule in turn activated the breakdown of glycogen in the liver supernatant fraction; it was soon identified as cyclic adenosine 3',5'-monophosphate (cyclic AMP). The group went on to identify a membrane-associated enzyme, adenylyl cyclase which was responsible for the synthesis of cyclic AMP from ATP, and a phosphodiesterase responsible for its breakdown. Cyclic AMP, the molecule synthesised in response to hormone, was designated the second messenger linking receptor/hormone interaction with increase in glycogenolysis.

Subsequently the transduction events in this response were further elucidated, with the discovery of protein kinase A in 1968 (Walsh et al., 1968) and that the activation of this enzyme by cyclic AMP led to phosphorylation of target enzymes which were thus activated and catalysed the reactions involved in glycogen metabolism. However, the question of how receptor occupation induced adenylyl cyclase activity was left unanswered until Rodbell showed that the hepatocyte response required the presence of guanosine triphosphate (Rodbell et al., 1971). By 1980 the GTP-binding molecule responsible for regulation of adenylyl cyclase activity had been purified (Northup et al., 1980) and became known as G\(_s\). At the same time, another GTP-binding protein, transducin, involved in retinal light perception was being studied and found to bear considerable similarity to the guanine nucleotide-binding protein associated with adenylyl cyclase (Kuehn et al., 1980).

These two proteins were the first to be discovered of a range of much studied and highly significant signal transduction components, the G proteins. It is now clear that the G proteins are members of a protein superfamily that includes tubulin (the protein of the cell cytoskeleton), soluble proteins involved in initiation and elongation during protein synthesis and lower molecular weight GTP-binding proteins derived from \(ras\) and \(ras\)-related genes. High affinity and high specificity GTP-binding is a
characteristic of all members of the superfamily, as is their GTP-ase activity. Depending on whether the protein is GTP or GDP-bound, the protein will exist in differing conformational states, vital in determining its functional status; (Bourne et al., 1991). Defective G proteins exist, and are important in a number of genetic diseases; it may be that the physiological importance of this class of protein will soon become a therapeutic importance as well.

1.1.2 G protein structure

Although transducin is activated by light and Gs by hormone/receptor interaction, both require GTP and both are heterotrimers composed of subunits designated α, β and γ. The α subunits were distinct from one another in the two G proteins whereas the β and small γ subunits seemed to be far more similar (Manning and Gilman, 1983; Fung, 1983). As more G proteins were described in other systems, it became clear that the basic structure of the molecule was similar, and that the majority of them, unlike transducin, was associated with membrane receptors. This and the heterotrimeric structure of the proteins, are features which set G proteins apart from other members of the GTP-binding protein superfamily.

It was soon established that it was the α subunit which binds to GTP on activation of the G protein, a step that requires the presence of magnesium ions. This in turn causes the dissociation of the G protein into the α-GTP complex and βγ subunit. The intrinsic GTPase activity of the activated α subunit leads to eventual hydrolysis of the nucleotide resulting in a loss of functional activity of the α subunit, and reassociation of the GDP-bound trimer, a sequence of events sometimes referred to as the GTPase cycle. The molecular studies revealed that the α subunit is a hydrophilic protein having a molecular mass of 39 to 52 kDa, ranging in length from 350 to 395 amino acid residues. α subunits share at least 40 per cent sequence homology, with the highest levels of conservation occurring in regions associated with guanine nucleotide binding.

The G protein is always in its trimeric form when associated with the receptor, the α subunit playing a major role in determining with which type of receptor the trimer associates. It is also believed in most cases that the activated α subunit is responsible for interaction with the effector or at least the next component of the signal transduction.
cascade (Gilman, 1987; Birnbaumer et al., 1990); however, controversy still surrounds this claim and it will be addressed later.

Biochemical and immunochemical studies have revealed that the amino terminal region of the α subunit protein (1 to 2 kDa) is important in βγ association; the carboxy terminus is associated with the receptor; single point mutations in a residue close to the C terminus result in a loss of receptor association (Bourne et al., 1991). Pertussis toxin (PTX), a bacterial toxin capable of uncoupling certain receptor/G protein interactions, ADP-ribosylates a residue near the C terminus of the α subunit. Similarly, antibodies raised against the C terminal decapeptide uncouple the G protein from its receptor. Work done on chimeric α subunits suggests that the domain concerned with effector interaction may also reside somewhere near, although not so close to, the C terminus. The α subunit also has a high affinity binding site for divalent cations; this area is probably important in magnesium binding which is required for GDP/GTP exchange.

A large domain located closer to the N terminus, known as the GAP (GTPase-activating protein) domain, shows considerable diversity amongst different α proteins. It has been postulated that this area of the molecule may be important in regulating or activating the GTPase activity. One piece of evidence in support of this is that cholera toxin (CTX), another bacterially-derived G protein-active toxin, ADP-ribosylates a residue within the GAP domain resulting in permanent activation of the α subunit, possibly by disrupting GTPase regulation in the GAP region. Certainly α subunits treated with CTX have considerably reduced GTPase activity. Similar effects are seen in G proteins in which the same residue acted on by CTX is mutated, and when certain residues of the GTPase region are changed. Activation of α subunits can also be brought about by mutations of a residue near the N terminus that may normally be responsible for maintaining the tight binding of GDP in the inactive α subunit.

As stated, the GDP-bound protein is inactive whilst the GTP-bound form causes a conformational change leading to dissociation of the trimer and activation of the effector. Fluoride and the aluminium tetrafluoride anions are able to activate the α subunit (Gilman, 1987; Antonny et al., 1990; Higashijima et al., 1991). The latter ion may work by mimicking one of the phosphate groups of GTP. Mutation of a glycine residue in the GTP-binding region prevents activation of α subunits either by the fluorides or GTP itself.
The hydrophobic β and γ subunits have masses of 39 and 11 kDa respectively. β subunits are about 340 amino acid residues long (Birnbaumer et al., 1990). The β subunit proteins have repetitive structures consisting of a recurring 43 amino acid domain. The functional role of this sequence repetition is not known. The βγ subunit, as well as the α subunit C terminus, plays an essential role in coupling the G protein trimer to the receptor, since only the intact G protein is able to interact with receptor. The γ subunit is around 70 amino acids in length, and it is not clear which regions of the protein are important in binding to the β subunit. Generally, the functional regions of the βγ dimer are poorly understood and it is not known which areas are important in association with the α subunit, interaction with the receptor or effector (if the last action does in fact occur). There is some suggestion however, based on the lipophilicity of the βγ subunit, that it may serve to anchor the G protein trimer in the plasma membrane.

There are no instances in which dissociation of the βγ heterodimer is known to occur. The tight association is non-covalent, and the molecular forces underlying it are not understood. The βγ subunit has the important role of binding to the α subunit after hydrolysis of GTP to form the G protein trimer anew; whether the subunit plays any stimulatory role itself is not clear. There is still some debate over whether the βγ subunit interacts with effector in a number of G protein systems.

Evidence from electrophoretic techniques has suggested that there may be some diversity amongst β subunits. There are now known to be four different genes encoding β subunit proteins. Similarly, six complementary DNAs have been cloned for γ proteins (Hepler and Gilman, 1992). How this diversity affects the affinities of various forms of the two subunits for one another, and for the α subunit, is not yet clear. α diversity is discussed in the next section.

1.1.3 The functions and classification of G proteins.

At the same time as biochemical studies into G protein structure, it was being shown that the distribution and diversity of function of G proteins was far greater than had been expected. Whereas certain G proteins could stimulate adenyl cyclase activity, others could inhibit it (Hildebrandt et al., 1983). Many other transduction mechanisms involving G proteins are now known to exist, such as the activation of phospholipase C.
resulting in inositol trisphosphate and diacyl glycerol production (Cockroft and Gomberts, 1985; Litosch et al., 1985); phospholipase A2 activation resulting in arachidonate metabolism (Murayama and Ui, 1985; Burch et al., 1986); the modulation of numerous potassium (Yatani et al., 1987a; VanDongen et al., 1988) and calcium channels (see Hescheler and Schultz 1993 for review).

G proteins are all members of family that is related to the ras oncogenes and distantly to the EF-Tu bacterial elongation factor. Around eighty per cent of hormones and neurotransmitters rely on various members of the G protein family to mediate their responses. The receptors with which they are coupled number about one hundred, and they are all of the family of membrane proteins having seven transmembrane domains, with extracellular amino termini and intracellular carboxy termini, all belonging to the opsin gene superfamily. The lengths of these terminus chains and of the intracellular chains that link the membrane domains vary considerably. Once receptor/agonist interaction has occurred, the GTPase cycle is initiated, and signal transduction commences. The precise mechanism of receptor-induced GTP binding is not fully clear. There is a nucleoside diphosphokinase in the membrane that is found complexed with certain G proteins; this enzyme may serve to maintain high levels of GTP in the locality of the G protein. It may even phosphorylate the bound GDP thereby negating the need for nucleotide exchange (Kikkawa et al., 1990).

There is evidence suggesting that G proteins can also exert negative feedback influences on the receptor, since GTP binding to the α subunit causes a reduction in the affinity of the receptor for its ligand. This phenomenon of desensitisation may well be mediated by receptor phosphorylation and has been exploited to determine which receptors are associated with G proteins. Desensitisation may be heterologous, in which case exposure to one agonist causes a reduction in the response to others which act at different receptors. There is evidence to suggest that phosphorylation sites on the third intracellular loop of the receptor protein may be important in heterologous desensitisation. Both protein kinases A and C have been implicated in this form of desensitisation (Johnson et al., 1990). In the case of homologous desensitisation agonist exposure reduces the magnitude of subsequent responses to that same agonist. A protein kinase named β-ark (β-adrenoceptor kinase) has been shown to phosphorylate only agonist-occupied receptors apparently allowing a second protein, β-arrestin, to
compete with G protein for receptor coupling. This mechanism has been proposed to account for homologous desensitisation (Hausdorff et al., 1990).

The diversity of the G proteins (based on the variety of α subunits) is comparatively small compared to the diversity of receptors associated with them, numbering fewer than twenty. Only about half of this number have been purified. Comparison of the α subunit homologies enables the following broad classifications (see Table 1.1.3.1 for summary):

The $G_S$ proteins exist in four isoforms deriving from a single gene. They are sensitive to the actions of the bacterial toxin cholera toxin (CTX). The toxin acts on the α subunit by an adenosine phosphate ribosylation mechanism which permanently activates the subunit by preventing it from hydrolysing the bound GTP. In addition to adenylyl cyclase activation, recent evidence suggests that $G_S$ proteins may activate L-type calcium channels of cardiac and skeletal muscle by directly interacting with the channel proteins themselves (Yatani et al., 1987b).

The $G_i$ proteins exist in three forms, this time all deriving from separate genes. They are all sensitive to pertussis toxin (PTX) which somehow prevents activation of the trimer by occupied receptor, although the G protein can still be activated by non-hydrolysable GTP analogues (such as GTP-γ-S and GMP-P(NH)P) in the presence of PTX. $G_i$ proteins directly activate potassium currents in the heart and pituitary gland leading to inhibition of cardiac output and secretory activity respectively (Codina et al., 1987a & b; Yatani et al., 1990). They also modulate the ATP-sensitive potassium channels of ventricular myocytes and pancreatic β cells, as well as being responsible for the inhibition of adenylyl cyclase (Ui et al., 1984; Gilman, 1984). The $\alpha_i$ subunit has a mass of 40 to 41 kDa.

Also considered to be part of the $G_i$ family, are the $G_o$ proteins. The two isoforms of $G_o$ are, like $G_S$, derived from a single gene, and are again PTX-sensitive. Like $G_i$, $G_o$ mediates inhibitory signals by activation of certain potassium channels, but it is confined to brain and peripheral neuronal tissues (VanDongen et al., 1988). It has also been reported that $G_o$ may be involved in the inhibition of neuronal calcium channels (Hescheler et al., 1987; Ewald et al., 1989). Gilman (1987) has also reported the activation of phospholipase C by $G_o$ and $G_i$ in pituitary cells and Xenopus oocytes. The $\alpha_o$ subunit has a mass of 39 kDa.
Two proteins Gq and G11 are derived from separate genes, but both are insensitive to PTX. The proteins show great sequence homology, and both are responsible for the activation of phospholipase C in a number of tissues. These proteins may also be involved in the inhibition of the M-current, a voltage sensitive potassium current of some amphibian and mammalian sympathetic neurones that is sensitive to certain agonists, typically muscarinic agonists.

Gt, the transducin proteins, are only found in photoreceptor tissue, one type occurring in rod cells, the other in the cones. During phototransduction, they activate cyclic GMP phosphodiesterase (Hurley, 1987). Both types are sensitive to PTX and CTX. Another sensory protein, Goif, associated with olfactory organs is more similar to the Gs group.

Finally, the mysterious Gz/x is not known to exist outside its gene form. The protein itself has yet to be found, and any physiological role it may play if it is expressed is even more enigmatic.

Of the various actions of G proteins outlined above, direct interactions between ion channels and G proteins will be discussed in the next section.
### Table 1.1.3.1: G proteins and their actions.

<table>
<thead>
<tr>
<th>G protein subtype</th>
<th>Properties and functions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_s$</td>
<td>CTX-sensitive; activates adenyl cyclase leading to increased cytoplasmic cyclic AMP levels; directly stimulates L-type calcium channels in cardiac and skeletal muscle.</td>
</tr>
<tr>
<td>$G_i$ and $G_o$</td>
<td>PTX-sensitive; $G_i$ inhibits adenyl cyclase and directly activates $K^+$ channels in cardiac and pituitary tissue and ATP-sensitive $K^+$ channels in pancreatic $\beta$-cells; $G_o$ activates neuronal $K^+$ channels and inhibits neuronal $Ca^{2+}$ channels; may also be linked to activation of phospholipase C in <em>Xenopus</em> oocytes.</td>
</tr>
<tr>
<td>$G_q$ and $G_{11}$</td>
<td>PTX- and CTX-insensitive. Activate phospholipase C; inhibit neuronal $M$-currents.</td>
</tr>
<tr>
<td>$G_t$ and $G_{olf}$</td>
<td>$G_t$ only found in retinal tissue; involved in phototransduction activating cyclic GMP phosphodiesterase on stimulation by photons. $G_{olf}$ only found in olfactory tissue. Has similarities with $G_s$.</td>
</tr>
<tr>
<td>$G_{2/3}$</td>
<td>Functions unknown. Only the genes for this protein have been found.</td>
</tr>
</tbody>
</table>

### 1.2 G protein-coupled potassium channels.

#### 1.2.1 The atrial muscarinic potassium channel.

In 1921 Otto Loewi published results obtained from his now famous experiments with two frog hearts which were cannulated such that the effluent bathing solution from one preparation perfused the other. Stimulation of the vagus nerve of the first heart resulted in the expected cessation of beating; once the perfusate reached the second organ however, it too stopped beating. An unidentified substance which he named vagusstoff, had been released by the vagus of the first heart and had an inhibitory
effect on the myocardia of both organs. Subsequent work identified the vagusstoff as acetylcholine (Loewi and Navratil, 1926), and its receptor as m2 muscarinic (Fukuda et al., 1987; Bujo et al., 1988; Fukuda et al., 1988), being located in the sino-atrial tissue of the heart. The result of muscarinic agonist action was increased potassium permeability and decreased adenylyl cyclase activity, accounting for decreased excitability of sino-atrial myocytes and an overall reduction in heart rate (Trautwein and Dudel, 1958). The acetylcholine-sensitive potassium conductance became known as $K_{\text{ACh}}$. Pfaffinger et al. (1985) showed that the cardiac potassium conductance was inwardly rectifying, whilst Horie and Irisawa (1989) determined the single channel conductance of rat $K_{\text{ACh}}$ at 41 pS in symmetrical $K^+$, and showed that the inward rectification was partly dependent on internal magnesium. The story of how channel and receptor were coupled was uncovered by a number of groups in the mid-1980s onwards; their experiments are outlined below.

It was already known that acetylcholine had an inhibitory effect on adenylyl cyclase of cardiac myocyte membranes (Murad et al., 1962; Kurose and Ui, 1983; Mattera et al., 1985). However, the rapidity with which the agonist acted on the $K_{\text{ACh}}$ channels (a delay of 100 to 300 ms, Purves, 1976; Glitsch and Pott, 1978; Hartzell, 1980; Osterrieder et al., 1982) was too fast for channel opening to be a consequence of reduction of cyclic AMP concentration (Trautwein et al., 1982; Nargeot, et al., 1983), but too slow to be due to an action on a ligand-gated ion channel. Later work by Brown et al., (1990) also ruled out a role for PKC when the group found phorbol esters, ATP and the non-hydrolysable ATP analogue APP(NH)P to have no effect on $K_{\text{ACh}}$.

In 1984 Soejima and Noma used patch clamp techniques to study $K_{\text{ACh}}$. Cell-attached patch recordings from atrial myocytes revealed active potassium channels when acetylcholine was included in the patch pipette, but not when acetylcholine was bath applied. They concluded that a soluble second messenger like cyclic AMP was not involved in the response, since channel activity was limited to the area of the membrane where agonist was applied. However, excision of a cell-attached patch to form an inside out patch resulted in the rapid loss of channel activity, suggesting that an essential cytoplasmic constituent was being lost.

Pfaffinger et al., (1985) found that the acetylcholine response was also short lived in whole cell configuration, but that inclusion of GTP in the pipette filling solution
could prevent rundown. This, and the sensitivity of the agonist-induced K\textsubscript{ACh} current to PTX strongly implied the involvement of a G protein in the response. At the same time it was shown that the non-hydrolysable GTP analogue GPP(NH)P could permanently activate the potassium conductance (Breitweiser and Szabo, 1985). Kurachi et al. (1986b) showed that K\textsubscript{ACh} activity could be restored in inside-out patches by the application of GTP to the cytoplasmic face of the membrane when acetylcholine was in the pipette; the actions of GTP were again blocked by PTX and GTP-\gamma-S (another GTP analogue resistant to enzymatic hydrolysis) also brought about permanent activation of the channels in a magnesium-dependent manner. The involvement of a G protein was put beyond doubt when Yatani et al. (1987a) showed that an activated G protein from human erythrocytes in the same size range as other PTX-sensitive G proteins, could activate the K\textsubscript{ACh} response in inside-out patches when applied at picomolar concentrations. The erythrocyte G protein was provisionally named G\textsubscript{k}. The evidence that had accumulated by that time suggested that the response was G protein-mediated, and that the G protein and channel were closely associated in the cell membrane. No major cytoplasmic components seemed to play any role as demonstrated by Pfaffinger's earlier finding that the response could operate unhindered in isolated membrane patches, provided that GTP levels were maintained on the cytoplasmic aspect. This type of membrane-confined response became known as 'membrane-delimited' (Brown and Bimbaumer, 1990).

Interest started to focus on which components of the G protein were important in activating the effector. Logothetis et al. (1987) reported that purified \(\beta\gamma\) subunits from bovine brain could also activate K\textsubscript{ACh} in inside-out patches. The corresponding \(\alpha\) subunits were without effect. Shortly afterwards Codina (1987b) found preactivated \(\alpha\) subunits of G\textsubscript{k} to open the atrial channels at picomolar concentrations, whilst the \(\beta\gamma\) subunits were inactive. Other groups also published results showing that it was the \(\alpha\) subunit that interacted with effector in the case of the K\textsubscript{ACh} response, and that the native G protein was G\textsubscript{i} (Cerbai et al., 1988; Logothetis et al., 1988; Brown et al., 1990). Codina (1988) showed that the response was at least one hundred times more sensitive to activated \(\alpha\) subunit than to \(\beta\gamma\). Anti-\(\alpha\) antibody disrupted the agonist-induced response, whereas anti-\(\beta\gamma\) did not (Yatani et al., 1988\textsuperscript{o}).
The controversy continued when Kurachi et al. (1989b) found $K_{ACh}$ to be sensitive to $\beta\gamma$ subunits purified from rat brain. $K_{ACh}$ channels were activated in inside-out patches by subunits in the 10 pM range. Activated $\alpha$ subunits ($\alpha_i$ and $\alpha_o$) were also found to have an effect at 1 nM, but only in the presence of magnesium. More evidence arguing in favour of the physiological role of the $\beta\gamma$ in the $K_{ACh}$ response came from two groups in 1994.

Wickman et al., (1994) obtained $\beta\gamma$ dimers composed of different $\beta$ and $\gamma$ subtypes purified from brain and recombinant bacterial sources. The proteins caused a half-maximal activation of the $K_{ACh}$ current at 3 to 30 nM in inside-out patches, comparing poorly with results obtained by Brown et al., (1990) in which $\alpha$ subunits were maximally active at 50 pM. Wickman also found that 100 nM $\alpha$-GTP-$\gamma$-S was active, but concluded that this was due to contamination of the activated $\alpha$ subunit preparation with free GTP-$\gamma$-S. These results again indicate that $\beta\gamma$ subunits are active at higher concentrations; whether these concentrations are reached physiologically during an agonist response is not clear. One possibility is that $\beta\gamma$ subunits act via an arachidonate mechanism since anti-phospholipid antibody has been shown to block $K_{ACh}$ activation by $\beta\gamma$ subunits but was without effect on agonist or GTP-$\gamma$-S-induced responses (Kim, et al., 1989, see section 1.3.1.2).

Reuveny et al., (1994) expressed a gene encoding the cardiac G protein-coupled $K^+$ channel, GIRK1 (see section 1.2.1.2), in Xenopus oocytes. When $\beta_1\gamma_2$ subunit cRNA was coexpressed, the channels showed constant activity, independent of cytoplasmic GTP, but when cRNA for the $\alpha_{i-2}$ subunit and $K^+$ channel were coexpressed, no such activity was apparent.

Excised membrane patches from oocytes in which GIRK1 and $\beta_1\gamma_2$ were expressed still showed $K^+$ channel activity when the cytoplasmic face was deprived of GTP and magnesium; this was done to rule out the possibility that GIRK1 was sensitive to activated oocyte $\alpha$ subunits. Patches from cells in which intact G protein trimers had been coinjected showed no $K^+$ channel activity in the absence of GTP; both results were taken as evidence that the channel is dependent on the free $\beta\gamma$ dimer for activation.

In cells expressing $\beta\gamma$ proteins with the channels, recombinant $\alpha_{i-2}$-GDP abolished the tonic $K^+$ channel activity, presumably by interacting with $\beta\gamma$ dimers and reducing
their free concentration. One possible difficulty in using recombinant α subunit protein was reported by Brown et al. (1990). The group found that native α subunits were considerably more active in inside out myocyte patches than were recombinant subunits; the loss of activity obtained with the recombinant proteins may explain the results obtained by some groups who used a bacterial source of α subunit. Furthermore the levels of expressed βγ subunit in the oocyte model may be much greater than those that occur physiologically. The debate over the roles of the G protein subunits in atrial K⁺ channel activation has not been resolved, as contradictory findings continue to be published.

As to the identity of the endogenous α subunit in the K_ACh response, cDNA cloning has shown that the erythrocyte G_k was encoded by an α; clone, α;·3 (Codina et al., 1988; Yatani et al., 1988b). α;·3 is one of three α; clones (Codina et al., 1988); all three are active at very similar concentrations on the K_ACh response and on the inhibition of adenylyl cyclase (Brown et al., 1990; Yatani et al., 1988b). Unfortunately these findings do not shed any light on the identity of the endogenous cardiac G protein. The three clones are therefore multifunctional, but the reason, if any, for their heterogeneity is not yet clear.

In addition to acetylcholine, adenosine receptors are also linked to an increased potassium conductance in mammalian atrial myocytes (Drury and Szent Gyorgyi, 1929; Hartzell, 1979; Belardinelli and Isenberg, 1983; West and Belardinelli, 1985). Belardinelli and Isenberg (1983) have suggested that adenosine may act via the same K⁺ channels that are sensitive to the action of acetylcholine. The receptor has been shown to have pharmacological characteristics of the adenosine A1 subtype (Evans et al., 1982; Hamilton et al., 1987; Paton and Kurahashi, 1981). Using whole-cell patched-clamped guinea-pig atrial myocytes, Kurachi et al., (1986a) showed that both adenosine and acetylcholine were capable of inducing outward currents with inwardly rectifying current/voltage characteristics. Maximally, adenosine was only able to elicit a current about sixty per cent of the size of that induced by maximal concentrations of acetylcholine. In cell attached patches, adenosine was active when included in the pipette filling solution but not when bath applied. Inside out patches showed the response to be membrane delimited, with a requirement for GTP on the cytoplasmic surface of the membrane; characteristics that are identical to those of the acetylcholine.
response. Furthermore, the adenosine response was sensitive to PTX pretreatment, and was permanently activated by GTP-γ-S. These results led the group to assume that, like the muscarinic response, the activation of atrial K⁺ channels was a G protein-dependent process, and the endogenous G protein was of the Gᵢ type.

1.2.1.1 Arachidonate metabolism and G proteins.

Studies by Kim and Clapham (1989) and Kurachi (1989a) have shown that leukotrienes and their congeners from 5-lipoxygenase arachidonate metabolism are also capable of activating the Kᵥ channels. Like acetylcholine, arachidonate in the pipette filling solution of cell attached patch recordings activates Kᵥ channels that cannot be distinguished from Kᵥ. This response is sensitive to NDGA, a lipoxygenase inhibitor, but unaffected by cyclooxygenase inhibitors such as indomethacin. More specifically, 5-lipoxygenase inhibition (by AA-861) attenuates the arachidonate response, whilst blockers of 12-lipoxygenase (such as baicalein) are without effect (Kurachi et al., 1989a). Consistent with these results, are those obtained from experiments using metabolites synthesised from the two lipoxygenase pathways. Thus Kurachi found that the 5-HPETE metabolites (such as 5-HPETE itself, and the leukotrienes LTA₄ and LTC₄ but not however LTB₄) activate Kᵥ. Furthermore, the 12-lipoxygenase metabolites (such as 12-HPETE, 12-HETE and 5-HETE) were without effect. The results of Kim et al., (1989) were broadly similar although they reported a couple of contrasting findings, namely, that 12-HETE and LTB₄ were capable of activating Kᵥ channels.

These results are interesting not just because they reveal a novel, and rather unexpected pathway for Kᵥ activation, but because they may throw some more light on the question of which G protein subunits interact with the Kᵥ channel during a receptor-mediated response. Kim et al., (1989) reported that βγ subunits are active on Kᵥ, but this response is sensitive to NDGA or anti-phospholipase A₂ antibody. The authors concluded that previous workers who found βγ subunits to be active at high concentrations were activating a phospholipase A₂-dependent mechanism rather than observing direct βγ/Kᵥ channel interaction. These results are consistent with work done on transducin (Jelsema and Axelrod, 1987) in bovine retinal outer rod segments in which it was reported that phospholipase A₂ was stimulated by βγ subunits, but
inhibited by activated \( \alpha \) subunit. The \( \alpha \) subunit does not seem to involve phospholipaseA\(_2\) in the cardiac muscarinic response since NDGA has no effect on acetylcholine or GTP-\( \gamma \)-S-elicited response. Thus the case for direct \( \alpha \) subunit/\( K^+ \) channel interaction is not weakened, but the G protein regulation of the response appears to be more intricate than originally believed, with the \( \beta \gamma \) subunit being active on an effector enzyme whose metabolites are important in channel modulation.

The precise nature of the interaction of \( K^+ \) channel with 5-lipoxygenase metabolites is not clear. Kurachi et al., (1989a) found that the activation of channel by 5-lipoxygenase metabolite was GTP-dependent, implying that there may be G protein activation by the metabolites, directly or indirectly. Kim et al., (1989) did not find a need for GTP in the response, and in any case neither NDGA nor anti-phospholipase A\(_2\) antibody had any effect on the acetylcholine-mediated response suggesting that 5-lipoxygenase metabolites do not play an important role in mediating the agonist-induced response, and possibly, by implication in the vagal inhibition of cardiac output. However, in some species, \( \alpha \)-adrenergic agonists can activate cardiac G protein-coupled channels, and Kurachi et al., (1989a) did find the cardiac adrenoceptor-mediated response to be sensitive to the actions of agents that disrupt the 5-lipoxygenase pathway.

1.2.1.2 Molecular cloning techniques and the study of \( K_{ACB} \) channels.

Molecular biology has enabled, through protein purification and the determination of complementary DNA sequences, the identification of three major superfamilies of membrane ion channels. The ligand-gated channels, such as the nicotinic and GABA\(_A\) receptors are composed of multiple subunits arranged in radial symmetry around a central ion pore. The next group comprises the voltage-gated ion channels such as sodium and calcium channels, and although these may also be composed of multiple subunits, the functional characteristics of voltage and toxin sensitivities reside in one large protein subunit. The large subunit in this class of ion channel has a sequence of three to four hundred amino acids that is repeated four times. Each such sequence traverses the membrane six times, and each has an ‘S4’ region which is rich in positively charged amino acids and may well serve as the voltage sensor (Stühmer et al., 1989).
The first potassium channels to be cloned were those from the *Shaker* mutant of *drosophila* (Tempel et al., 1987), an A-type channel, which enabled the identification of the A-channel gene locus. cDNA fragments from this area were injected into oocytes which then expressed A currents. The gene from which these fragments came was found to be large, and cDNAs were obtained from it by alternative splicing that yielded distinct A-currents on oocyte expression, many or all of which may be expressed in *drosophila* in a tissue-specific fashion. These proteins bore similarities to sodium and calcium channels, but were simpler; the sequence that was analogous to the repeated domains of the Na\(^+\) and Ca\(^{2+}\) channel proteins, occurred only once in the *shaker* protein, i.e. just one group of six membrane-spanning amino acid chains (S1 to S6), with the S4 region again being rich in charged residues and probably serving as the voltage sensor.

The third class is the inwardly rectifying channels, discussed later on in this section.

The *shaker* probe has enabled a whole family of related K\(^+\) channel genes to be identified, *Shal, Shab* and *Shaw* (Wei, et al., 1990). The expressed channels vary in characteristics from A current to delayed rectifier, some having properties of both types of conductance. There is evidence that the *shaker* family of channels is expressed as a tetramer, i.e., that there are four subunits containing the S1 to S6 membrane-spanning regions arranged symmetrically around the ion pore (MacKinnon, 1991). An hydrophobic loop running from the S5 to S6 regions, known as H5, dips part of the way into the membrane and forms a portion of the lining of the pore (Durell and Guy, 1992).

More recently, two inwardly rectifying K\(^+\) channel genes have been cloned; ROMK1 isolated from a kidney cDNA library (Ho et al., 1993) and IRK1 from a macrophage library (Kubo et al., 1993b). Unlike the voltage-gated channels derived from the *shaker* gene however, ROMK1 and IRK1 have only two membrane-spanning regions, M1 and M2; between these runs a chain similar to the H5 region of the *shaker* family. As with *shaker* channels, H5 may dip into the membrane to form part of the ion pore. The C and N termini probably reside within the cytoplasm. This arrangement is similar to that found in the other voltage-gated channels, except that their extra four membrane-spanning regions (S1 to S4) form an ‘outer shell’ and do not occur in ROMK1 or IRK1. Thus the S4 region, which is believed to be a voltage-sensor, is absent from the inwardly rectifying channels. This is consistent with the different voltage-sensitivity characteristics of this type of channel. Like the voltage-gated
channels however, it has been suggested that the inwardly rectifying channels may exist as tetramers.

A very interesting development occurred when Kubo et al. (1993a) isolated a complementary DNA for the G protein-coupled K⁺ channel of rat heart based on sequence homologies with ROMK1 and IRK1 by screening a rat heart cDNA library. They called the new gene GIRK1, and found it to have roughly a forty per cent sequence homology with the cDNAs of the other two inward rectifiers. GIRK1 is a 4200 base gene and encodes a protein structurally similar to the IRK1 and ROMK1. All three channels have two hydrophobic membrane spanning domains (M1 and M2) with the H5 loop running between them. The derived cRNA from GIRK1 was injected into *Xenopus* oocytes and K⁺ channels equivalent to Kᵦᵡ of rat heart were expressed.

If muscarinic m2 complementary RNA was coinjected into the oocytes, then carbachol could induce an inwardly rectifying K⁺ current. The K⁺ current tended to increase slightly after the beginning of a hyperpolarising voltage step; a characteristic of the cardiac current. No carbachol-activated currents were recorded from cells that had not been treated with both GIRK1 and m2 receptor cRNA. Kubo concluded that the G protein mediating the response would have to be endogenous. When GTP-γ-S was applied to the cytoplasmic aspect of excised membrane patches, single channel openings were observed; confirming that channel activation was a membrane delimited response. The single channel conductance was 42 pS in symmetrical K⁺ (c.f. 41 pS of the rat cardiac channel) and the inward rectification was dependent on intracellular magnesium ions such that when magnesium was removed from the cytoplasmic side of inside-out patches, inward rectification was lost. The group also found GIRK1 in rat brain where other G protein-coupled K⁺ channels are known to occur (see later).

The N- or C-termini of GIRK1 share little homology with the termini of IRK1 or ROMK1 and it has been proposed that they may represent 'autoinhibitory' domains, and G protein interaction with these regions may remove this inhibition leading to a dramatic increase in the open probability of the channel (Lester and Dascal, 1993). Although it is not certain that GIRK1 is tetrameric, there is interesting evidence to suggest that channel gating may require four G protein subunits to interact with the channel to initiate channel opening (Ito et al., 1991). The cloning of the atrial channel
can pave the way to similar approaches to G protein-coupled channels in the brain (see section 1.2.4).

Shortly after Kubo’s work, Dascal et al. (1993) also published results on the cloning of the rat atrial muscarinic channel based on sequence homologies with ROMK1 and IRK1, which they called KGA. The channel was again expressed in *Xenopus* oocytes with a 5HT1a receptor cDNA which apparently coupled to the channel to give larger inwardly rectifying potassium currents than when the m2 muscarinic receptor was coexpressed. Again the group found about a forty per cent homology with the cDNAs of IRK1 and ROMK1, and the predicted structure was similar, with a pore-forming region flanked by two transmembrane domains; both the C- and N-termini were believed to reside on the cytoplasmic side of the membrane. The pore-forming region bore a high level of homology with ROMK1 and IRK1, roughly seventy per cent. The cloned channel had unique sequences at the C and N termini; either of these regions, Dascal proposed, could be involved in G protein interaction.

One interesting observation made by Reuveny et al., (1994) concerns the enzyme β-adrenergic receptor kinase 1 (βARK). βARK may play a role in β receptor desensitisation through receptor phosphorylation, and has been shown to interact with βγ subunits (Inglese et al., 1993). Some limited sequence similarity was found between the C terminus sequences of the βARK protein (where the βγ binding site resides (Koch et al., 1993)) and GIRK1. Deletions of amino acid sequences from the C terminus did result in a loss of channel activity when the channel was expressed in oocytes in which βγ subunits were also present. Although this points towards the C terminus region having a role in interaction with G protein, it is possible, as the group acknowledged, that these deletions affected the ability of the mutant channels to act as functional ion pores.

One of the most recent and interesting reports on the molecular structure of the channel protein and the genes encoding them is by Krapivinsky et al., 1995. The group argued that although GIRK1 gave rise to functional K⁺ channels when injected into *Xenopus* oocytes (Kubo et al., 1993a) there remained some unexplained differences between this channel and the native K⁺ channel of atrial myocytes. Evidence for this includes the unexpectedly high open probability of the expressed channel in the absence of agonist; the relative insensitivity of the expressed channel to exogenously-applied βγ
subunits in inside-out patch experiments and the fact that coexpressed \(\beta\)-adrenoceptors are capable of coupling to the GIRK1 channel. When preparations of atrial tissue were treated with antibodies against the predicted sequence for the GIRK1 channel protein, a second peptide coimmunoprecipitated. This was named CIR (cardiac inward rectifier) and its amino acid sequence bore extreme similarity to an ATP-sensitive \(K^+\) channel protein designated RNKATP (Ashford et al., 1994). Low stringency screening of a rat atrial cDNA library with IRK1 led to the identification of the CIR gene.

Injection of the CIR cRNA by itself into a number of different expression systems did not however produce ATP-sensitive \(K^+\) channels. When CIR and m2 muscarinic receptor cRNAs were coexpressed in oocytes it was found that acetylcholine could evoke a small \(K^+\) current; this response did not occur when the muscarinic receptor was expressed on its own. The CIR channel was also expressed in Sf9 cells and inside-out patch experiments revealed that \(K^+\) channel activity could be greatly increased by application of GTP-\(\gamma\)-S to the cytoplasmic membrane aspect. When ATP was applied to channels activated in this way, it had very little effect on channel gating. Thus for all the similarity of CIR to RNKATP, the expressed channels showed CIR to have more characteristics in common with \(G\) protein-coupled channels rather than ATP-sensitive \(K^+\) channels.

However, coexpression with GIRK1 gave rise to \(G\) protein-coupled inwardly rectifying \(K^+\) channels bearing greater resemblance to the native atrial myocyte channels than were obtained when either CIR or GIRK1 were expressed alone. When CIR and GIRK1 were coexpressed with muscarinic receptor cDNA, acetylcholine elicited much larger \(K^+\) currents than were obtained when either CIR or GIRK1 were expressed individually with the receptor gene. Western blots showed that the larger currents were not due greater GIRK1 protein expression. The single channel conductance of the CIR/GIRK1 channel was found to be 37 pS in symmetrical 140 mM \(K^+\) at -70 mV; this compares with 15 to 30 pS obtained with CIR alone and 42 pS of GIRK1 (Kubo et al., 1993) and 41 pS of the atrial muscarinic channel (Horie and Irisawa, 1989). However, it was also found that the agonist-independent \(K^+\) channel activity observed with the CIR/GIRK1 conductance was even greater than seen in oocytes expressing GIRK1 only; in this respect CIR/GIRK1 differs from the atrial \(G\) protein-coupled channel.
In systems where both proteins were expressed, the resulting channels showed greater sensitivity to $\beta\gamma$ subunits than observed with GIRK1 alone. The sensitivity compares well with earlier reports testing $\beta\gamma$ subunit concentrations on the atrial myocyte current (Wickman et al., 1994); Krapivinsky also lends weight to the belief that $\beta\gamma$ dimers are responsible for G protein/K$^+$ channel interaction, showing that CIR/GIRK1 channel activity induced by GTP-\(\gamma\)-S or $\beta\gamma$ subunits can be reduced by application of $\alpha$-GDP subunits.

Chromatographical work showed that the CIR/GIRK1 channel was a heteromultimeric complex of both GIRK1 and CIR proteins. Their data showed that if the channel was composed purely of the two expressed proteins it would be comprised of more than the expected four subunits. Regardless of this, the work is important in being one of the first of its kind to address the possibility of K$^+$ channels existing as heteromultimers. The group points out that the ability of GIRK1 alone to produce functional K$^+$ channels with many of the characteristics of the atrial G protein-coupled channel may be due to the GIRK1 protein combining with a native oocyte protein similar to CIR. This is because GIRK1 does not yield functional channels in other expression systems such as the HEK or CHO cells and that a CIR homologue does indeed exist in the oocyte genome.

As previously mentioned, the CIR protein is very similar to the RNKATP protein associated with K$\textsubscript{ATP}$ channels described by Ashford et al., 1994. It is also suggested that CIR, which occurs in ventricular myocytes as well, may combine with other K$^+$ channel proteins to form the ATP-sensitive channels present in ventricular myocytes. This principle of the combination of different protein subunits to form heteromultimeric K$^+$ channels with varying properties may be of great importance in the understanding of K$^+$ channel stoichiometry and structure.

1.2.2 The G protein-coupled K$^+$ channel of submucous plexus neurones of guinea-pig intestine.

1.2.2.1 The enteric nervous system.

The autonomic nervous system has been divided into three categories; the sympathetic, the parasympathetic and enteric (Langley, 1921). The enteric nervous
The enteric nervous system is relatively independent of central control; although sympathetic and parasympathetic tracts do run to the gastrointestinal tract, they mediate their effects on digestive processes through the enteric nervous system itself. When these pathways are severed however, there is surprisingly small effect on digestive function. The enteric nervous system contains complete reflex pathways, comprised of sensory neurones, interneurones, and motor neurones which can be excitatory or inhibitory. Peristaltic and vasodilator reflexes are both under the control of the enteric nervous system.

Abrahamsson (1973) estimated that there are between ten and one hundred million neurones in the enteric nervous system, and it is also believed that there are at least ten distinct neurone types within it. Structurally, interconnected nerve strands and ganglia are arranged in plexuses that occur in layers within the gut wall, and this arrangement extends from the oesophagus to rectum. Enteric neurones are also to be found in the gall bladder, extrahepatic biliary tract and pancreas.

In most mammalian species, the arrangement of the enteric plexuses is very similar, and the plexuses are broadly classified as follows:

The subserous plexus lies closest to the outer gut wall, has very few ganglia, and serves as the interface between external and enteric neurones. It contains numerous neuronal processes of extrinsic origin which project into the gut wall and synapse with enteric neurones lying deeper within the intestine.

The myenteric plexus is rich in ganglia and contains the majority of cell bodies of the enteric nervous system. There are also numerous axons running between the longitudinal and circular muscularis externa layers. Sympathetic and parasympathetic axons run into this plexus, as well as projections from enteric sensory neurones. In the guinea-pig duodenum, these nerve strands serve to connect the ganglia, which may contain anything from ten to one hundred neurones. The deep muscular plexus contains very few ganglia, but consists of nerve bundles running along the inner face of the circular smooth muscle, with branches that run deep into the muscle tissue. Neurones of the circular muscle are small and may have several axons. Other fibres connecting the submucous and myenteric plexuses run through this layer.

The submucous plexus occurs within the submucosal layer, a diffuse layer of connective tissue. Axons from submucous neurones project into the mucosa, occurring in both the villi and glandular components of the gut lining. Submucous plexus (SMP)
neurones of guinea-pig duodenum tend to be smaller than those of the myenteric plexus, and there are altogether fewer neurones than in the latter. Projections from the SMP run to the myenteric plexus, to the mucosa, and to paravascular nerves which run alongside arteries and arterioles of the mesentery and gut wall. The SMP itself has a markedly profuse network of arterioles.

Electrophysiological studies have suggested that whilst many sensory neurones lie in the myenteric plexus, a large proportion of enteric motoneurones are to be found in the SMP governing peristaltic motility (Hirst, 1979; Hirst and McKirdy, 1975; Wood, 1981). It has been suggested that neuronal interactions controlling the ‘wave’ of smooth muscle contraction during peristalsis may require a number of spontaneously active motoneurones. Whilst it is accepted that the majority of SMP neurones receive both inhibitory and excitatory synaptic inputs (Hirst and McKirdy, 1975; Hirst and Silinsky, 1975), Surprenant (1984a) reported that some neurones of the SMP lack such inputs. Intracellular recording experiments revealed that one class of these cells had spontaneously occurring action potentials and may therefore be important in the regulation of peristalsis; the electrophysiological properties of these cells resembled AH neurones of the myenteric plexus as described by (Nishi and North, 1973; Hirst et al., 1974). The latter cell type, Surprenant proposed, may be sensory neurones.

Surprenant (1984b) also recorded potentials from SMP neurones having synaptic inputs, both excitatory and inhibitory. These are discussed in the next two sections.

1.2.2.2 G protein-coupled K⁺ channels in SMP neurones.

In 1984 A. Surprenant studied the effects of transmural stimulation on SMP neurones with synaptic inputs. SMP tissue was dissected from the duodenum and pinned out in an organ bath; intracellular voltage recordings were then obtained from the neurones of the plexus. Transmural stimulation was found to evoke three types of potential in post-synaptic neurones: a fast nicotinic excitatory post synaptic potential (e.p.s.p.) of 50 to 80 ms duration recorded in nearly eighty per cent of cells; a slow e.p.s.p. of 15 to 20 seconds, and an inhibitory post synaptic potential (i.p.s.p.) of 1 second which occurred in just over fifty per cent of cells.
Pharmacological approaches revealed that SMP neurones also hyperpolarised in response to exogenously applied noradrenaline and other agonists with selectivity for \( \alpha_2 \) receptors such as UK 14304 (North and Surprenant, 1985; Surprenant and North, 1988). In addition to the adrenergic response, somatostatin has been shown to cause membrane hyperpolarisation in guinea-pig SMP neurones as have agonists of the \( \delta \) opioid receptor (Mihara et al., 1987; Mihara and North, 1986; Tatsumi et al., 1990). Certain sympathetic and enteric nerves that have synapses with neurones of the SMP are known to contain both noradrenaline and somatostatin; it is likely that the release of these neurotransmitters accounts for the i.p.s.p. observed during transmural stimulation experiments (Costa et al., 1977; Hockfelt et al., 1977; Costa et al., 1980; Schultzberg et al., 1980; Costa and Furness, 1984; Keast et al., 1984). The resulting inhibition would cause a reduction in the activity of secretomotor neurones of the mucous plexus, leading to water and electrolyte reabsorption.

Whole-cell voltage-clamp techniques employed by (Tatsumi et al., 1990) on SMP neurones revealed an outward \( K^+ \) current induced by noradrenaline which was responsible for the membrane hyperpolarisations and i.p.s.p.s observed in voltage recordings. Pressure ejection of noradrenaline caused the current to occur with a latency of 30 to 60 ms which compares with the 100 ms latency of the atrial muscarinic response (Surprenant and North, 1987). In the case of the latter tissue, the delay in onset of response was considered too slow to be mediated via a fast synaptic mechanism, but too fast to involve a soluble cytoplasmic second messenger.

In an attempt to determine the transduction mechanisms at work in the SMP response, Surprenant and North used strategies designed to alter intracellular cyclic AMP levels such as application of forskolin and CTX; cells treated in this way did not show abnormal resting currents, and the interventions had no effect on agonist-induced \( K^+ \) currents. Similarly, bath application of phorbol esters was ineffective indicating that protein kinase C was unlikely to play any role. Intracellular recordings using microelectrodes containing 20 mM GTP-\( \gamma \)-S, however, showed a gradual hyperpolarisation and simultaneous reduction in input resistance, suggesting the involvement of a G protein in the response.

Pretreatment of the cells with PTX abolished responses to noradrenaline, [D-Pen\(^{2,5}\)]-enkephalin (a \( \delta \) opioid agonist) and somatostatin; i.p.s.p.s evoked by transmural
stimulation of presynaptic neurones were also sensitive. Other SMP currents, such as the fast and slow e.p.s.p.s were insensitive to PTX treatment (Surprenant and North, 1988). Tatsumi et al. (1990) confirmed these findings using the whole cell recording configuration, and showed that the response in PTX-treated cells could be reconstituted over about 15 minutes, by including preactivated G<sub>i</sub> or G<sub>o</sub> proteins in the pipette filling solution. As with the cardiac K<sup>+</sup> current, agonist-induced K<sup>+</sup> channel activity in SMP neurones was shown to be membrane delimited (Shen et al., 1992) and a similar transduction mechanism involving direct G protein/K<sup>+</sup> channel coupling was strongly implied.

The agonist-induced outward current in SMP neurones showed inward rectification at a number of different extracellular potassium concentrations, with reversal potentials around the potassium equilibrium potential (E<sub>K</sub>). The agonist-induced current showed sensitivity to K<sup>+</sup> channel blockers such as caesium and barium. Tatsumi et al. (1990) also reported that there was a time-dependent component to the agonist-evoked current, that is to say hyperpolarising voltage steps performed in the presence of noradrenaline induced currents that took some time (in the order of tens of ms) to reach their maximal values.

Shen et al. (1992) studied outside-out patches from SMP neurones. In symmetrical 160 mM K<sup>+</sup>, and at a holding potential of -70 mV, three 'background' potassium channels were observed; a smaller 30 to 65 pS channel, an intermediate channel of 120 to 160 pS and a larger one of 220 to 260 pS single channel conductance. The small conductance channel was detected in over ninety per cent of recordings. The open probabilities (Po) of all three channels was greatly increased by noradrenaline provided GTP was maintained at the cytoplasmic face of the membrane. Furthermore, all three channels were active in the absence of agonist, as has been observed in the atrial channel (Okabe et al., 1991; Ito et al., 1991). However, the atrial conductance is mediated by only one channel of around 40 pS conductance. Somatostatin too, caused a marked increase in Po of all three channels.

1.2.2.3 Other synaptically-modified currents occurring in SMP neurones.

Surprenant et al. (1990) reported that noradrenaline, somatostatin and met-enkephalin were capable of inhibiting a voltage-dependent calcium current in SMP
neurones at around 100 ms after application. The current was found to be insensitive to nifedipine, but blocked by ω-conotoxin. Intracellular dialysis with GTP-γ-S also caused inhibition of the calcium current, and pretreatment of cells with PTX prevented inhibition by agonists. Inclusion of Go or Gi proteins in the pipette filling solution restored the ability of agonists to block the current after PTX treatment. One conclusion was that the G proteins that mediate the increase in potassium conductance of SMP neurones also inhibit an ω-conotoxin-sensitive calcium current.

Akasu and Tokimasa (1989) described a tonic calcium-activated potassium conductance in SMP neurones. In their studies however, the calcium-activated K+ current was insensitive to apamin (see section 1.3.2) and the calcium channel blockers ω-conotoxin and nifedipine, implying that the underlying calcium conductance was due neither to N- or L-type channels. Tachykinins such as substance P, substance K and neurokinin B caused slow inward currents, probably underlying the slow e.p.s.p. described by Surprenant (1984a), as did forskolin and 8-bromo cyclic AMP, suggesting that there was a cyclic AMP-dependent transduction mechanism at work. Akasu and Tokimasa suggested that the slow e.p.s.p. was due to the release of a tachykinin from other enteric neurones, which caused an inhibition of the calcium-activated potassium current, possibly by a cyclic AMP-dependent mechanism.

One possibility, therefore, is that there are two calcium conductances in SMP neurones; one that is sensitive to ω-conotoxin and inhibited by agonists that also activate the G protein-coupled potassium current, and another which is tonically active and maintains a calcium-sensitive potassium conductance that contributes to membrane potential. The K+ current is inhibited by neurokinins which results in the slow e.p.s.p. observed in SMP neurones. Calcium-activated K+ currents (IK(Ca)) have also been reported in myenteric plexus neurones. In AH neurones of the myenteric plexus, this current was shown to be tonically active and to contribute to prolonged after hyperpolarisations (Kunze et al., 1994). Like the IK(Ca) of SMP neurones, the myenteric conductance was apamin-insensitive, but blocked by charybdotoxin and iberiotoxin. It may be that the SMP calcium-activated K+ conductance is also sensitive to these blockers.

Some of the voltage-gated K+ currents of SMP neurones are discussed in chapter five.
1.2.2.4 G protein-coupled potassium channels in other tissues.

It is becoming clear that a wide range of neurotransmitters exert inhibitory effects via receptors linked to G protein-coupled K⁺ channels (North, 1989). Agonists at muscarinic M2, adrenergic α₂, 5HT-1a, dopamine D₂, adenosine A₁, GABAβ, somatostatin and δ and µ opioid receptors have all been shown to hyperpolarise neurones, many of them central neurones, by increasing permeability (Nicoll 1988; Mihara et al., 1987a; Tatsumi et al., 1990; Nicoll, 1988; North et al., 1987; Andrade et al., 1986; Trussel and Jackson, 1987; Gähwiler and Brown, 1985; Sasaki and Sato, 1987; Nakajima et al., 1988; Aghajanian and Wang, 1986; North and Williams, 1985; Yakel et al., 1988; Moore et al., 1988). Agonists are rendered ineffective by PTX treatment, and the K⁺ current is permanently activated by GTP-γ-S.

Results from a number of experiments suggest that the endogenous G protein in neurones is of the Gα variety rather than Gβ which mediates the cardiac response. Anti-Gα antibody has been used in a number of tissues to abolish agonist-induced K⁺ currents, (Nicoll, 1988; Stemweis and Robishaw, 1984; Huff et al., 1985; Gierschik et al. 1986; Gilman, 1987; Brabet et al., 1988; Itoh et al., 1986; Holz et al., 1986; Dunlap et al., 1987; Scott and Dolphin, 1987 a&b; Wanke et al., 1987; Hescheler et al., 1987; Ewald et al., 1988). In rat hippocampal pyramidal cells VanDongen et al., (1988) found that recombinant activated α₉ subunits increased the open probability of four K⁺ channels in excised inside out patches. In contrast to the atrial channel, VanDongen found very little tonic activity of the α₉-sensitive channels in the absence of G protein, but like K⁺, the channels were activated by picomolar concentrations of α₉ (c.f. Brown et al., 1990; maximal activation of K⁺ occurred with 50 pM α₉). Such experiments indicate that neuronal inhibition in the central nervous system is likely to be mediated by membrane-delimited K⁺ channel activation in a great many instances.

There is also evidence that adenosine triphosphate (ATP)-sensitive K⁺ channels may also be subject to direct G protein gating. In pancreatic β cells, ATP-sensitive K⁺ channels regulate insulin secretion through their effect on membrane potential which in turn governs the activity of voltage-sensitive calcium channels; as intracellular calcium rises, secretory activity is promoted. Insulin secretion is inhibited by circulating
adrenaline, locally released somatostatin, and noradrenaline from sympathetic nerves. One of the mechanisms by which these agents act, is activating ATP-sensitive K⁺ channels in a PTX-sensitive manner (Ribalet and Ciani, 1987; Ribalet et al., 1989).

Sulphonylurea-sensitive channels that are gated by G proteins have been reported in other tissues. Thus Lin et al. (1993) performed patch clamp experiments on isolated neurones from rat corpus striatum. In cell attached patches, an 85 pS channel was observed when dopamine or D₂ agonists were included in the pipette filling solution. The open probability of these channels was close to zero however, when agonists were omitted from the filling solution. When a mixture of agonist and sulphonylurea (tolbutamide or glibenclamide) was included in the filling solution, the open probability of the 85 pS channel was less than that of a channel exposed to agonist alone. The reduction of open probability was dose-dependent, with tolbutamide being active at surprisingly low concentrations (10 nM producing over fifty per cent reduction in open probability), between ten and one hundred times more potent than glibenclamide. This is in obvious contrast with the ATP-sensitive (K_{ATP}) channel of pancreatic β cells in which glibenclamide has been shown to be one hundred times more potent than tolbutamide (Ashcroft and Ashcroft, 1992). Furthermore, the dopamine-sensitive channel was not affected by diazoxide, which is known to activate K_{ATP} conductances in other tissues (Ashcroft and Ashcroft, 1992). However, in cell-attached patch recordings the 85 pS channel was activated when intracellular levels of ATP were reduced by inducing metabolic depletion with the NADH dehydrogenase inhibitor rotenone. Lin concluded that the dopamine-sensitive channel was a novel class of K_{ATP} channel, but did not conjecture as to the nature of the receptor/channel coupling. The only piece of evidence from this work that points towards direct G protein/K⁺ channel coupling, is the ability of agonists in the pipette filling solution to open the 85 pS channel in cell-attached patch experiments, but the possibility exists that central K_{ATP} channels are also subject to direct G protein gating. Similarly there have been reports of tolbutamide-sensitive hyperpolarisations in guinea-pig substantia nigra neurones (Roeper, et al., 1990). These potential changes are induced by agonists of D₂ and GABA₉ receptors. As more interest focuses on the diversity of neuronal G protein-coupled K⁺ channels, the study of their pharmacology may well become more important in classifying them and perhaps even in the development of therapeutic strategies.
In addition to neurones and cardiac myocytes, direct G protein/K\(+\) channel interactions are known to exist in secretory tissue. One particularly widely studied cell is the GH3 clone, a cell line derived from a rat pituitary lactotroph tumour. Muscarinic agonists and somatostatin have been shown to open K\(+\) channels in GH3 cells in a PTX-sensitive manner (Yatani et al., 1990), and such an action could underlie the inhibition of hormone secretion produced by these agonists in native lactotrophs. The response to carbachol was found to be membrane delimited, with a requirement for GTP on the cytoplasmic face of inside-out patches. The channels could be opened by GTP-\(\gamma\)-S and purified human erythrocyte G protein; preactivated erythrocyte \(\alpha\) subunits activated a single species of 55 pS K\(+\) channel (measured in symmetrical K\(+\)), whilst the \(\beta\gamma\) dimer was ineffective. As well as widening the diversity of known G protein/K\(+\) channel interactions, the GH3 response lends weight to the argument that the \(\alpha\) subunit is the G protein component that interacts with the K\(+\) channel.

One of the aims of the present study is to compare the pharmacologies of G protein-coupled K\(+\) channels with that of other K\(+\) channels. A brief review of this diverse family of ion channels is presented in the next section.

### 1.3.1 The voltage-gated K\(+\) channels.

In their study of the action potential of squid giant axons, Hodgkin and Huxley (1952) described a potassium current that was responsible for the repolarisation of the membrane potential and was activated after a delay on depolarisation. This current, being responsible for the outward rectification of this tissue, became known as the “delayed rectifier” frequently abbreviated to “I\(_{\text{K}}\)”\. I\(_{\text{K}}\) is widely distributed amongst excitable and non-excitable tissues. The characteristics of the current vary in different tissues in terms of kinetics of gating, the “threshold” potential at which the conductance begins to occur and the sensitivity of I\(_{\text{K}}\) to channel-blocking drugs. There is also evidence, discussed in chapter 5, that more than one type of I\(_{\text{K}}\) may coexist in the same cell. Of the potent peptide toxins that are of value in K\(+\) channel classification, dendrotoxin isolated from the venom of African mambas has been shown to be active on a number of different I\(_{\text{K}}\)s such as those of the guinea-pig dorsal root ganglion.
neurone (Penner et al., 1986) and rat nodose ganglia (Stansfeld et al., 1986). Blockers of cardiac I_{KS} are also of value clinically in the treatment of certain heart dysrhythmias.

The A current (I_A) was originally described in molluscan neurones by Connor and Stephens (1971) and it is now known to exist in numerous excitable tissues, including those of mammal, insect and amphibian. Like the delayed rectifier, the A current is activated by depolarising voltage steps. In rat sympathetic neurones for instance, its threshold of activation is around -60 mV, so it is in fact activated at potentials negative to those needed to observe delayed rectification. Furthermore, it activates rapidly and inactivates after only a few tens of milliseconds making it clearly distinguishable from the delayed rectifier or indeed from any other K^+ current. I_A will normally be inactivated at resting potentials of -50 to -60 mV. The hyperpolarisation following an action potential is enough to remove inactivation however causing the transient outward A current to arise and temporarily keep the cell membrane hyperpolarised. Only after inactivation can the potential begin to depolarise again, eventually leading to another action potential. In this way, I_A helps to maintain interspike intervals.

In nearly all the tissues studied, I_A is sensitive to the convulsant compound 4-aminopyridine (4-AP), at concentrations in the range 1 to 20 mM depending on the tissue. Although heterogeneity is believed to exist amongst the A currents of various tissues, Rudy (1988) has pointed out that there are miscellaneous channels that carry transient outward currents that differ markedly from I_A described above in their sensitivity to 4-AP, the threshold potential of channel opening and the time constant of inactivation. Examples of these are the conductances of larval muscle of drosophila, in which the A-like current is activated at -40 mV and more positive, and the sensory neurones of the rat nodose ganglion, in which the "A current" has a time constant of inactivation of one to two seconds, and is blocked by only 30 μM 4-AP.

The M current (I_M) is a K^+ first described in frog sympathetic neurones (Brown and Adams, 1980) showing both time and voltage-dependent characteristics. It is activated with slow kinetics on depolarisation (in the order of tens to hundreds of ms) and also inactivates slowly when the membrane potential is stepped back to a more hyperpolarised level. I_M is important in the regulation of cell excitability and was one of the first K^+ currents found to be subject to modification by neurotransmitters; it is
inhibited by acetylcholine acting via muscarinic receptors (hence M current), substance 
P and luteinizing hormone releasing hormone leading to an increase in excitability. Since 
its discovery, $I_M$ has been found in mammalian peripheral neurones such as rat sympathetic 
neurones (Constanti and Brown, 1981) and central neurones in human neocortical cells 
(Halliwell, 1986). It also exists in certain smooth muscle cells such as that of the toad 
stomach. The list of neurotransmitters known to inhibit $I_M$ has grown as knowledge of 
its distribution has increased; in the example of the toad gastric smooth muscle cell, $I_M$ 
is actually augmented by β-adrenoceptor agonists (Sims et al., 1988).

The modification of $I_M$ by neurotransmitters acting at G protein-coupled 
receptors is insensitive to pertussis toxin (PTX) and may well involve the activation of 
phospholipase C and the phosphoinositil pathway (Berridge and Irvine, 1984). However, the identity of the component responsible for interaction with M channels is 
still unclear, although there are several reports that agents which activate protein kinase 
C can inhibit $I_M$ in some but not all tissues in which the current occurs (Moore et al., 
1988; Marrion, 1987; Brown and Higashida, 1988). In hippocampal neurones however, 
Dutar and Nicoll (1988) have found $I_M$ to be sensitive to IP3, suggesting that both 
halves of the phosphoinositol cascade may be important in the regulation of this rather 
mysterious current.

1.3.2 The calcium-activated K\(^+\) channels.

Calcium-activated K\(^+\) channels were also first described in molluscan neurones 
(Meech and Strumwasser, 1970), and have since been found in excitable and secretory 
cells. They are believed to be important in spike frequency regulation and contribute to 
the resting potentials of some cells. They may also play a role in the homeostasis of 
intracellular calcium since their activation by raised levels of the ion will lead to 
hyperlporisation favouring the closing of voltage-gated calcium channels. This effect is 
important in secretory cells and possibly in neuronal cells as well. Of the 
calcium-activated K\(^+\) channels, two types are discussed below.

The ‘BK’ channel or maxi-K channel is so called because it has a large single 
channel conductance. Single channel recordings using equal potassium concentrations 
on either side of the patch have revealed channel conductances of 150 to 250 pS 
(Pallotta et al., 1981). Pallotta et al. used cultured rat myotubes to show that this
channel is also strongly voltage-gated. In addition to being sensitive to membrane potential the gating of these channels has is also dependent on intracellular calcium concentrations (Barrett et al., 1982). Proposed state diagrams are complicated with two or three calcium ions binding to the channel to increase the likelihood of channel opening. Barrett’s study suggests, from single channel recordings, that depolarising to +50 mV would bring about less than a ten per cent open time if the calcium concentration on the intracellular side of the membrane is maintained at 100 nM.

BK channels are believed to be responsible for very rapid after hyperpolarisations (AHP) in many tissues. Barrett and Barrett (1976) showed an AHP of 1 to 2 ms occurring after an action potential in frog spinal motoneurone attributable to the BK conductance. The BK current is also believed to be contributory in action potential repolarisation in certain tissues such as the rat hippocampal neurone (Lancaster and Nicoll, 1987).

BK channels are sensitive to external tetraethyl ammonium at 2 to 5 mM and to the scorpion venom extract charybdotoxin in nanomolar concentrations.

SK channels (small conductance) by contrast have single channel conductances of between 10 and 14 pS. They are insensitive to membrane potential and their gating depends only on intracellular calcium levels. In many neuronal tissues they are responsible for slow AHPs seen after an action potential. The duration of these AHPs is presumably dependent on the rate of removal of internal calcium. During a train of action potentials the intracellular calcium concentration builds up and the slow AHP that results may slow the rate of firing or even hyperpolarise the cell such that it cannot reach the threshold potential and the action potentials stop altogether, a phenomenon known as spike frequency adaptation.

Apamin, a toxin derived from bee venom, is a potent blocker of the SK channel. It has been of value in identifying SK channels in such tissues as the neurones of rat superior cervical ganglion (Kawai and Watanabe, 1986) and guinea-pig hepatocytes (Burgess et al., 1981). The channel has also been described in cultured rat skeletal muscle and has also been shown to be apamin-sensitive but insensitive to externally-applied tetraethyl ammonium (Blatz and Magelby, 1986).

There is however a calcium-activated potassium conductance that produces prolonged AHPs in some tissues, that is not sensitive to apamin. Apamin-insensitive
AHPs have been found in central neurones such as in the rat hippocampus (Lancaster and Nicoll 1987), and peripheral cells like the guinea-pig myenteric neurones (Kunze et al., 1994). Kunze et al., using intracellular recording techniques, showed that the apamin-insensitive AHP of guinea-pig myenteric neurones was sensitive to charybdotoxin and iberotoxin and concluded that BK channels were responsible for this AHP although slow AHPs are not normally associated with BK channels. Constantin et al. (1993) described a slow AHP in cultured rat skeletal muscle which was not sensitive to apamin, charybdotoxin or TEA. It would seem then, that the SK channel is just one of possibly a number of calcium-activated potassium channels that produce long AHPs, and that the apamin-insensitive currents are also found in a variety of neuronal and non-neuronal tissues.

Another Ca-activated K+ current that is voltage insensitive is the potassium current found in most animal erythrocytes, is that discovered in the '50s by Gardos, and which has an intermediate single channel conductance lying between the values for SK (<15 pS) and BK channels (150-200 pS). Unitary conductances measured in frog erythrocytes using media with high concentrations of symmetrical K+ revealed a calcium-activated K+ conductance of 50 pS (Hamill, 1983). Intracellular calcium concentrations in the range 0.1 to 10 µM have been shown to activate the erythrocyte conductance (Hamill, 1983; Grygorczyk and Schwarz, 1983). Grygorczyk and Schwarz also proposed that two Ca2+ ions are required to open the K+ channel on the basis of the steep correlation between open probability and calcium concentration observed in single channel recordings. Charybdotoxin has been shown to block the erythrocyte channel potently as well as blocking BK channels (Wolff et al., 1988).

1.3.3 K+ currents gated by other factors.

The gating of some K+ channels is not dependent on membrane potential or intracellular calcium levels, the G protein-coupled channel being an example. Similarly, a K+ conductance that is insensitive to voltage and calcium has been reported in guinea-pig hepatocytes; instead activation accompanies cell swelling and is believed to be sensitive to cell volume (Sandford et al., 1992). The volume-activated current may well play a homeostatic role in the maintenance of cell volume via an effect on intracellular solute concentration. Spectral analysis studies performed by Sandford et al. revealed a
single channel conductance of 7 pS. The precise nature of the mechanism linking changes in cell volume to the gating of the channel is not yet understood.

ATP-regulated K+ channels (K\textsubscript{ATP}) of pancreatic β-cells may also play an important homeostatic role in determining insulin secretion. When treated with glucose, β-cells depolarise due to the blockade of K\textsubscript{ATP} channels by intracellular ATP leading to action potentials and hormone release (Ashcroft et al., 1984; Cook and Hales, 1984). K\textsubscript{ATP} channels have also been found in cardiac myocytes (Noma, 1983; Trube and Hescheler, 1984), skeletal muscle (Spruce et al., 1985), and neurones (Ashford et al., 1988) but their physiological roles in these tissues is not so obvious. Sulphonylurea drugs are therapeutically useful in the management of diabetes and are blockers of K\textsubscript{ATP} in β-cells (Trube et al., 1986). They are also of interest pharmacologically since they bind with high affinity to the cell membranes of CNS neurones (Geisen et al., 1985). As may be expected of such a widely occurring channel, K\textsubscript{ATP} currents have differing single channel properties depending on the tissue in which they are found.

The S potassium current of *aphysia* sensory neurones provides an example of a current under inhibitory and stimulatory control. The current is normally tonically active, but 5-hydroxytryptamine inhibits the S current via a G\textsubscript{s} protein which acts via increased cyclic AMP leading to increased protein kinase A activity (Siegelbaum et al., 1982). The effect is to sensitise the sensory neurones by increasing their excitability. S current stimulation, however, reduces excitability and neurones sensitivity. This is induced by a tetrapeptide called FMRFamide which stimulates the current via an action on arachidonate metabolism.

1.4 Aims of the present study.

1.4.1 The pharmacological characterisation of G protein-coupled channels.

One of the main aims of the present work is to study the pharmacology of two G protein-coupled K+ channels, namely the rat atrial muscarinic and guinea-pig SMP neurone conductances. Given that this class of K+ channel is rapidly expanding, ion channel pharmacology could prove to be a useful tool in classifying G protein-coupled K+ channels. Some highly potent toxins have been of great value in the classification and identification of other K+ channels, for example apamin has enabled the identification of
small conductance calcium-activated K⁺ channels in both excitable and non-excitabie
tissues. In this study, it is proposed to use more simple organic compounds, known to
be active at other K⁺ channels, to establish a pharmacological profile of the G protein-
coupled channels in the above tissues, and where possible investigate the mechanism of
block (see next section).

The first set of confounds to be studied have been shown to be blockers of the
calcium-activated K⁺ conductance of rabbit erythrocytes. Cetiedil was originally
developed as a potential therapy for sickle cell anaemia; it has since been found to be a
blocker of the calcium-activated erythrocyte conductance (Christopherson and
Vestergaard-Bogind, 1985) and of the volume-activated K⁺ conductance of rat
hepatocytes (Sandford et al., 1992). Analogues of cetiedil, designated UCL 1495 and
UCL 1269 have been found to have much greater selectivities for the erythrocyte and
hepatocyte channels respectively (D. Benton, pers. comm.; K. Burke, pers. comm.). The
antifungal agent clotrimazole has also been found to have blocking activity at the
erthrocyte channel (Alvarez et al., 1992).

The anti-arrhythmic agent propafenone has class I and III activities (Dukes and
Vaughan Williams, 1984), and has more recently been shown to be a blocker of K_\text{ACH} in
guinea-pig atrial myocytes, making it of immediate interest in the present study
(Inomata et al., 1993).

Two other compounds, 1,3-di-o-tolylguanidine (DTG) and (+)-3-(3-
hydroxyphenyl)-N-(1-propyl)piperidine (3PPP) were reported to block ligand-activated
hyperpolarisations in rat locus coeruleus, dorsal Raphe nucleus and in guinea-pig
submucous plexus neurones (Bobker et al., 1989). These and the sulphonylurea,
tolbutamide, which, as already mentioned is active on certain agonist-induced potassium
conductances of central neurones, were studied for their actions on the SMP
rat atrial conductance. The structures of the above compounds are given in appendix 1.

Some of the more active compounds are also tested on voltage-gated potassium
and sodium currents in SMP neurones, partly to elucidate further their selectivity as ion
channel blockers, and partly to examine mechanisms of channel block.
1.4.2 Some characteristics of channel block relevant to the present study.

The interaction of channel blockers with channel macromolecules can be compared to the interaction of hormones with receptors. Unlike neurotransmitter/receptor interactions, channel blocking compounds can act on channel proteins from within the cell as well as from outside it. A classical example of this is the block of the delayed rectifier of squid giant axon by quaternary ammonium compounds. Armstrong and Binstock (1965) bathed the tissue in high K⁺ and found that the current-voltage relation (cvr) of the open channels was almost linear over a large voltage range. Injection of tetraethyl ammonium (TEA) into the axoplasm resulted in a change in the cvr such that the channels conducted inward current far more readily than outward; a finding that led to the conclusion that TEA was acting at an intracellular site on the channel protein. The voltage dependence of block was attributed to the ability of inward moving K⁺ ions to expel TEA molecules from the ion pore whilst depolarising voltage steps favoured the movement of internal TEA towards their receptor on the axoplasmic face of the channel.

Ammonium compounds having larger alkyl moieties such as tetranonyl ammonium (TNA) have been found to be more potent than TEA at blocking the delayed rectifier of squid giant axon when applied intracellularly (Armstrong, 1966, 1969) indicating that there are hydrophobic interactions involved in the binding of the quaternary ammonium molecule. Using TNA, Armstrong found that depolarising voltage steps elicited outward currents that showed the delayed onset typical of control currents, but which then began to decay at rates dependent on the concentration of TNA, a phenomenon ascribed to open channel block. Local anaesthetic block of neuronal sodium channels is similar. These amino compounds are also believed to act from within the cell although they do not bear a permanent charge. The uncharged form is lipid soluble and capable of crossing neuronal membranes, but it is the protonated form of the molecule that blocks Na⁺ channels, a principle that may be relevant to the present study. Quaternary analogues of the local anaesthetic molecule such as QX-314 are ineffective when applied extracellularly, and therefore useless clinically, but are active when injected into axons (Frazier, et al., 1970). Repeated depolarising voltage steps yield progressively smaller Na⁺ currents implying that the drug is only able to act
on the open or inactivated channel and that each voltage step enables more drug/open channel binding to occur, a phenomenon known as use-dependent block (Courtney, 1975). The degree of block is dependent on the number of channels that open during a depolarising pulse; however the block becomes more pronounced when the cell is repeatedly stepped to potentials more positive than those needed for maximal Na\(^+\) channel opening (Strichartz, 1973). This is because at more positive potentials the QX-314 is itself drawn into the channels by the electric field across the membrane, towards its binding site. These phenomena have led to the postulation that the channel gate is located near the cytoplasmic end of the channel, a hydrophobic local anaesthetic receptor exists some distance within it, and the ion selectivity filter is nearest the extracellular end of the channel protein. The open channel block of delayed rectifier channels observed with quaternary ammonium compounds implies a similar type of channel topology.

Hydrophobic local anaesthetics also show use-dependent unblocking, suggesting that the molecule remains trapped within the channel and can only leave when the channel opens, hence full recovery of the sodium current may require several depolarising steps. Other local anaesthetics, if sufficiently lipophilic, like benzocaine or lidocaine, may be able to leave the channel by passing laterally through the channel protein and possibly into the membrane lipid as well. This constant 'leakage' of molecules from the binding site within the channel means that there is not necessarily an increase in the level of block observed with successive depolarising steps. At low frequencies hydrophobic anaesthetic molecules that have reached the receptor from the aqueous cytoplasmic phase may 'leak' away whilst the channel is shut so that little or no use-dependence is observed over successive depolarising steps (Hille, Courtney and Dum, 1975).

Such principles may be equally relevant in the mechanisms of K\(^+\) channel block.
CHAPTER 2

MATERIALS AND METHODS
2.1 Tissue Culture.

2.1.1 Preparation of isolated guinea-pig submucous plexus neurones.

Hartley guinea-pigs of either sex (150 to 400 g) were killed by a blow to the head followed by exsanguination, the abdomen was opened and the caecum removed. The submucous plexus tissue was dissected from the caecum as described by Hirst and McKirdy (1975) and Surprenant (1984). Briefly, the caecum was washed out using a physiological saline solution of the following composition (mM): NaCl 137, KCl 4, CaCl₂ 2, MgCl₂ 1, N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES) 10 and glucose 10 adjusted to pH 7.4 with NaOH. The physiological saline had previously been sterilized through a 0.22 μm millipore filter. The caecum was opened longitudinally and kept in physiological saline containing 50 units/ml penicillin and 50 μg/ml streptomycin and kept at 4°C prior to dissection. Portions of the tissue, roughly 4 cm², were then pinned out onto a sylgard-coated petri dish containing Leibovitz L-15 tissue culture medium at room temperature with the mucosal side of the tissue facing up; the mucosa was then removed using fine forceps and spring bow scissors. This exposed the submucosa which was peeled from the underlying smooth muscle and placed in Leibovitz L-15 medium containing penicillin and streptomycin (50 units/ml and 50 μg/ml) until sufficient submucosal tissue had been collected.

A tissue culture procedure similar to that described by Tatsumi et al., (1990) was then adopted. The tissue was transferred to a collagenase solution (Worthington class II) containing 1.5 mg/ml enzyme in Hanks’ balanced salt solution (calcium- and magnesium-free) and kept at 37°C for forty minutes. After incubation, the tissue was spun at 900 to 1000 revolutions per minute for five minutes and the supernatant removed. The pellet was resuspended in Hanks’ balanced salt solution containing 1 mg/ml trypsin (Sigma, from bovine pancreas) and incubated at 37°C for fifteen minutes. The tissue was spun again, resuspended in 1 ml neuronal tissue culture medium and gently triturated with a fire-polished Pasteur pipette of 0.5 mm internal diameter ten to fifteen times. This tissue culture medium was based on Leibovitz L-15 enriched with the following agents: NaHCO₃ (25 mM), glucose (30 mM), penicillin (100 international units/ml), streptomycin (100 μg/ml), glutamine (2 mM), foetal calf serum (5 ml per 50 ml culture medium),

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neuronal growth factor (5-50 ng/ml final concentration). The suspension was made up to 5 ml volume with Leibovitz L-15 medium and spun as before. The pellet was resuspended in 1 ml of neuronal tissue culture medium and plated out onto six 35 mm culture dishes that had been treated with laminin (50 μg/ml) two hours previously. The dishes were incubated at 37°C in humidified atmosphere of 5 per cent CO₂ in air overnight and 2 ml neuronal tissue culture medium was added to each dish the following morning. By this stage the cells had adhered to the bottom of the culture dishes either individually or in groups which were assumed to be the remnants of incompletely dissociated ganglia. A successful preparation could give a yield of up to one hundred cells per dish. The cells were used experimentally up to three days after isolation.

2.1.2 Preparation of neonatal rat atrial myocytes.

A similar protocol was adopted in the preparation of atrial myocytes from neonatal rat. A one day old rat was killed by decapitation and the atria removed. A few radial cuts were made in either atrium with a pair of spring bow scissors to enhance enzyme access, and the tissue was transferred to a solution containing 2 mg/ml collagenase (Worthington class II) in Hanks’ balanced salt solution (calcium and magnesium-free). After twenty minutes incubation at 37°C, the enzyme solution was removed and replaced with 2.5 mg/ml trypsin (Sigma, from bovine pancreas) in Hanks’ balanced salt solution, and incubated for a further 15 minutes. The tissue was not spun at any point.

The enzyme solution was removed and replaced with a solution containing 6 mg/ml bovine serum albumin to arrest further enzyme activity and 10 mM magnesium to protect the cells from any build-up in intracellular calcium. After 3 minutes this was removed and then replaced with a tissue culture medium of the same composition as that used in the preparation of the submucous plexus neurones. (In later procedures 2 mM EGTA was added to this medium, to buffer calcium down to the micromolar level as this seemed to make the myocytes more robust). The tissue was then triturated with a wide bore fire-polished Pasteur pipette of 2 to 3 mm internal diameter to yield a translucent cell suspension which was diluted five- to ten-fold in tissue culture medium (again containing 2 mM EGTA in later experiments). The suspension was plated out onto 35 mm culture dishes pretreated with laminin (50 μg/ml).
The cells normally adhered to the culture dishes within three hours and were ready for experimental use on the same day. Initially they appeared spherical and phase-bright, but by the second day after tissue culture, the cells began to flatten and contract spontaneously and were no longer used.

2.2 Intracellular Recording Techniques.

2.2.1 Recording medium.

Before recording procedures began, the culture medium was removed and cells perfused whilst still in the culture dish with a physiological saline of the following composition (mM): NaCl 137, KCl 4, CaCl₂ 2, MgCl₂ 1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10 and glucose 10 adjusted to pH 7.4 with NaOH. The flow rate was set to 5 ml per minute.

The cells were constantly superfused with physiological saline which was stored in a 100 ml reservoir and flowed under gravity through a glass coil encased in a heated water jacket. The temperature of the saline in the culture dish was 30°C and the fluid level was kept constant with a suck-off tube operated by a vacuum pump.

Drugs were applied dissolved in the physiological saline kept in reservoirs and could be introduced into the culture dish using a multiway tap.

2.2.2 Recording conditions.

The culture dish was inserted into a hollow in a perspex block and mounted onto the stage of an inverted Nikon TMS phase contrast microscope and viewed under 400x magnification.

The microscope and recording set up were housed in a Faraday cage to exclude 50 Hz electrical interference from near by appliances, and mounted on steel and concrete slabs supported on a Micro-g vibration isolation table (Technical Manufacturing Corporation) to minimize the transmission of externally-caused vibration.
2.2.3 Microelectrodes.

Microelectrodes were pulled from 1.0 mm borosilicate glass capillaries containing a filament (Clark Electromedical GC-100F-15) using a Narashige PN-3 horizontal puller. Molar potassium citrate filling solution of pH 7.2 was introduced into the lower third of the electrode using a 1 ml syringe and fine stainless steel needle immediately prior to use. 10 mM KCl was included in the microelectrode filling solution to ensure stability of the half cell. Microelectrodes with resistances of between 80 and 120 MΩ were used. A bath electrode consisting of a silver/silver chloride pellet (Clark Electromedical E205) set in Krebs solution was inserted into the cell perfusate to make electrical contact with the microelectrode.

The microelectrode holder and headstage were mounted on a hydraulic fine manipulation system (Narashige MO-203) which was used to manipulate the microelectrode towards the cells at higher magnifications.

2.2.4 Cell impalement and the measurement of cell properties.

The single microelectrode ‘bridge’ circuit of an Axoclamp 2A amplifier (Axon Instruments) was used, with an HS-2 headstage (Axon Instruments) usually with a gain of 0.01. After insertion into the bathing solution, current pulses of 100 pA were passed every 5.4 seconds enabling the microelectrode resistance to be measured and offset with the ‘bridge’ balance control. Any standing potentials were also offset before cell impalement. The microelectrode was advanced so as to make contact with the cell membrane and the circuit oscillated for 1 ms to achieve impalement. The best recordings were obtained from spherical phase-bright neurones. Both single cells and cells in clusters were used. In the case of the myocytes, the cells were also at their best when phase-bright and spherical. After 48 hours the cells began to flatten and become less bright. Myocytes would also form gap junctions with one another which could have enabled them to couple electrically. When myocyte cultures took on this appearance, they were usually discarded.

Voltage and current outputs from the amplifier were amplified five-fold, digitized (with an Instrutech VR-10 digital data recorder) and then simultaneously stored on tape (Panasonic video cassette recorder NV-L20 HQ) and displayed on an oscilloscope (Gould 20 MHz digital storage Type 1421) and pen chart recorder (Gould Easy Graf 13-
6615-10A and Devices chart recorder). Signals recorded on the Gould Easy Graf recorder were filtered through a -3db 20 Hz low pass filter to remove excessive noise from experimental traces. Data were then subjected to computer-aided analysis (see section 2.3.3).

During intracellular recordings regular microelectrode current injections of between 5 and 50 pA were made, the frequency and duration of which were controlled using a Digitimer D4030 pulse generator. The electrotonic changes in membrane potential produced were used to measure cell resistance and capacitance.

2.2.5 Tip potentials.

Physico-chemical interactions on the surface of the microelectrode tip and diffusion potentials lead to the establishment of microelectrode tip potentials. These tip potentials occur in most microelectrodes, generally being proportional to microelectrode resistance and inversely proportional to the ionic strength of the external solution. Thus the tip potential of a microelectrode may be offset in bathing solution but when the microelectrode is introduced into the different ionic environment of the cell cytoplasm, the tip potential will fall leading to a measurement of membrane potential which is more positive than the true value. In the present study capillary-containing microelectrodes are filled with electrolyte solution immediately prior to use. Both the presence of the capillary and the rapidity of use of the microelectrode after filling have been shown by Okada and Inouye (1975) to reduce the magnitude of tip potentials. Furthermore, the measurement of conductance changes should not be affected by tip potentials although absolute potential measurement will be subject to error.

2.3 Whole Cell Recordings.

2.3.1 Patch pipettes and filling solutions.

Patch pipettes were pulled from borosilicate glass tubing GC 150TF -15 (Clark Electromedical Instruments) which has a thin wall with inner filament, using an L\M-3P-A puller (List Medical). Immediately prior to use they were back filled with filling solution delivered from a 1 ml syringe delivered through a 0.22 µm millipore filter. All patch pipette filling solutions were based on a solution of the following composition
(mM): KCl 150, N-2-hydroethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5 and NaCl 2, adjusted to pH 7.2 with NaOH. To this stock solution 40 μM CaCl₂ and 150 μM EGTA were added to give a free calcium concentration of 100 nM. Also, given the potassium concentration of the extracellular bathing medium used in all experiments (4 mM, section 2.2.1) this pipette solution gave a calculated value of -94.7 mV as the potassium equilibrium potential at 30°C.

In most experiments ATP (2 mM), and MgCl₂ (1 mM) were included in the filling solution and in some GTP (100 μM) was used to help prevent run down of the G protein-coupled response, or GTP-γ-S (50 μM) was used to bring about a permanent activation of the G protein-sensitive currents.

2.3.2 Discontinuous single electrode voltage clamp recordings.

The majority of whole cell recordings were performed using higher resistance patch electrodes of around 8 to 10 MΩ with the discontinuous single electrode voltage clamp mode (switch clamp) to prevent dialysis of cellular constituents into the patch pipette in an effort to avoid run-down of the G protein-coupled potassium current. For switch clamping, an HS-2 headstage (with a gain of 0.1L) was used with the Axoclamp 2A amplifier.

Initially, gigaohm seals were obtained in bridge balance mode; once the whole cell configuration had been achieved (usually by gentle suction) the recording mode was switched to discontinuous current clamp. A second Scopex 4S 6 oscilloscope was used to monitor the events at the headstage during switch clamp experiments; this enabled the microelectrode capacitance to be offset using the negative capacitance control of the Axon amplifier. Normally, the fluid level in the culture dish was kept to a minimum to reduce pipette capacitance as much as possible. It was not normally necessary to coat patch electrodes with sylgard since the maintenance of low fluid levels seemed to be adequate for minimising capacitance during whole cell recordings. The second oscilloscope also enabled the sample rate to be set to an optimal level before switching to voltage clamp mode. Once in discontinuous single electrode voltage clamp mode, the current was filtered at 0.1 kHz to reduce noise, or 0.3 kHz in experiments where a sharp voltage step resolution was needed, i.e. when studying the neuronal sodium current. The switch clamp had a duty cycle of 30 per cent current passing and 70 per cent voltage.
measuring. Typically, sample rates of 5 kHz could be achieved. Because the cell is not a perfect capacitor in the sense that current and voltage are not precisely 90 degrees out of phase with each other, the phase shift control of the Axon amplifier could be used to compensate for this phenomenon which in turn enabled the clamp gain to be increased thereby improving the quality of the voltage clamp.

The axoclamp voltage and current outputs were stored and displayed in the same way as described for microelectrode recordings (section 2.2.4). Initially, the ‘bridge’ mode was used to measure patch electrode resistance and after attainment of the whole cell configuration the discontinuous current clamp mode was used to set the negative capacitance to its optimal level prior to voltage clamping.

Any resistance occurring between the membrane and the patch pipette (series resistance) can distort the results obtained in whole-cell recordings. One of the advantages of the discontinuous voltage clamp is that any breakdown in the quality of the clamp is easy to detect.

2.3.3 Data analysis.

Results were analysed either from tape or during an experiment using a Dell System 200 computer with Pclamp 5.5.1 program operating through a TL-1 interface. This enabled membrane potentials and currents to be measured. The program also fitted single exponentials to the electrotonic potentials allowing an estimate of membrane time constant and capacitance to be made, and to the decaying phase of time-dependent currents (such as the A current of the neurones) to give time constants for the inactivation of the current.
All graphs and figures were prepared using the Sigmaplot scientific graph plotting package, and the error bars presented in all subsequent graphs indicate the standard error of the mean. Curves were fitted to sets of data points using Sigmaplot which employed an iterative regression procedure to obtain the best fit as determined by the method of least squares. Dose-response data were usually fitted with curves deriving from the Hill equation (see section 3.2). From the best fits, such parameters as the IC$_{50}$ or EC$_{50}$ values and Hill slopes could be obtained. In some figures the maximum response ($B_{\text{max}}$) of the curve fit was constrained to one hundred per cent, in others it is an unconstrained parameter. For example, when curves were fitted to agonist dose-response data (such as conductance increase produced by noradrenaline and somatostatin in SMP neurones) maximum responses were left unconstrained. Sufficiently high concentrations of agonist were used to establish a clear maximum. Other figures however (such as those showing block of agonist-induced conductance increase by various concentrations of a test compound) do not show clear maximal effects since sufficiently high concentrations were not tested. Initially, when fitting curves to dose-inhibition data as illustrated in figure 3.3.1.1, curves were generated with unconstrained maxima and maxima constrained to 100 per cent, although the maximum block obtained with the top doses of some of these compounds was in the region of 80 to 90 per cent. It was found however that the IC$_{50}$ values obtained from constrained and unconstrained curves were very similar and from then on curve fitting was done with maxima constrained to 100 per cent. In all cases but one, drugs were applied at sufficient concentrations to produce at least an 80 per cent block of the various potassium conductances studied, implying that the maximum inhibition is not likely to be significantly different from 100 per cent. However, if the maximum inhibition produced by a compound was for instance 60 per cent, then the IC$_{50}$ value would become misleading as a measurement of the concentration at which the drug produced half of its maximal effect. Again, since curves were normally only fitted to data sets in which at least an 80 per cent inhibition of the potassium conductance was obtained at the top drug concentration (with the exception of propafenone acting on $I_K$ of SMP neurones), this consideration is less likely to distort the significance of the calculated IC$_{50}$ value. Furthermore, if a drug were acting selectively at a subset of potassium channels and incapable of producing 100 per cent block of the whole cell $K^+$ conductance, this would be reflected in the Hill slope which would be expected to be less than unity. This is the case with the block of the delayed rectifier of SMP neurones by propafenone which produced 60 per cent block at the highest concentration tested (20 $\mu$M) but the fitted curve (having a $B_{\text{max}}$ value constrained to 100 per cent) gave a Hill slope of 0.6 (see sections 5.3 and 5.4).
2.4 Sources of Materials.

Aldrich Chemical Company Ltd.: Tetraethylammonium, 1,3-di-o-tolylguanidine (DTG).

BDH Laboratory Supplies: NaCl, KCl, MgCl₂, CaCl₂ and glucose.

Calbiochem: Somatostatin.

GIBCO Reagents Ltd.: Hanks' balanced salt solution, Leibovitz L-15 culture medium.

Innothera: Cetiedil.

Research Biochemicals Incorporated: (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine, (3PPP).

Sigma Chemical Company: ATP, GTP and GTP-γ-S. Bovine pancreatic trypsin. (-) Noradrenaline, adenosine, fluconazole, propafenone, clotrimazole, tolbutamide.

Worthington: Class II Collagenase.

UCL 1269 synthesised by Craig Roxburgh, UCL 1495 and UCL 1559 synthesised by Salah Athmani.
2.5 Composition of tissue culture media.

Table 2.5.1: Hanks’ balanced salt solution:

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<th>Reagent</th>
<th>Concentration (mg/L)</th>
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<tr>
<td>KH$_2$PO$_4$</td>
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<tr>
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</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
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<tr>
<td>D-Glucose</td>
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</tr>
<tr>
<td>Phenol Red</td>
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</table>

Table 2.5.2: Leibovitz L-15 culture medium:

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</thead>
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<tr>
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CHAPTER 3

THE USE OF INTRACELLULAR RECORDING TECHNIQUES TO STUDY THE PHARMACOLOGY OF G PROTEIN-COUPLED POTASSIUM CONDUCTANCES IN GUINEA-PIG SUBMUCOUS PLEXUS NEURONES AND NEONATAL RAT ATRIAL MYOCYTES.
3.1 Intracellular recordings from isolated submucous plexus neurones.

The neuroD cultures prepared as described in chapter two occurred either as single cells or as groups of four to fifteen cells, presumably the remnants of ganglia that had not been completely dissociated. Initially the cells appeared phase-bright and spherical, but by three days after the preparation they had begun to develop processes.

Intracellular recording experiments usually began the day after the tissue culture of the submucous plexus neurones had been prepared. The yield of neurones was variable, and there were always cells from the mucosal endothelium present in the preparation, although the neurones could readily be identified from other cells by their comparatively large size. Ten isolated spherical cells were measured using a graticule at x 400 magnification and gave a mean diameter of 24 ± 1 μm.

After impalement, the hyperpolarising current injections were reduced in magnitude to give electrotonic potential changes of about -20 mV every 5.4 seconds. The duration of the current pulses was adjusted so that the electrotonic potential had time to settle to its final value, depending on the membrane time constant. Current pulse duration was typically in the range of 200 to 500 ms. The shape of the electrotonic potential was regularly checked to ensure that the microelectrode resistance did not change during an intracellular recording. The bridge balance control was adjusted when necessary so that just the slow exponential portion of the potential was recorded. Cell with membrane potentials positive to -30 mV or with input resistances less than 0.5 GΩ were rejected. Normally the potential drop and resistance increase from a good cell would be instantaneous; very little time was taken for the ‘sealing in’ of the microelectrode.

From a sample of forty intracellular submucous plexus neurone recordings, the resting potential was -58.3 ± 1.8 mV (mean ± standard error). Tip potentials arising after impalement were not offset; see chapter 2. The cell input resistance measured from the electrotonic potentials, was 1.33 ± 0.18 GΩ from the same sample of forty cells. By fitting single exponentials to the electrotonic potentials obtained from the same recordings, the membrane time constant (τ) was found to be 65 ± 10 ms.
Treating the membrane as a parallel RC circuit, membrane capacitance was calculated as the ratio of the resistance of a cell membrane to its time constant:

\[ C = \frac{\tau}{R} \]

where \( C \) is the membrane capacitance, \( R \) the input resistance and \( \tau \) the membrane time constant. The mean capacitance of the forty recordings was 49.4 ± 2.6 pF.

Depolarising current pulses of sufficient magnitude induced action potentials in successfully impaled cells. Figure 3.1.2 shows a typical SMP neurone action potential induced by a 15 pA depolarising pulse.

In some recordings, action potentials occurred immediately after the current pulse. These 'break spikes' were observed in about twenty per cent of cells, especially those with more positive resting potentials, and they tended to diminish in frequency as the recording progressed. The likelihood is that hyperpolarising current removed some of the inactivation of the sodium channels which caused action potentials to occur after the hyperpolarising pulse.

In all seventy-six intracellular recordings were obtained from SMP neurones. An additional seven cells were successfully impaled, but these cells showed spontaneous excitatory activity, giving 2 to 3 action potentials a second when no current pulses were being made; these cells were not used in the study of the pharmacology of the G protein-coupled channel (see next section). Thus just over 8 per cent of cells showed this type of activity. This is consistent with the findings of Annmarie Surprenant (1984) who found that five per cent of SMP neurones showed spontaneous activity. Some of them lacked synaptic input, whereas others were stimulated by cholinergic inputs. In the present study, two cells were found to exhibit long after hyperpolarisations (AHP) in the order of ten seconds' duration. Again Surprenant reported the existence of such SMP neurones which were not believed to have synaptic inputs and whose AHPs were sensitive to cobalt and extracellular calcium levels. Surprenant reported that twenty per cent of SMP neurones lacked synaptic input; in this study, cells having long AHPs and cells displaying spontaneous activity accounted for just under eleven per cent of SMP
Figure 3.1.2: An action potential recorded from an SMP neurone. The cell was depolarised by a 15 pA current pulse and had a resting resistance of 1.12 GΩ and resting potential of -69 mV.
cells. It may be that the tissue culture procedures (not employed in Surprenant’s study) caused the loss of this type of neurone accounting for this discrepancy.

3.2 The actions of agonists on the submucous plexus neurone.

Surprenant and North (1983) had already shown that α2 agonists are capable of inducing increases in G protein-coupled potassium conductances in guinea-pig submucous plexus neurones, and Mihara et al. (1987a) showed that somatostatin was also capable of activating this conductance. Initially, experiments with noradrenaline and somatostatin were performed to determine dose-response relationships for these agonists.

Figure 3.2.1 shows changes in membrane potential in response to 1 μM noradrenaline and 20 nM somatostatin in a guinea-pig SMP neurone. The downward deflections are electrotonic potentials caused by current injections of 10 pA made every 5.4 seconds. The input resistance of the cell as calculated from the size of the electrotonic potentials is 2 GΩ and resting potential about -50 mV. In part A, the bathing solution was switched from control to solution containing 1 μM noradrenaline for 50 seconds. The resulting increase in potassium permeability caused the membrane potential to increase to -85 mV, and the electrotonic potentials to fall in magnitude as the input resistance decreased to 0.8 GΩ. The response reversed about 40 seconds after switching back to drug-free bathing solution. In part B, 20 nM somatostatin is seen to do the same as noradrenaline again with a 50 second application, with the extent of the hyperpolarisation and fall in resistance being very similar. However, the response took some three minutes to reverse after switching solutions. This slow time-course of recovery from somatostatin was characteristic of the agonist, possibly due to high affinity for its receptor. Of the seven cells that showed spontaneous excitatory activity, noradrenaline was tested on three. None of these responded to the agonist.

The magnitude of agonist responses such as those illustrated in figure 3.2.1 was measured as the conductance increase induced by agonist. Using Pclamp 5.5.1, four consecutive electrotonic potentials were averaged, and the potential measured at a point when it had reached its maximum value. Membrane conductance was calculated as current pulse magnitude divided by the electrotonic potential. Resting membrane
Figure 3.2.1: Responses to 1 μM noradrenaline and 20 nM somatostatin. An intracellular recording showing two responses from the same SMP neurone.
conductances before and after the response were measured in this way, meaned and then subtracted from the conductance measurement taken during the response, to give the agonist-evoked conductance increase. In all, 84 per cent of the neurones responded to the two agonists; no recordings were made in which a cell responded to one agonist but not the other.

Conductance increase was chosen in preference to the degree of hyperpolarisation produced, since the latter parameter is also dependent on the resting membrane potential of the cell. A cell with a more negative resting potential (i.e. one close to the potassium equilibrium potential) would only give a relatively small change in membrane potential even in the presence of a maximal concentration of agonist. Conductance increase however gives a more linear representation of the magnitude of agonist-induced membrane currents. Over the course of the intracellular recording experiments, the mean conductance increase produced by 1 μM noradrenaline was 2.7 ± 0.3 nS (59 cells) and the 20 nM somatostatin response 3.3 ± 0.6 nS (38 cells). An unpaired t test did not reveal any significant difference between the sizes of the response to the two agonists.

In Figure 3.2.2 concentration-response curves for the two agonists are shown in which the responses obtained during each recording have been expressed as a percentage of the response to 1 μM noradrenaline or 20 nM somatostatin depending on the agonist being tested, which in each case was within ten per cent of the maximum response. The data points have been fitted, using the method of least squares, with an equation of the form:

\[
\text{Percentage Response} = \frac{B_{\text{max}} \times a^n}{a^n + k^n} \quad \text{Equation 3.2.1}
\]

where \(a\) = the concentration of agonist, \(n\) = the Hill slope and \(k\) = the concentration of drug producing 50 per cent of the control response (EC\(_{50}\)) and \(B_{\text{max}}\) is the maximum response, which has not been constrained to one hundred percent.

From these curve fits, the EC\(_{50}\) for noradrenaline was calculated as 130 ± 25 nM with a Hill slope of 1.2 ± 0.3, whilst the EC\(_{50}\) for somatostatin came to 3 ± 1 nM with a slope of 1.1 ± 0.4.
Figure 3.2.2: Dose–response curves for conductance increases induced in SMP neurones by noradrenaline (○) and somatostatin (▼). Somatostatin data are expressed as a percentage of the 20 nM response, noradrenaline data expressed as a percentage of the 1 μM response.
Tatsumi et al., (1990) contrastingly found noradrenaline to produce a larger current response than somatostatin at supramaximal concentrations of each agonist in whole cell voltage clamp experiments although did not state whether the difference was of statistical significance. Furthermore, they reported that whilst 59 per cent of cells were responsive to noradrenaline, only 36 per cent responded to somatostatin. In this study however, relatively few recordings were made in which a cell was exposed to both noradrenaline and somatostatin; of these however it was found that the neurone either responded to both or did not respond at all.

Whilst Tatsumi obtained maximum outward currents with 10 μM noradrenaline, Sarfert et al. (1987) found 1 μM to be a near-maximal response. Furthermore, Mihara et al., (1987) also found somatostatin to have an EC$_{50}$ of 3 to 5 nM from both voltage recording and voltage clamp experiments.

3.3 The effects of cetiedil, clotrimazole and propafenone on agonist responses in the guinea-pig submucous plexus neurone.

For the purpose of studying the actions of channel blocking compounds on the agonist-evoked conductance increase of submucous plexus neurones, standard doses of both noradrenaline and somatostatin were chosen. It was decided that the lowest dose of agonist that elicited a near maximum response would be used; excessively high doses of agonist were avoided in case this lead to the desensitisation of the response. Thus 1 μM noradrenaline and 20 nM somatostatin were routinely used. It was also found that responses evoked with somatostatin tended to run down more rapidly than those obtained with noradrenaline, which were frequently well maintained, so most experiments testing new blockers were first performed using 1 μM noradrenaline as agonist, and somatostatin was used to confirm that compounds which appeared to block the noradrenaline response were also active on the response evoked with somatostatin. After obtaining a control response to agonist, the cell was bathed in the test compound for five minutes and the agonist then re-applied in the presence of blocker. The blocker was removed and a third dose of agonist tested after five minutes to test for the
reversibility of any blocking action and confirm that the response had not 'run down'. The agonists and channel blocking compounds were dissolved in the physiological bathing solution and bath-applied. It was noted that cells which had become desensitised to somatostatin still responded to noradrenaline. Agonist applications of sixty to ninety seconds’ duration were routinely used.

Figure 3.3.1 illustrates the above procedure showing consecutive responses to three applications of noradrenaline in a submucous plexus neurone in which the electrotonic potentials have been induced with a 10 pA current pulse. The first part is an initial control response to 1 μM noradrenaline. The cell was then bathed in a blocking compound (10 μM propafenone; see next section) for five minutes and the agonist re-applied. The extent of the conductance increase and hyperpolarisation were both attenuated. The blocker was washed out and the agonist re-applied five minutes later; the response showed almost complete recovery. Note that propafenone caused some depolarisation and decrease in resting conductance; this phenomenon is discussed in section 3.3.4.

Whenever possible, the block produced by a drug would only be calculated from recordings in which the agonist response recovered appreciably after the removal of test compound. The control and recovery conductance increases would be meaned, and from this value was subtracted the conductance increase obtained in the presence of blocker. This figure was then expressed as a percentage of the mean of the control and recovered responses to get the percentage inhibition of agonist-induced conductance increase produced by the blocker.

3.3.1 Inhibition of the response to 1 μM noradrenaline.

Amongst the first compounds found to be active against noradrenaline-induced responses were the agents cetiedil, clotrimazole and propafenone (see section appendix 1 for structures).

Figure 3.3.1.1 shows concentration-inhibition curves for these three drugs, plotting percentage inhibition of the 1 μM noradrenaline response against log concentration of drug. Again the data points have been fitted with an equation of the sort given in section 3.2.1 except that the maximum effect (B_max) has been constrained to 100 per cent since there was no reason to believe that the compounds could produce
Figure 3.3.1: Three responses to 1 μM noradrenaline from an SMP recording. A control response, followed by a response obtained in the presence of 10 μM propafenone and the recovery of the noradrenaline response after washing out propafenone. See text for details.
Figure 3.3.1.1: Inhibition of the 1μM noradrenaline-induced conductance increase by cetiedil (▼), clotrimazole (○) and propafenone (□).
a level of block of the conductance that was greater than 100 per cent. From these fitted curves, IC$\text{S}_{50}$s were calculated for the three drugs and given in Table 3.3.1.1. As can be seen, all three compounds are active at low micromolar concentrations, making them comparable in activity with their actions at other potassium channels such as the erythrocyte $K_{(Ca)}$ and hepatocyte volume-activated conductances.

Table 3.3.1.1: IC$\text{S}_{50}$s and Hill slopes for three K$^+$ channel blockers on the K$^+$ conductance induced by 1 µM noradrenaline in guinea-pig submucous plexus neurones. The values are obtained from the fitted curves shown in figure 3.3.1.1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$\text{S}_{50}$ (µM)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.5 ± 0.1</td>
<td>1.4 ± 0.04</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>5.9 ± 0.4</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

Even at top concentrations the effects of propafenone and cetiedil had largely reversed five minutes after washing out the drug. Clotrimazole at 10 µM however, normally took fifteen minutes to reverse fully.

3.3.2 Inhibition of the response to 20 nM somatostatin.

That the response to noradrenaline was sensitive to the above compounds was not proof of channel block. In order to rule out the possibility that the blockers had antagonist activity at the $\alpha_2$ receptor, experiments were performed to ensure that the response to somatostatin was also sensitive; the results are summarised in figure 3.3.2.1.

At 10 µM clotrimazole and propafenone produced near maximal inhibitions of the control conductance increase as they did in experiments which used 1 µM noradrenaline as agonist. The dose response points for cetiedil were fitted with a curve that gave an IC$\text{S}_{50}$ of 9.00 ± 0.52 µM, slightly higher than value obtained using noradrenaline. Again the maximum had been constrained to 100 per cent. It is possible that this difference is due to antiadrenergic actions of cetiedil but the discrepancy is
Figure 3.3.2.1: The inhibition of the 20 nM somatostatin response by cetiedil ( ▼ ), clotrimazole ( ○ ), propafenone ( □ ) and quinine ( ◊ ) in intracellular voltage recordings.
small and experiments described in the next section show that cetiedil acts in a receptor-independent manner on this response. This question is discussed further in chapter 4.

Also 30 μM quinine is shown to produce a block of around 70 per cent. Quinine is known to be a blocker of a number of K⁺ channels such as the volume-activated K⁺ channel of guinea-pig hepatocytes, where it acts with an IC₅₀ value of 12 μM (Sandford et al., 1992). Because it is also an adrenoceptor antagonist (de Zoeten et al., 1983), it was not tested on the noradrenaline response.

3.3.3 The noncompetitive nature of block.

If a compound were acting as a reversible receptor antagonist rather than a blocker of the G protein-coupled channel one might expect its actions to be competitive; a channel blocker on the other hand should produce a level of block that is not reversed by increasing agonist concentrations. An early set of experiments was performed to study this aspect of the block produced by cetiedil. Figure 3.3.3.1 shows a control dose-response curve to noradrenaline and responses to three maximal concentrations of noradrenaline in the presence of 5 μM cetiedil. There is clearly no evidence for surmountability of the blocking action over the range of noradrenaline concentrations employed.

Further evidence indicating that these compounds were not acting as receptor antagonists is discussed in the next chapter.

3.3.4 The effects of blockers on resting conductance.

During experiments designed to study the effects of the above compounds on the agonist-induced response, it was noticed that when a cell was bathed in higher concentrations of cetiedil, clotrimazole or propafenone prior to the application of agonist, the resting membrane potential fell to a more depolarised level, and the membrane conductance also decreased. It was as if a resting conductance that contributed to the resting potential were being blocked. Akasu and Tokimasa (1989) reported a tonically-active calcium-activated K⁺ conductance in SMP neurones. It may be that the drugs were having some effect on this conductance, or possibly even on the
Figure 3.3.3.1: Dose–response curves for noradrenaline control (O) and in the presence of 5 μM cetiedil (▽) in SMP neurones. All responses are expressed as a percentage of the control response to 1 μM noradrenaline.
Figure 3.3.4.1: The blockade of the resting membrane conductance of guinea-pig SMP neurones by cetiedil (▼), clotrimazole (○) and propafenone (□). Data obtained from intracellular recordings.
resting calcium conductance responsible for its activation. Figure 3.3.4.1 shows the effects of the three drugs on the resting membrane conductance of submucous plexus neurones. Clotrimazole again produced the most pronounced effects but propafenone did comparatively little at the concentrations employed. Cetiedil produced a maximal thirty per cent reduction of resting conductance at concentrations of 20 μM and above.

3.3.5 Possible use-dependence of the block by clotrimazole.

Another observation made during the testing of the compounds was that clotrimazole not only blocked the response to noradrenaline, but seemed to alter the shape of the response as well. Figure 3.3.5.1 shows another voltage trace from a cell of 1.3 GΩ resting resistance, with control and partially blocked responses. In the presence of 5 μM clotrimazole, the agonist causes an initial rapid hyperpolarisation and conductance increase which then fall to steady levels. A possible explanation for this was that clotrimazole was blocking the G protein-coupled channels in a use-dependent way, so that the agonist was causing the channels to open normally, and only once they had done so was the clotrimazole able to act on them, accounting for the delayed onset of block. A necessary corollary would be that the kinetics of open channel block are slow enough to enable it to be seen in this type of experiment. The “shape” of the response recovered after washing out the clotrimazole. The distinctive shape of an agonist response in the presence of clotrimazole was not observed using cetiedil or propafenone (see figure 3.3.1). Whether this was because they were not use-dependent in their mode of block or that they were use-dependent and acting with fast kinetics, can not be determined from these results. Conductance measurements were made only after the response had settled to its steady level, and for this reason ninety second agonist applications were used to ensure that the clotrimazole had fully equilibrated with the open channels.

Contrastingly, evidence obtained from experiments on the pharmacology of the rabbit erythrocyte calcium-activated potassium conductance (I_{K(Ca)}) suggests that cetiedil is more likely to be a use-dependent blocker than clotrimazole (D. Benton, pers. comm.). In these experiments, I_{K(Ca)} is induced by treating the erythrocytes with calcium ionophore. If the cells have previously been bathed in cetiedil, the potassium loss will be smaller and the rate of K⁺ loss will rapidly decrease suggesting that open
Figure 3.3.5.1: Responses to 1 μM noradrenaline from an impaled SMP neurone. A control is followed by a response in the presence of 5 μM clotrimazole and a recovery response after washing out clotrimazole. The gradual increase in the extent of the inhibition by clotrimazole is possibly due to open channel block. See text for details.
channels are equilibrating with the cetiedil. This phenomenon is not noted with clotrimazole however, and treatment of erythrocytes prior to ionophore application elicits K⁺ loss that does not decline so rapidly implying that this drug is not an open-channel blocker of the erythrocyte \( K_{(Ca)} \) conductance.

As mentioned earlier, clotrimazole was one of the slowest compounds to reverse after removal from the bathing solution. If it were acting as an open channel blocker, then the possibility exists that it could become trapped in the channel after the agonist application. Once the cells were bathed in control saline solution again, several agonist applications may be required to 'release' the clotrimazole from the channels before the response recovered to control level. Normally, complete recovery from 10 \( \mu M \) clotrimazole required three agonist applications of 90 seconds’ duration every five minutes, covering at total of fifteen minutes after washing out the blocker. Two experiments were performed after exposure of a cell to 10 \( \mu M \) clotrimazole in which 90 second noradrenaline applications were made every 2.5 minutes. This revealed that the time taken for complete recovery was still 12.5 to 15 minutes. That recovery time was independent of the frequency of agonist application argued against the idea of trapping to explain the long wash-out time of clotrimazole.

3.4 Time course of the block produced by clotrimazole and cetiedil.

In experiments designed to test the actions of blockers on agonist-induced responses, the cell was bathed in physiological saline containing test compound for five minutes. In experiments by other workers studying the block of \( K_{(Ca)} \) in rabbit erythrocytes, it was found that the length of time for which certain blockers were exposed to the blood cells prior to measuring ionophore-induced K⁺ efflux was critical in determining the level of block of the \( K_{(Ca)} \) channel (D. Benton, pers. comm.).

Time course experiments were performed with 3 \( \mu M \) clotrimazole and 5 \( \mu M \) cetiedil, concentrations at which roughly a 50 per cent level of block would be expected. Using 1 \( \mu M \) noradrenaline as agonist, it was found that with 2, 5 and 10 minutes' exposure to either drug, significant changes in the level of block did not occur (figure 3.4.1). It seems from these experiments that cetiedil and clotrimazole equilibrate within the first two minutes of application so the five minute exposure time
Figure 3.4.1: The effect of exposure time on the blocking actions of cetiedil and clotrimazole on the G protein–coupled conductance of SMP cells. 1 µM noradrenaline was used as agonist.
routinely used was adequate for the measurement of effects of these compounds. However, these experiments do not rule out the possibility that the level of block may increase after ten minutes.

These results again contrast with the findings from the rabbit erythrocyte in which 30 μM cetiedil has a time constant of 5.6 minutes for the rate of onset of block of the $K_{(Ca)}$ conductance (D. Benton, pers. comm.). Time course data for clotrimazole on other $K^+$ channels are not available.

3.5 Other blockers tested on the noradrenaline response in submucous plexus neurones.

Several other compounds were tested for their ability to block the 1 μM noradrenaline response in the submucous plexus neurone, and their structures are given in appendix 1.

Amongst them were two analogues of cetiedil, UCL 1269 and UCL 1495. These compounds had originally been synthesised as potential blockers of the $K_{(Ca)}$ in erythrocytes. UCL 1495 was particularly active on the erythrocyte channel (IC$_{50}$ of 1.2μM, D. Benton, unpublished), whilst UCL 1269 was far less potent (IC$_{50}$ of 150 μM, D. Benton, unpublished). However, UCL 1269 was found to be active as a blocker of the volume-activated $K^+$ channel in guinea-pig hepatocytes acting with an IC$_{50}$ of 2 μM (K. Burke, pers. comm.). The selectivity of these compounds for other types of potassium channel made them interesting drugs to try on the G-protein-coupled channel.

Tolbutamide the oral hypoglycaemic is best known as a blocker of ATP-sensitive potassium channels in pancreatic β cells (Trube et al., 1986; Zunkler et al., 1988) and it has also been shown to be a blocker of G protein-coupled $K^+$ channels occurring in guinea-pig substantia nigra and rat corpus striatum neurones (Roepel et al., 1990; Lin et al., 1993). The drug was studied on hyperpolarisations and conductance increases elicited by agonists of dopamine D$_2$ and GABA$_B$ receptors. It is possible that these tolbutamide-sensitive channels, as well as being coupled to G proteins, are also sensitive to intracellular levels of ATP and ADP.
The compounds 1,3-di-o-tolylguanidine (DTG) and (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine (3PPP) were also studied after reports that they inhibited agonist-induced hyperpolarisations in guinea-pig submucous plexus neurones and rat locus coeruleus (Bobker et al., 1989). Using somatostatin, noradrenaline and UK 14304 as agonists in SMP neurones, DTG was reported to have an IC$_{50}$ of 32 nM although maximally it was only capable of blocking 53 per cent of the UK 14304-mediated hyperpolarisation. 3PPP produced a maximum inhibition of 32 per cent when tested against the UK 14304-mediated hyperpolarisation in the same tissue. The IC$_{50}$ of the 3PPP was 140 nM. Both drugs were also reported to inhibit somatostatin-induced hyperpolarisations.

Fluconazole, like clotrimazole, is an anti-fungal agent, although the structures of the two compounds are quite different. UCL 1559 is a quaternary derivative of clotrimazole.

As can be seen from figure 3.5.1, the above compounds had differing actions on the G protein-coupled conductance of SMP neurones. UCL 1495 and 1269, whilst they showed differing selectivities for other types of K$^+$ channel, both produced about a sixty per cent inhibition of the control noradrenaline-induced conductance increase at 10 μM. Tolbutamide (0.5 mM) was without effect suggesting that this channel does not have the pharmacological characteristics of the K$_{ATP}$ channel, and therefore differs from those centrally-occurring G protein-coupled channels which have been reported to be tolbutamide-sensitive.

DTG (10 μM) and 3PPP (30 μM) were used at concentrations that would be expected to be supramaximal from Bobker's work. In the present study 3PPP and DTG produced a 21 and 24 per cent inhibition of the control conductance increase respectively, slightly less active than reported by Bobker.

Fluconazole (10 μM) produced only a ten per cent inhibition of control whilst quaternisation of clotrimazole totally abolished its activity. Possibly the lipophilicity of the drug is important in determining its activity. UCL 1559 is also inactive as a blocker of the erythrocyte K$_{(Ca)}$ channel (D. Benton, unpublished) and it is likely that lipophilicity is an important factor with blockers of this channel.
Figure 3.5.1: Histogram showing the effects of various compounds on the noradrenaline-evoked conductance change in SMP neurones impaled with sharp microelectrodes.

Percentage inhibition of conductance increase induced by 1 μM noradrenaline

-20  0   20   40   60   80   100

(4)  3PPP 30μM
(5)  DTG 10μM
(4)  Tolbutamide 500μM
(8)  UCL 1269 10μM
(4)  UCL 1495 10μM
(4)  Fluconazole 10μM
(3)  UCL 1559 10μM

Figures in brackets are the number of observations.
3.6 Intracellular recordings from isolated rat atrial myocytes.

Intracellular recordings from atrial myocytes were usually performed on the day of preparation and the next day. Two days after culture they tended to become flat and elongated and successful impalements were more difficult to attain. The culture technique yielded large numbers of cells although they did not stick to the culture dish as readily as the neurones.

During the first day in culture the myocytes were spherical in shape and phase bright; after two days they became more elongated and flatter. These older cells typically showed spontaneous contractile activity which made stable intracellular recordings from them difficult to maintain. The cells that were chosen for impalement were usually phase bright and spherical. The mean diameter of ten cells measured shortly after cell culture was 18 ± 1 μm.

As with the SMP neurones, regular hyperpolarising current pulses were used to measure cell resistance after achieving a stable intracellular recording. From a sample of twenty-five cells a mean resting potential of -47.0 ± 3.1 mV (± standard error of the mean) was measured, and an input resistance of 1.85 ± 0.31 GΩ. Membrane time constants were measured as described for the neurones, giving a mean value of 47 ± 8 ms, from which the mean cell capacitance came to 26.3 ± 2.4 pF (n=25).

Atrial myocytes showed far more spontaneous excitatory activity during intracellular recordings although there was no accompanying contraction. Figure 3.7.1 shows a voltage recording from a cell with a resting input resistance of 1.6 GΩ and a resting potential of -45 mV; 20 pA hyperpolarising current pulses of 150 ms duration are being injected every 5.4 seconds. In this recording, excitatory potentials are regularly occurring although no depolarising current pulses are being passed as would be necessary to elicit an action potential from an SMP neurone. The figure shows two types of excitatory activity; rapid ‘break spikes’ of about 100 ms duration occurring after each hyperpolarising current pulse and much slower depolarisations lasting for 1.5 to 2 seconds, arising spontaneously about every 25 seconds. The two types of potential are shown inset on expanded time scales. About 20 per cent of myocytes exhibited the
Figure 3.7.1: A voltage recording showing a response to 1 μM adenosine in a rat atrial myocyte. The recording also shows spontaneous depolarisations which were typical of these cells. A rapid 'break spike' (A) and a slow potential (B) are shown inset on faster time bases; see text.
slow excitatory activity whilst nearly all of them showed the rapid 'break spikes'. Figure 3.7.1 also shows a response to 1 μM adenosine (section 3.7); both types of excitatory potential are abolished by the drug.

The frequency of the spontaneous potentials varied from cell to cell, possibly depending on whether they were myocytes of the sino-atrial node which would be expected to show spontaneous pacemaker activity. Generally the depolarisations seen in these cells decreased in frequency as the recordings progressed.

3.7 The actions of adenosine on the atrial myocyte.

The G protein-coupled conductance of atrial myocytes can be activated by adenosine acting at the A1 receptor (Evans et al., 1982; Hamilton et al., 1987; Paton and Kurahashi, 1981) as well as the muscarinic M2 receptor (Fukuda et al., 1988). The results presented in this and the following sections are based on data obtained from twenty-three intracellular myocyte recordings. Figure 3.7.1 shows a typical response to 1 μM adenosine, which comprises a conductance increase and hyperpolarisation similar to that observed with agonists acting on the G protein-coupled conductance of SMP neurones. Conductance increase was again used to measure the magnitude of response, and figure 3.7.2 shows the concentration-response relationship for adenosine in atrial myocytes. Again the data points have been fitted to a curve based on the equation of the form given in section 3.2 using an iterative curve fitting routine.

In figure 3.7.2 the response obtained from each cell has been expressed as a percentage of the 1 μM adenosine response. The curve fit yielded an IC₅₀ value of 155 nM for adenosine on this tissue, and the conductance increase obtained with 3 μM adenosine, the largest dose of agonist employed, was 0.8 ± 0.2 nS (n=4).

3.8 The use of adenosine as agonist.

Adenosine was chosen in preference to a muscarinic agonist for pharmacological studies of the K⁺ conductance because cetiedil has been reported to be quite a potent antimuscarinic agent acting with an IC₅₀ of 10 nM in gut bath.
Figure 3.7.2: The log concentration response curve for adenosine in one day old rat atrial myocytes. Responses from each recording are expressed as a percentage of the response to 1 μM adenosine.
experiments with guinea-pig ileum (D. Benton, pers. comm.). Again a near maximal
dose of adenosine was chosen to study the pharmacology of the conductance to avoid
possible run-down of the response, in this instance 1 µM. The agonist was bath-applied
for 25 to 30 seconds, and the cell then bathed in blocker for five minutes before
reapplication of agonist. After washing out the blocker, adenosine was applied every
five minutes until the inhibition of the response had fully reversed.

3.8.1 Inhibition of the response to 1 µM adenosine.

Cetiedil, clotrimazole, propafenone and UCL 1495 were all found to be active
inhibitors of the G protein-coupled conductance of atrial myocytes as well, and
concentration-inhibition curves for these drugs are shown in figure 3.8.1.1. The IC\textsubscript{50}
values and Hill slopes obtained from these fitted curves with maxima constrained to 100
per cent, are summarised in table 3.8.1.1.

Table 3.8.1.1: IC\textsubscript{50}s for four channel blockers on the G protein-coupled
conductance induced by 1 µM adenosine in one day old rat atrial myocytes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (µM)</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 1495</td>
<td>0.4 ± 0.1</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>0.9 ± 0.01</td>
<td>1.6 ± 0.02</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>1.8 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.3 ± 0.6</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

Although cetiedil, clotrimazole and propafenone are active in the same
concentration range as was found with the neurones, the order of potency has changed
from clotrimazole>propafenone>cetiedil to clotrimazole>cetiedil> propafenone.
Furthermore, UCL 1495, which at 10 µM produced 63 per cent inhibition of the 1 µM
noradrenaline response in SMP cells is actually a little more active than clotrimazole in
the myocytes. These contrasting observations are considered in more detail in the
discussion section of chapter 4.
Figure 3.8.1.1: Inhibition of the conductance increase induced in rat atrial myocytes by 1 µM adenosine by UCL 1495 ( ◊ ), cetiedil ( ▽ ), propafenone ( □ ) and clotrimazole ( ○ ).
As with the neurones, cetiedil and propafenone reversed rapidly, having washed out fully after five minutes at top concentrations. Clotrimazole and UCL 1495, however took ten to fifteen minutes to reverse fully when used at 2 and 1 μM respectively.

3.8.2 The effects of blockers on resting conductance.

As with the neurones, blockers of the agonist-induced conductance also had an effect on the resting conductance of atrial myocytes in the absence of agonist. There was a fall in resting conductance and an accompanying depolarisation as if a resting potassium conductance was being blocked. Evidence exists to suggest that the G protein-coupled conductance may contribute to the resting potential of the tissue (Okabe et al., 1991; Ito et al., 1991). This may account in part for the actions of blockers on the resting properties of the cells. The reduction of resting conductance produced by clotrimazole, cetiedil, propafenone and UCL 1495 in atrial myocytes is shown in figure 3.8.2.1. The order of drug potencies on the inhibition of resting conductance and and its correlation with the IC$_{50}$ values calculated for the inhibition of the adenosine-induced response, would indeed suggest that the same channel is involved in both resting conductance and adenosine response in these cells.

3.8.3 Possible use-dependence of block of G protein-coupled conductance.

As was the case with the SMP neurones, clotrimazole was found to alter the shape of the adenosine response in atrial myocytes. Again, the initial hyperpolarisation and conductance increase produced by adenosine sagged or decayed to a lower level in the presence of clotrimazole. This too suggested that the channels, once opened by agonist were being blocked by clotrimazole in a use-dependent manner accounting for the slow reduction in the magnitude of the response. Figure 3.8.3.1 is a voltage recording from a cell with resting resistance of 1.9 GΩ, showing a control response to 1 μM adenosine, a response obtained in the presence of 1 μM clotrimazole, and a recovered response obtained ten minutes after washing the drug out. In this example, the initial agonist-induced hyperpolarisation, falls by 25 mV before reaching its final value. The relaxation took about 10 seconds to decay to half of the final value. A similar sequence of responses is shown in figure 3.8.3.2 in which 10 μM propafenone has been
Figure 3.8.2.1: The reduction of the resting conductance of atrial myocytes by cetiedil ( ▼ ), clotrimazole ( ○ ), propafenone ( □ ) and UCL 1495 ( ◊ ).
Figure 3.8.3.1: A voltage recording showing three responses to 1 μM adenosine, a control (A), followed by a response obtained in the presence of 2 μM clotrimazole (B) and a response obtained five minutes after washing out clotrimazole (C).
Figure 3.3.2: A voltage recording from an atrial myocyte showing three responses 1 μM adenosine: a control (A) followed by a response obtained in the presence of 10 μM propafenone (B), and a recovery response (C) obtained five minutes after the propafenone was washed out.
used instead; it can be seen that propafenone does not alter the adenosine response in the same way as clotrimazole. Again, care was taken when using clotrimazole, to measure the membrane conductance after it had settled to its final level. Other blockers were also checked to see if they had the same effect on the response to agonist as clotrimazole: only UCL 1495, the most active compound on the atrial conductance showed any similar action. The effect was only observed with top concentrations of UCL 1495 (1μM) and it was not always repeatable. Also the effect, when it did occur, did not reverse completely, so control agonist applications made up to fifteen minutes after washing out UCL 1495 still showed a slight sag although the attenuation of response had largely reversed. It may be that the drug was remaining in the cell membrane to some extent, since it is a lipophilic molecule and likely to be soluble in the lipid phase of the membrane.

3.9 Other blockers tested on the adenosine response in atrial myocytes.

Single doses of DTG (10 μM), fluconazole (10 μM) and UCL 1269 (10 μM) were also tested on the myocytes (figure 3.9.1). As was the case with neurones, fluconazole was inactive. UCL 1269 also produced a negligible six per cent inhibition of control response to adenosine whilst it had produced 66 per cent inhibition of the noradrenaline response in the neurones at the same concentration. This makes it the only compound that was active in the neurones but inactive in the myocytes.

Intracellular recording experiments were not performed with atrial myocytes to ensure that active compounds were not acting through a receptor-blocking mechanism. The fact that some of the compounds had actions on the resting conductance of the myocyte does however suggest that they may be active as K⁺ channel blockers.

More persuasive evidence is described in the next chapter based on experiments designed to show that the compounds active on the myocyte response were working through a receptor-independent mechanism.

The data presented in this chapter are discussed along with results obtained from voltage-clamp experiments, at the end of chapter 4.
Figure 3.9.1: Percentage inhibition of control adenosine response by three compounds in one day old rat atrial myocytes.

Percentage inhibition of 1 μM response

![Diagram showing percentage inhibition of 1 μM response for UCL 1269 10 μM, DTG 10 μM, and Fluconazole 10 μM.](image_url)

- UCL 1269 10 μM
- DTG 10 μM
- Fluconazole 10 μM

Number of observations in brackets:
- (4) for UCL 1269 10 μM
- (4) for DTG 10 μM
- (3) for Fluconazole 10 μM
CHAPTER 4

THE USE OF THE VOLTAGE CLAMP TECHNIQUE TO STUDY THE BLOCK OF THE G PROTEIN-COUPLED CONDUCTANCE IN GUINEA-PIG SUBMUCOUS PLEXUS NEURONES AND RAT ATRIAL MYOCYTES; GENERAL DISCUSSION OF RESULTS.
4.1 Introduction.

One of the problems of voltage recording studies designed to look at channel pharmacology, is the rectification of the G protein-coupled $K^+$ channel. Thus the extent of the conductance increase produced by a given concentration of agonist will depend on the resting membrane potential and how close it is to the potassium equilibrium potential ($E_K$). If the resting potential changes during the course of a recording then so will the conductance increases produced by the same dose of agonist. Such considerations could distort measurements of the actions of blockers in intracellular recordings.

Furthermore, during agonist-induced hyperpolarisations other conductances may well be affected, such as the tonic $K_{Ca}$ conductance which is maintained by calcium entry via voltage-gated calcium channels. The conductance change measured in this case may not be directly attributable to the activation of G protein-coupled channels by agonist. Also, blockers tested in intracellular recording experiments may have actions on other voltage-gated conductances that are activated on hyperpolarisation, producing changes in the response that are not due to the block of G protein-coupled channels. Another criticism could be that the blocker may be voltage dependent, producing different levels of block at different membrane potentials.

Despite being a convenient and often very fruitful technique, the findings obtained using intracellular recordings had to be confirmed with voltage clamp experiments. So having established some basic data concerning the pharmacology of the G protein-coupled channels using intracellular recording techniques, it was decided to continue the work using patch clamp methods to enable more detailed study. Initially experiments were performed to confirm that the G protein-coupled conductances of the two tissues were inwardly rectifying, and then GTP-γ-S was used during whole cell recording experiments to activate the G protein-coupled conductances permanently, negating the need for agonists. This had the added advantage of demonstrating whether or not the blockers were acting as receptor antagonists.
Some preliminary switch clamp recordings were obtained from either cell type in control conditions, i.e. with control physiological saline solution (see section 2.2.1) and 100 μM GTP (rather than GTP-γ-S) in the pipette solution. A sample of six SMP recordings gave a mean input resistance of 1.12 ± 0.21 GΩ (mean ± s.e.m.) and a sample of four myocytes gave figures of 1.91 ± 0.38 GΩ. These results are summarised in table 4.1.1 along with cell resistance measurements obtained from intracellular current clamp experiments (chapter 3).

Table 4.1.1: A comparison of the resting resistances of SMP neurones and atrial myocytes obtained from both voltage clamp and current clamp recordings. Means and standard errors of the mean. Figures in brackets indicate the number of observations.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Voltage clamp</th>
<th>Current clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP neurones</td>
<td>1.12 ± 0.21 GΩ (6)</td>
<td>1.33 ± 0.18 GΩ (40)</td>
</tr>
<tr>
<td>Atrial myocytes</td>
<td>1.91 ± 0.38 GΩ (4)</td>
<td>1.85 ± 0.31 GΩ (25)</td>
</tr>
</tbody>
</table>

The figures obtained from either recording mode are in quite close agreement with one another. One may have expected the values obtained from intracellular impalements to be smaller since the resistance of the membrane/glass seal is generally not as great as it is with patch pipettes in whole cell configuration. Tatsumi et al. (1990) reported a mean resting resistance of 590 MΩ for a sample of eight voltage-clamped SMP neurones in whole cell configuration, whilst intracellular voltage recordings performed by Mihara et al. (1987a) gave values of 60 to 250 MΩ. Clearly both values are considerably less than those obtained in the present study. The microelectrodes and patch pipettes used in this work did not vary significantly in terms of their resistances from those used in other studies of SMP neurones. Thus the reason for the greater recording resistances in this study is unclear.
4.2 Inward rectification of the G protein-coupled currents.

The first voltage-clamp experiments were performed to confirm reported current-voltage relationships (c.v.r.) in the G protein-coupled conductances. Surprenant and North (1988) and Tatsumi et al. (1990) reported that the α2-coupled K⁺ conductance of guinea-pig submucous plexus neurones was inwardly rectifying, a property most easily seen with voltage clamp. Initially cells were switch-clamped using patch pipettes of 5 to 10 MΩ resistance, and switching rates of 3 to 5 kHz. The pipette filling solution contained 100 nM free calcium buffered with EGTA and 10 μM GTP as described in chapter 2. Figure 4.2.1 shows a switch-clamped SMP neurone held at -60 mV (very close to the resting membrane potential) and stepped to various test potentials for 150 ms. This was performed first in normal bathing solution, and again one minute after the application of 1 μM noradrenaline. The two current-voltage relations are shown in part A; current measurements were made 120 ms after the beginning of the voltage step. Subtracting the control isopotential current values from those obtained in the presence of agonist, gives the current voltage relation for the agonist-induced conductance alone; this has a reversal potential of -90.0 mV. This is close to the theoretical value for the potassium equilibrium potential (E_K) calculated from the K⁺ concentrations in the bathing and pipette solutions (-94.7 mV), suggesting that the outward agonist-induced current is carried predominantly by potassium ions. Larger currents are recorded at potentials negative to E_K when the potassium current is inward confirming the findings of Tatsumi and others with this tissue. The neurones showed very little inward rectification in the absence of agonist even at -130 mV. This is again in keeping with Tatsumi, although Surprenant and North reported inward rectification occurring markedly in control conditions at potentials of -110 mV and below.

Figure 4.2.2 shows the current traces obtained from an SMP neurone during a c.v.r. performed one minute after the application of 1 μM noradrenaline. The cell was held at -55 mV (the resting potential before agonist application) at which potential there is a standing outward current due to agonist application. Trace A shows that the inward current occurring during a 150 ms voltage step to -140 mV is time dependent.
Figure 4.2.1: A. Current voltage relations in an SMP neurone, control (O) and in the presence of 1 μM noradrenaline (●).

B. A plot of the control current subtracted from current measured in the presence of agonist to show the current-voltage relation of the agonist-induced conductance.
Figure 4.2.2: Whole cell currents obtained from an SMP neurone held at -55 mV and stepped to various test potentials in the presence of 1 μM noradrenaline. In addition to agonist-induced inward rectification (A), there are also A-type currents occurring after the hyperpolarising steps (B) and delayed rectification occurring during depolarisation (C).
This is a phenomenon frequently reported with inwardly rectifying conductances, and Tatsumi et al. (1990) have observed it with agonist-induced $K^+$ currents in guinea-pig SMP cells. A-type currents occur after hyperpolarisation (B), and a sufficiently large depolarising step elicits a delayed outward current (C). The voltage-dependent currents of SMP neurones are discussed in the next chapter.

At a holding potential of -60 mV, 1 μM noradrenline evoked an outward current of $232 \pm 62 \text{ pA (n=10)}$, similar to values obtained by Tatsumi et al. who reported currents in the range of 200 to 250 pA in response to maximal doses of noradrenaline. Other agonists linked to the G protein-coupled $K^+$ current were not applied to voltage-clamped neurones in the present study.

The G protein-coupled conductance of atrial myocytes can be activated by adenosine and acetylcholine, and has also been reported to be inwardly rectifying (Kurachi et al., 1986; Pfaffinger et al., 1985). Figure 4.2.3 illustrates a c.v.r. performed in a rat atrial myocyte one minute after the application of 1 μM adenosine, showing a reversal potential of -86.5 mV, again fairly near $E_K$. The protocol is the same as that used with the neurones. Inward rectification is also apparent, as can be seen from the subtracted curve (B).

Typical current traces obtained from a myocyte during a c.v.r. are shown in figure 4.2.4. Although there is inward rectification, time-dependent development was not seen in the present study though it was reported to occur with this current (Kurachi et al., 1986a; Sakmann et al., 1983). The outward currents obtained on depolarisation reach maxima at 30 ms after the voltage step, and then decay over the rest of the duration of the step. It resembles the transient outward current reported by Dukes and Morad (1990) in rat ventricular myocytes, having similar onset and inactivation time courses. The outward conductance is probably a mixture of transient outward and delayed rectifier currents.

In the myocytes 1 μM adenosine produced an outward current of $156 \pm 64 \text{ pA (n=10)}$ at a holding potential of -50 mV. One source of concern with the myocyte preparations was that in older cultures the cells may have become electrically coupled so there was a danger that agonist-induced currents may have been recorded from more than one cell. Care was taken during patch clamp experiments with atrial myocytes to use
Figure 4.2.3: A. Current–voltage relations in an atrial myocyte, control (O) and in the presence of 1 μM noradrenaline (●).

B. A plot of the control current subtracted from current measured in the presence of agonist to show the current–voltage relation of the agonist–induced conductance.
Figure 4.2.4: Current traces obtained during a voltage step protocol (inset) performed on a rat atrial myocyte in the presence of 1 μM adenosine. The cell was held at -50 mV: See text for details.
spherical phase bright single cells from fresh cultures before gap junctions had time to develop between the myocytes.

4.3 Activation of the outward current with GTP-γ-S.

The non-hydrolysable GTP analogue GTP-γ-S has been used to activate permanently K⁺ conductances both in guinea-pig SMP neurones (Tatsumi et al., 1990) and rat atrial myocytes (Kurachi et al., 1986). The analogue presumably binds irreversibly to the active G protein subunit and the α subunit and the in this work 50 μM GTP-γ-S was used in the pipette filling solution in place of the GTP and used to study both neurones and myocytes. Initially whole cell recordings began in bridge balance mode and agonist applied until a maintained hyperpolarisation and conductance increase were observed which persisted for at least thirty seconds after the removal of agonist. Then, in discontinuous voltage clamp mode, blockers were applied. The cells were held at -50 mV and stepped to -80 mV for 250 ms every 5.4 seconds. Frequently the neuronal G protein-coupled conductance activated spontaneously almost as soon as whole cell configuration had been achieved. Application of 1 μM noradrenaline did not increase the level of hyperpolarisation or membrane conductance.

4.3.1 The SMP neurone.

A recording from an activated SMP neurone is shown in figure 4.3.1.1 in which the outward current and membrane conductance are both permanently increased by an initial dose of 1 μM noradrenaline. Cetiedil (5, 10 and 20 μM), subsequently applied, produces a reversible dose-dependent reduction in outward current and conductance. Similar experiments were performed using other drugs that had been active on the noradrenaline-evoked response; these results are summarised in figure 4.3.1.2.

A dose-response curve was constructed for cetiedil and the IC₅₀ calculated at 10.2 ± 0.3 μM corresponding well with the value of 9.0 ± 0.5 μM obtained for the inhibition of the somatostatin-induced conductance increase (section 3.3.2). The value obtained with
Figure 4.3.1.1: Actions of cetiedil on a guinea-pig SMP neurone. The cell is being stepped from −50 to −80 mV every 5.4 seconds and the pipette filling solution contains 50 μM GTP-γ-S to activate the G protein-coupled conductance. The activation was initiated with a dose of 1 μM noradrenaline.
noradrenaline, 5.9 ± 0.4 μM, in which cetiedil is slightly more potent, may reflect some slight α2 receptor block produced by cetiedil.

Propafenone (3.5 μM), clotrimazole (2 μM), UCL 1269 (10 μM) and UCL 1495 (10 μM) are all shown producing appropriate levels of block with respect to intracellular recording experiments performed using noradrenaline, about fifty per cent inhibition of control. Figure 4.3.1.2 however, does not show inhibition produced by tetraethyl ammonium (TEA). At 20 mM it had negligible effect on the GTP-γ-S-stimulated conductance. This is in keeping with the work of Shen et al., (1992) in which three single G protein-coupled channels of guinea-pig SMP neurones were reported in outside out patch experiments. 10 mM TEA applied to the extracellular face of the excised patches was found to have no effect on any of the three channels described.

The value of these findings is two-fold. First, more evidence has been obtained to rule out receptor blockade by the compounds, and also these results agree with those using voltage recordings. Also the possibility that rectification may have distorted the results described in chapter 3 can be ruled out. This suggests that the potential difficulties which arise in non-voltage-clamped experiments had not produced misleading results.

4.3.2 The atrial myocyte.

As with the neurones, cetiedil, clotrimazole, propafenone and UCL 1495 were all tested on the GTP-γ-S-induced response at concentrations close to their IC50s determined using adenosine during the voltage recording experiments (figure 4.3.2.1). Once again they produced expected levels of block and there was no reason to assume that any receptor blockade or misleading rectification had occurred during the intracellular recordings using adenosine as agonist.
Figure 4.3.1.2: The inhibition of $K^+$ conductance increase induced by 50 $\mu$M intracellular GTP–$\gamma$–S, by cetiedil (▼), clotrimazole (●), propafenone (□), UCL 1495 (♦) and UCL 1269 (◇) in SMP neurones. The number of cells tested is shown in brackets.
Figure 4.3.2.1: The effects of blockers on the G-protein-coupled conductance increase of rat atrial myocytes activated with 50 μM GTP-γ-S in the patch pipette. Figures in brackets represent the number of observations.
4.4 The effects of blockers on the current voltage-relation of SMP neurones.

Current-voltage relations were also performed in the presence of cetiedil and clotrimazole on SMP neurones. During experiments in which the K⁺ conductance was permanently activated by inclusion of GTP-γ-S, voltage step protocols were performed in control conditions and then in the presence of the blockers. Some results for 2 μM clotrimazole are shown in figure 4.4.1. The results indicate that inward rectification was also attenuated by clotrimazole and that inward K⁺ currents carried by G protein-coupled channels were also subject to block by these compounds. Similar results were also obtained using cetiedil. Analysis of c.v.r.s performed in the presence of blockers revealed that the percentage inhibition of inward current produced by cetiedil and clotrimazole was indistinguishable from the inhibition of outward current at IC₅₀ concentrations. Thus the rectification properties of the G protein-coupled conductance were not altered, in that both inward and outward currents are sensitive. This is in contrast to the way that quaternary ammonium compounds change the c.v.r. of delayed rectifiers in squid giant axon blocking outward K⁺ currents whilst leaving the inward conductance comparatively unaffected (Armstrong and Binstock, 1965). The apparent insensitivity of block to membrane potential was studied in more detail in section 4.6 and the implications covered in the final section.

Figure 4.4.1 also shows that outward rectification in SMP neurones is sensitive to these compounds, a property that is studied in greater detail in chapter five.

4.5 UCL 1559 applied intracellularly in SMP neurones.

Intracellular recording experiments had shown the quaternised analogue of clotrimazole, UCL 1559, to be inactive when tested on the noradrenaline response of SMP neurones. One explanation for this is that clotrimazole acts on the K⁺ channel from the cytoplasmic side of the membrane and quaternisation removes the lipophilicity of the compound preventing it from crossing the membrane. This is analogous to some of the examples of channel block mentioned in chapter one; QX-314, a quaternary local
anaesthetic derivative is inactive when applied extracellularly, but blocks Na⁺ channels
when injected into the axon; TEA is a far more effective blocker of delayed rectifier
channels when applied intracellularly.

A series of voltage-clamp experiments was performed using a control pipette filling
solution and a filling solution containing 50 μM UCL 1559. Whole-cells responses were
obtained to noradrenaline using the two solutions alternately. Responses were obtained
every five minutes, and only recordings that lasted for a minimum of twenty minutes were
used for data analysis. Low resistance patch pipettes of 2 to 3 MΩ were used to try and
ensure that the filling solution could adequately dialyse into the cytoplasm. With a holding
potential of -60 mV, a mean agonist-induced outward current of 217 ± 46 pA was
obtained from nine cells using the control filling solution, and a mean response of 264 ± 78
pA obtained from nine cells dialysed with 50 μM 1559.

Thus UCL 1559 is as inactive when applied intracellularly as extracellularly; this
suggests that quaternisation disrupts the ability of the molecule to interact with the channel
macromolecule. The question of which side of the membrane clotrimazole acts is not
answered.

4.6 The voltage-dependence of block of the neuronal conductance.

A series of experiments were performed to test the voltage-dependence of the
block of the G protein-coupled conductance in SMP neurones. If any such voltage-
dependence could be observed it may give insights into the mode of action of the blockers.
A blocker that is sensitive to membrane potential might well be in its charged form during
interaction with the channel. If one assumes that a compound containing a tertiary amino
group like cetiedil would become positively charged if it were ionised, then the nature of
the voltage-dependence may give some clue as to the side of the membrane on which the
drug was acting. For instance, the block produced by a positively charged compound
acting extracellularly may be expected to be enhanced by a hyperpolarising voltage step as
is the case with tubocurarine and the nicotinic receptors of the frog neuromuscular
junction (Colquhoun et al., 1979).
As Colquhoun pointed out, the enhanced block of open channels by tubocurarine during a voltage step, indicated that tubocurarine was acting at least partially, through an open channel block mechanism. Thus observations on the mechanism of action of blockers in SMP neurones may also be made using similar protocols as those employed by Colquhoun and colleagues.

To this end, switch clamp recordings with SMP neurones were made using 50 μM GTP-γ-S in the pipette solution to activate the G protein-coupled conductance permanently. The cells were held at -55 mV, close to the zero current potential and stepped to -130 mV for 6 seconds. The current records were later studied to see if any relaxations occurred after the voltage jump which would be indicative of voltage-dependent changes in the level of block.

Figure 4.6.1 shows a typical result; the standing currents have been offset to zero to enable clearer comparison of the three traces. The first step (A) is a control performed after the activation of the G protein-coupled conductance; an instantaneous inward current of -0.25 nA occurs, equivalent to a conductance of 3.55 nS. This rather large conductance value reflects the inward rectification of the G protein-coupled current. Five minutes after the bath application of 5 μM cetiedil, the protocol was repeated (B). This time the inward current only reached -0.12 nA equivalent to a 61 per cent inhibition of the membrane conductance which is consistent with this concentration of cetiedil. There were however, no relaxations in the current level during the six seconds of the voltage jump. The current traces from these and other experiments were examined on an expanded time scale and again no relaxations were found to occur immediately after the step. Trace C shows the recovery of control conductance five minutes after removing cetiedil from the bathing solution. The conductance has returned to the control level.

Altogether, this type of experiment was performed five times for 5 μM cetiedil, 2 μM clotrimazole and 3.5 μM propafenone, concentrations at which a fifty per cent inhibition of the GTP-γ-S-sensitive current would be expected. No evidence for voltage-dependence of block could be found with any of these compounds. It may have been that six seconds was not adequate to reveal current relaxations or that they occurred too rapidly to be observed depending on the time scale of blocking kinetics. If however the
actions of the blockers was genuinely not sensitive to membrane potential, it may be that they are not in a charged form when they interact with the channel protein. UCL 1559, the quaternary derivative of clotrimazole, was ineffective in experiments using agonists; this is consistent with the idea that only the uncharged form of clotrimazole is active on the channel. The same may be true of cetiedil and propafenone as well.
Figure 4.4.1: Two current−voltage relations performed on an SMP neurone activated with 50 μM GTP−γ−S. The cell was held at −55 mV and stepped to a variety of test potentials.

○ a control c.v.r.  ● a c.v.r. performed one minute after the bath-application of 2 μM clotrimazole.
Figure 4.6.1: Results obtained from voltage jump experiments (stepping from –55 to –130 mV) performed on SMP neurones. A, control, B, in the presence of 5 μM cetiedil and C, recovery after washing out cetiedil.
4.7 Discussion.

4.7.1 Characteristics of the block of the G protein-coupled conductance in atrial myocytes and SMP neurones.

Experiments outlined in this and the previous chapter point to the action of blockers being on the potassium channel rather than on the receptors linked to the G protein-coupled conductance. Briefly, compounds found to be active on the noradrenaline response in SMP cells were also active at the same concentrations on the somatostatin response, considerably weakening the likelihood of receptor blockade as a mechanism of inhibition of the response. This was so for clotrimazole, cetiedil and propafenone. It was also found that the block of the noradrenaline response by 5 μM cetiedil in the neurones was noncompetitive, such that increasing doses of agonist produced no reduction in the level of block, again arguing against reversible receptor antagonism. Finally, compounds found to have activity on responses recorded in SMP neurones and atrial myocytes were later tested on preactivated conductances in whole cell voltage clamp experiments. The use of GTP-γ-S enabled the receptor to be bypassed altogether in activating the G protein-coupled conductances. This technique enabled cetiedil, propafenone, clotrimazole, UCL 1269 and UCL 1495 to be shown to act in a receptor-independent manner in SMP neurones, and cetiedil, propafenone, clotrimazole and UCL 1495 in atrial myocytes. TEA was also tested in this way on the neurones. At 20 mM it was inactive, a result in keeping with the earlier report by Shen et al., 1992.

However, it should be pointed out that there is some evidence suggesting that cetiedil was more active on the noradrenaline-induced response (having an IC₅₀ of 5.9 ± 0.4 μM) than on the responses induced by somatostatin or GTP-γ-S (in which the IC₅₀ values were 9.0 ± 0.5 and 10.2 ± 0.3 μM respectively). This small discrepancy may be due to some weak α-adrenoceptor blocking activity of cetiedil.

One of the findings of the intracellular experiments was the slow onset of the block of the neuronal and atrial responses by clotrimazole, which was interpreted as possibly reflecting a use-dependent mechanism of block. Assuming use-dependent block, the kinetics of interaction of drug with open channels were slow enough to enable the
phenomenon to be observed during these experiments. Of the seven compounds that had some degree of activity on the conductances of the two tissues, clotrimazole was the only one to exhibit this distinctive action. This of course does not give any indication of whether the other compounds were acting as use-dependent blockers or not. If they were however, the rapidity of onset of block after agonist application would have been too great to observe the onset.

Clotrimazole was also one of the slowest drugs to reverse after removal from the bathing solution; 10 μM clotrimazole required some 15 minutes before agonist applications elicited fully-recovered responses. A cursory study described in chapter three (section 3.3.5) yielded results suggesting that clotrimazole was unlikely to become trapped in the channels on removal of noradrenaline. The time taken for the full recovery of the noradrenaline response after the removal of 10 μM clotrimazole from the bathing solution was not affected by increasing the frequency of agonist application as may be expected for a trapped channel-blocking compound. It is possible that clotrimazole, one of the more lipophilic of the compounds studied, was able to dissolve in the membrane lipid to some extent, rather like certain hydrophobic local anaesthetics which can move from sodium channel protein to lipid bilayer and vice versa. The lipophilicity of clotrimazole may account for its longer reversal time.

Another finding was that the compounds active on the agonist-induced conductance in both tissues also reduced resting membrane potential and decreased membrane conductance, as if blocking a tonic potassium conductance underlying resting membrane potential. In the case of the myocytes, it is known that there is a small basal level of G protein-coupled K⁺ channel activity, and this may well contribute to resting potential (Okabe et al., 1991; Ito et al., 1991). In keeping with this, the drugs which had proved to be the most active on the agonist-induced response seemed to produce the most pronounced effects on resting conductance, with UCL 1495 being most active, cetisedil and clotrimazole less so, followed by propafenone which had very little effect.

Shen et al., (1992) also showed that G protein-coupled channels in SMP neurones have a small open probability in the absence of agonist. However, Akasu and Tokimasa (1989) described a basal calcium-activated K⁺ conductance that contributed to membrane
potential. The effects of the blockers on the neuronal resting conductance would probably involve an action on $K_{Ca}$ as well, so compounds active on the agonist-induced conductance may not necessarily be active on the resting potential. In fact, clotrimazole was the most effective drug on $G_{K\text{(resting)}}$ followed by cetiedil; propafenone which had been more potent a blocker of the $G$ protein-coupled conductance than cetiedil, had negligible effect.

Voltage-clamp experiments designed to study possible voltage-dependence of the block of the neuronal conductance by cetiedil, clotrimazole and propafenone did not reveal any current relaxations during six second hyperpolarising voltage steps. One possibility is that the kinetics of voltage-dependence were too rapid or indeed too slow to enable current relaxations to be observed during these experiments. If however the block produced by these compounds was genuinely insensitive to membrane potential, it may be that the drugs were in a non-ionised form when interacting with the $K^+$ channel.

UCL 1559, the quaternised derivative of clotrimazole, was found to be inactive when applied extracellularly during voltage recordings, and when applied intracellularly by inclusion in the patch pipette filling solution during whole cell experiments. This finding is in keeping with the idea that $K^+$ channel block was brought about by drugs acting in their uncharged forms. If this is so, then it contrasts with the block of sodium channels by local anaesthetics and delayed rectifiers by quaternary ammonium compounds.

4.7.2 The comparative pharmacology of $G$ protein-coupled $K^+$ channels in guinea-pig SMP neurones and rat atrial myocytes; results obtained from intracellular recordings.

The preliminary work in this study has shown that the $G$ protein-coupled conductances conform in all important respects with those described in previous reports. Agonists produced membrane hyperpolarisation with an associated increase in membrane conductance. In patch clamp experiments outward currents induced by agonists in both neurones and myocytes have been shown to be inwardly rectifying and have reversal potentials close to the expected potassium equilibrium potential. The currents could also be activated by GTP-$\gamma$-S.
However, results obtained in chapter 3 showed some differences between the pharmacological profiles of the two tissues studied. The IC\textsubscript{50} values of the compounds that were found to be active on both tissues are summarised in Table 4.7.2.1. Propafenone is equally active on both tissues; clotrimazole and cetiedil are slightly more active on the myocytes than neurones; UCL 1495 is more than ten-fold more active on the myocytes than the neurones. The order of blocker potencies with the neurones is clotrimazole>propafenone>cetiedil=UCL 1495; with the atrial myocytes this becomes UCL 1495>clotrimazole>cetiedil>propafenone. Generally speaking the IC\textsubscript{50} values of these compounds do not differ dramatically from one another; however, they are all active in the low micromolar range, making them quite potent when compared for instance with the block of delayed rectifiers by TEA or the block of peripheral K\textsubscript{ATP} conductances by tolbutamide.

Of the other compounds tested, UCL 1269 (hydroxycetiedil) produced 60 per cent inhibition of the noradrenaline-induced neuronal conductance at 10 \textmu M but only 7 per cent block of the adenosine-induced conductance of the myocytes at the same concentration making it the only compound that was markedly more effective on the neurones than on the myocytes. 500 \textmu M tolbutamide produced a 5 per cent block of the noradrenaline response in SMP neurones suggesting that none of the channels underlying this conductance are of the K\textsubscript{ATP} class of potassium channel. As mentioned in chapter one, certain G protein-coupled K\textsuperscript{+} channels of central mammalian neurones have been shown to be very sensitive to tolbutamide; this does not seem to be the case with the SMP neurone.

Although clotrimazole was one of the most active compounds to be tested on either tissue, UCL 1559, its quaternary derivative was inactive on the neurones at 10 \textmu M. Furthermore, fluconazole, another antifungal agent, produced only 9 per cent block of the noradrenaline-induced conductance, and was totally without effect on the myocyte conductance. Quinine, a relatively non-selective blocker of most K\textsuperscript{+} channels, produced a 68 per cent inhibition of the noradrenaline response in SMP neurones at 30 \textmu M.

DTG and 3PPP produced similar levels of block of the neuronal conductance although DTG was slightly more active when tested on the myocytes.
These results differ somewhat from the findings of Bobker et al., 1989. In his work DTG at concentrations of 3 μM and above produced a maximal 50 per cent inhibition of the hyperpolarisation induced in SMP neurones by UK 14304. In the present study 10 μM DTG produced a 24.2 ± 3.2 inhibition of the noradrenaline-induced response; the compound was thus found to be slightly less active. When tested on the atrial myocytes however, 10 μM DTG produced a 66.7 ± 16.1 per cent inhibition of the adenosine-induced conductance increase, more active than it had been on the neurones.

Bobker also found 3PPP to produce a 30 per cent maximal inhibition at 10 μM and above in SMP cells. In the present study 30 μM 3PPP produced 21.6 ± 9.7 per cent block; again rather less active than that reported by Bobker.

The results summarised in table 4.7.2.1 suggest that there may be some differences between the two tissues on pharmacological grounds. In keeping with this, Shen et al., (1992) have proposed that there are four potassium channels in SMP neurones that are gated by pertussis-sensitive G protein, one of them with a large unitary conductance in the same range as the BK channel; however, Sakmann et al., (1983) report only one channel coupled to G protein in rat atria.

Table 4.7.2.1: Comparison of the pharmacologies of the G protein-coupled conductances of rat atrial myocytes and guinea-pig SMP neurones. Data from experiments in which agonists were used to activate the conductance.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ when tested on guinea-pig submucous plexus neurones (μM).</th>
<th>IC₅₀ when tested on rat atrial myocytes (μM).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>1.7 ± 0.1</td>
<td>0.9 ± 0.01</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>5.9 ± 0.4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.5 ± 0.1</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>&lt; 10</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>UCL 1269</td>
<td>&lt; 10</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>DTG</td>
<td>&gt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>
In the light of voltage clamp experiments using GTP-γ-S described in this chapter, the above values obtained using agonists, may be taken as representing channel block and distortions due to membrane rectification can be largely ruled out. The single channel studies of Shen and Sakmann indicate that different K⁺ channels may underlie the G protein-coupled conductances in the two tissues; this is reflected in their differing pharmacologies. A comparison with the pharmacologies of other K⁺ channels is outlined in section 4.7.3.

Table 4.7.2.2 shows calculated Hill slopes from experiments described in chapter 3; the values are all greater than unity. These high Hill slopes indicate that the channel and blocker may not interact on a simple one to one basis; there may be multiple drug binding sites on each channel. The block of KCa by cetiedil and its congeners in rabbit erythrocytes also produces dose-inhibition curves with Hill slopes considerably greater than unity (D. Benton, pers. comm.); this too may be due to complex interactions between the blockers and the channels on which they act, again with the possibility of positive cooperativity.

Table 4.7.2.2 Comparison of the Hill slopes of the K⁺ channel blockers in rat atrial myocytes and guinea-pig SMP neurones.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hill slope tested in SMP neurones</th>
<th>Hill slope tested in atrial myocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetiedil</td>
<td>1.54 ± 0.17</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>1.60 ± 0.13</td>
<td>1.60 ± 0.02</td>
</tr>
<tr>
<td>Propafenone</td>
<td>1.36 ± 0.04</td>
<td>1.48 ± 0.36</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>-</td>
<td>1.97 ± 0.69</td>
</tr>
</tbody>
</table>

(Krapivinsky et al., 1995)

A recent study outlined in chapter one has shown that the G protein-coupled channel of atrial myocytes may be a heteromultimer, with protein subunits encoded by different genes, GIRK1 and CIR. The precise stoichiometry remains unclear, and the value of pharmacology as a tool for comparing the properties of expressed and native K⁺

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channels may now start to be realised. As further attempts are made to express G protein-coupled channels in various systems, such compounds as clotrimazole and UCL 1495 may be of use in determining the properties of expressed channels in an effort to deduce the precise composition of the atrial channel where other criteria, such as inward rectification or the level of agonist-independent channel activity are no longer useful.

4.7.3 The actions of blockers of the G protein-coupled conductance on other K\(^+\) channels.

Many of the above compounds have been shown to be quite non-specific blockers of several other potassium channels. Table 4.10.2 shows data obtained from studies performed on the calcium-activated K\(^+\) conductance of rabbit erythrocytes (K\(_{(Ca)}\), r.b.c.), the volume-activated conductance of guinea-pig hepatocytes (K\(_{vol}\)) and the cromakalim-sensitive ATP-gated channels of the vascular smooth muscle of rat aorta (K\(_{KCo}\)).

Table 4.7.3.1. IC\(_{50}\) (\(\mu\)M) for K\(^+\) channel blockers acting on other channels; see text. Data from D. Benton (pers. comm.).

<table>
<thead>
<tr>
<th>Drug</th>
<th>K(_{(Ca)}), R.B.C.</th>
<th>K(_{vol})</th>
<th>K(_{KCo})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetiedil</td>
<td>26</td>
<td>2.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UCL 1269</td>
<td>150</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>1.2</td>
<td>-</td>
<td>&gt;&gt;10</td>
</tr>
</tbody>
</table>

In the tissues studied, cetiedil is least active as a blocker of K\(_{(Ca)}\) but around ten times more potent on the volume-activated and ATP-sensitive channels. Its level of activity on the G protein-coupled channels falls in between. Clotrimazole and UCL 1495 were two of the most potent compounds studied on K\(_{(Ca)}\) and likewise with the two G protein-coupled conductances. UCL 1269 however has a different selectivity; it was
weakly active on both $K_{Ca}$ and the myocyte conductance (10 μM having negligible effect on the latter) but far more active on the volume-activated hepatocyte channel (an IC$_{50}$ of 2 μM) and SMP conductance (having an IC$_{50}$ of less than 10 μM). UCL 1495 active on $K_{Ca}$ and G protein-coupled channels in the low micromolar range was however far less active on the ATP-sensitive channel. Fluconazole and UCL 1559, as well as being inactive on the G protein-coupled conductances, had no effect on $K_{Ca}$ of erythrocytes. It is also of note that results obtained in erythrocyte experiments indicated that cetiedil was acting in a use-dependent manner whilst clotrimazole was not, in contrast with results obtained in experiments on the G protein-coupled conductances. It seems that the mechanism of action of these compounds also varies with the different channels on which they have been tested.

In summary, it can be seen that compounds are emerging with selectivities for various $K^+$ channels or at least groups of channels, suggesting that the task of finding selective $K^+$ channel blockers is now under way and these compounds could assist with the classification and identification of the diverse and ubiquitous class of $K^+$ channels.
CHAPTER 5

THE ACTIONS OF CHANNEL-BLOCKING COMPOUNDS ON OTHER CURRENTS IN GUINEA-PIG SMP NEURONES.
5.1 Introduction.

The membranes of most excitable cells contain a variety of different potassium channels. Frequently, these channels are classified according to whether they are gated by changes in membrane potential or by changes in intracellular calcium levels. Since calcium channels are themselves sensitive to changes in membrane potential, voltage-gated and calcium-activated K⁺ conductances often occur simultaneously on depolarisation of the cell membrane.

The physiological roles of these conductances differ as well. The voltage-gated delayed rectifier for instance, which tends to open at depolarised potentials, is normally associated with the repolarisation phase of an action potential. However, the voltage-gated A current and calcium-activated SK current are believed to be responsible for regulation of excitability and the rate of firing of a neurone. This chapter deals with the whole cell conductances in SMP neurones which were observed on depolarisation, whether voltage-gated or activated by the increase in intracellular calcium that occurs on membrane depolarisation. Voltage-gated and calcium-activated potassium conductances of excitable tissues are discussed in section 1.3 of chapter one.

5.2 Calcium-activated currents in SMP neurones.

The outward currents induced by depolarisation in SMP neurones were studied by switch-clamping the cells in whole cell configuration with high resistance patch electrodes (8 to 10 MΩ) that had not been treated with sylgard. Standard pipette filling solutions were employed (see chapter 2). In nearly all recordings, switching rates of 5 kHz or more were achieved and the amplifier output was filtered at 0.3 kHz.

The cells were held at -60 mV and stepped to -20 mV every 5.4 seconds for 150 ms. In order to observe the delayed rectifier current uncontaminated by calcium-sensitive potassium currents, the standard bathing solution containing 2 mM calcium was replaced with a calcium-free saline of the following composition (mM): Sodium chloride (137), potassium chloride (4), magnesium chloride (5), HEPES (10), glucose (10), EGTA (0.5).
and titrated to pH 7.4 with 1 M sodium hydroxide solution. The magnesium concentration was increased to avoid the loss of membrane stability that might otherwise have resulted with the removal of calcium.

Figure 5.2.1 shows two typical current traces obtained from the same cell during a voltage step from -60 to -20 mV for 150 ms in which the baseline currents of either trace have been offset to zero although leak currents and capacity transients have not been subtracted. The first step is performed in the presence of 2 mM calcium (A) and the second in calcium-free solution (B). Both traces show an initial transient inward current (later found to be tetrodotoxin-sensitive, see section 5.9) followed by prolonged outward current. In the presence of calcium, the outward current first rises to a small peak, then falls to a steadier level, possibly some A current. The small peak does not occur in the calcium-free trace, and may possibly be due to the A current having some calcium-sensitivity. Although the A current is normally thought of as an entirely voltage-gated conductance, Dukes and Morad (1991) found that the transient outward current in rat ventricular myocytes was sensitive to extracellular calcium levels; substitution of calcium with strontium caused a suppression of the transient outward $K^+$ current in this tissue and a shift in its voltage-dependent activation. It may be that the A current in SMP neurones also has some sensitivity to extracellular calcium, if indeed the small peak seen in figure 5.2.1 is due to A current.

After the small peak in trace A, the outward current begins to rise again slowly. This current is probably a mixture of calcium-activated current and delayed rectifier. In trace B, the magnitude of the outward current has fallen probably due to the loss of the calcium-activated currents, leaving a slowly activating outward conductance which was probably the uncontaminated delayed rectifier. A small tail current is also apparent after returning to the holding potential in trace A. This was observed in most cells and is again lost in trace B. It may be due to a calcium-activated AHP current. It is shown more clearly in figure 5.2.2.

In a sample of twenty-three SMP cells, the outward current elicited by the depolarising voltage steps from -60 to -20 mV fell from $699 \pm 72 \ pA$ to $357 \pm 62 \ pA$ on the removal of extracellular calcium, a reduction of 49 per cent.
These results are consistent with the findings of Dunn (1994) who observed a similar reduction in outward rectification in rat superior cervical ganglion neurones when the cells were bathed in 100 μM cadmium, presumably due to the block of calcium channels and the subsequent block of calcium-activated K⁺ conductances. In cadmium-free solution, the shape of the current was not dissimilar from that in figure 5.2.1 in that there was a small peak of outward current followed by a sustained outward conductance. The addition of cadmium abolished this, leaving only the slowly-activating delayed rectifier.

Figure 5.2.2 shows a current trace from a recording in which the cell has been held at -60 mV and stepped to -20 mV for 150 ms every 5.4 seconds, revealing tail currents occurring after each step. This trace is from a recording in which the tail currents were well-pronounced, although tail currents of this kind were seen in nearly all neurones. The delayed rectifier currents have been truncated to enable the current axis to be expanded. The current level at the beginning is uncontaminated with AHP current because no depolarising steps had been performed prior to those shown. There is an inward current at the -60 mV holding potential, which makes this recording slightly atypical (see later). The current trace was sampled at 70 Hz using the Axotape computer program.

The currents clearly have a rising phase reaching a mean peak of 111 pA as reckoned from the current level at the beginning of the recording. They peak about one second after the potential is returned to -60 mV. Clearly the tail currents are not fully decaying before the next voltage step. The currents decayed to half their peak level (i.e. half the distance between peak $I_{\text{AHP}}$ and the current level at the beginning of the trace which was uncontaminated by any AHP current) 2.3 seconds after the peak. Removal of extracellular calcium brought about complete abolition of these tail currents.

These currents resemble the AHP currents recorded by Sah and McLachlan (1991) from neurones of the dorsal motor nucleus of guinea pig vagus. They voltage-clamped the currents that followed an action potential and recorded an apamin-sensitive current without a rising phase after a depolarising step and a longer lasting AHP current that was apamin-insensitive. The apamin-insensitive current clearly reached a peak after the action potential that generated it. They concluded that the calcium influx during the action potential triggered calcium
Figure 5.2.1: Currents obtained from the same SMP neurone held at —60 mV and stepped to —20 mV. A. Performed in normal saline containing 2 mM calcium and B. Performed in calcium—free saline. The shape and magnitude of the outward current is considerably changed on removal of extracellular calcium, but the initial transient inward current is comparatively unaffected.
Figure 5.2.2: A portion of a current trace from a switch-clamped SMP neurone held at -60 mV and stepped for 150 ms to -20 mV every 5.4 seconds. Long outward currents occur after each step until the bathing solution is switched from control (containing 2 mM calcium) to calcium-free solution. The delayed rectifier currents have been truncated to enable expansion of the current axis. The trace was sampled at 70 Hz.
release from intracellular stores, and this secondary increase in intracellular calcium accounted for the delayed peak of the apamin-insensitive current. It may be that the AHP current in SMP neurones was similarly sensitive to calcium-induced calcium release. The effects of apamin on the AHP current was not determined in SMP neurones.

The removal of extracellular calcium also caused a fall in the resting current at -60 mV from 59 ± 24 pA to -52 ± 23 pA (n=17). This figure was measured from recordings in which there were no depolarising voltage steps, i.e. there were no AHP currents contributing to the standing outward current in calcium-containing bathing solution. This is in keeping with the findings of Akasu and Tokimasa (1989) who found a resting K(Ca) in guinea pig SMP neurones. Whether this current is carried by the same channels as the AHP current is not clear, but Akasu and Tokimasa showed that the tonically active K(Ca) current was insensitive to apamin (300 nM), but blocked by TEA (10 to 40 mM) and barium (3 mM). Furthermore, the current was insensitive to the calcium channel blockers nifedipine (1 μM) and ω-conotoxin (0.5 μM). This implies that the resting calcium conductance responsible for potassium channel activation is mediated by a calcium channel that is neither of the L- nor of the N-type.

Figure 5.2.3 shows the effects of removing calcium on the current-voltage relationship. The cell was held at -60 mV and stepped to various test potentials for 250 ms; the steady-state currents were then measured. The resting current at -60 mV in calcium-free solution has been subtracted from the test potentials of both I-V curves; in practice the resting current at -60 mV was very close to zero in this cell.

Outward rectification was considerably reduced by the removal of calcium as can be seen at potentials of -20 mV and more positive. The residual outward current seen in the calcium-free curve was assumed to be due solely to the voltage-gated delayed rectifier.
Figure 5.2.3: Steady-state current-voltage relations in an SMP neurone bathed in physiological saline containing 2 mM calcium (○) and in calcium-free saline (△). Holding potential was −60 mV. See text for details.
5.3 The pharmacology of the delayed rectifier.

The delayed rectifier was routinely studied by patching the cells in calcium-containing solution, holding at -60 mV and stepping for 150 ms to -20 mV. The bathing solution was then switched to calcium-free (described in section 5.2) and the remaining outward current induced by the depolarising steps was treated as pure delayed rectifier (as illustrated in figure 5.2.1). The outward currents produced by four consecutive voltage steps were averaged using the Pclamp program and the current measured 140 ms after the beginning of the voltage step, i.e., when $I_K$ had reached its maximum level. Drugs were then bath-applied, dissolved in the calcium-free solution. The depolarising steps continued during drug application. After the cell had been bathed in the drug-containing solution for five minutes, $I_K$ was measured as described above. The bathing solution was switched back to calcium-free control and $I_K$ was measured five minutes after the drug had been washed out.

Cetiedil, clotrimazole, propafenone and UCL 1495, when bath-applied, were all found to block $I_K$ measured in calcium-free saline reversibly (figure 5.3.1), such that the current measured five minutes after washing out the drug had recovered. Inhibition was calculated as the delayed current in the presence of blocker subtracted from the mean of the control current and recovered current (after washing out drug) divided by the mean of the control and recovered currents. Each point given in figure 5.3.1 is the mean of four to six observations.

The IC$_{50}$ values for clotrimazole, cetiedil and propafenone are summarised in table 5.3.1 along with the Hill coefficients also calculated from the above curve fits.
Table 5.3.1: The blockade of the delayed rectifier in SMP neurones by four K+ channel-blocking drugs. Figures show the IC50 values and Hill slopes ± standard errors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 ± standard error (μM)</th>
<th>Hill slope ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>2.7 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>5.0 ± 0.6</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Propafenone</td>
<td>11.3 ± 2.4</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>&gt;5 μM</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.3.1: Dose–response curves showing the effects of blockers on the delayed rectifier current of guinea-pig submucous plexus neurones. The maxima have been constrained to one hundred per cent.
5.4 The inactivation of the delayed current.

In addition to the well documented delayed activation characteristics of $I_K$, it is also known that the delayed rectifier in certain tissues also inactivates over relatively long periods of maintained depolarisation. Nakajima et al. (1962) and Adrian et al. (1970) reported the phenomenon of inactivation of $I_K$ in frog skeletal muscle, in which the inactivation proceeds exponentially (with a time constant of 600 ms) and almost all of the current eventually inactivates. In frog myelinated nerve $I_K$ inactivates incompletely along a non-exponential time course (Schwarz and Vogel, 1971). It may be that inactivation characteristics could be an important feature for identifying differences in the delayed rectification of different tissues.

Initially, experiments were performed to find whether $I_K$ inactivated in SMP neurones. The cells were bathed in calcium-free solution containing 300 nM tetrodotoxin (TTX) so that calcium-activated potassium currents and sodium current would not occur when the cells were depolarised. Again in whole cell configuration, the cells were held at -50 mV and stepped to 0 mV for 16 seconds. This protocol revealed distinct inactivation of the delayed rectifier although it was never complete in all cells tested. Figure 5.4.1 shows a typical current trace.

From a sample of fifteen cells the mean of the peak outward current measured at 160 ms after the beginning of the voltage step was 2.3 nA ± 0.5 nA (n=15), whilst the mean steady state current measured at 15.8 s after the step began was 0.5 ± 0.1 nA (n=15). The current inactivated to 24 per cent of its peak level. Single exponentials were fitted to the inactivating currents giving a mean time constant ($\tau$) of 1376 ± 163 ms (n=15). The average regression coefficient of the exponential fits was 0.9949.

The long steps were also performed in the presence of 5 µM cetiedil and 10 µM propafenone, concentrations at which roughly a fifty per cent block was expected. The drugs were found to be more active on the peak current measured 160 ms after the beginning of the voltage step than on the steady state current measured after 15.8 seconds (table 5.4.1).
Table 5.4.1: The actions of propafenone and cetiedil on the peak and steady-state (inactivated) delayed rectifier currents.

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Percentage inhibition of peak current ± standard error.</th>
<th>Percentage inhibition of steady state current ± standard error.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propafenone 0 μM</td>
<td>71 ± 9 (n=4)</td>
<td>27 ± 8 (n=4)</td>
</tr>
<tr>
<td>Cetiedil 5 μM</td>
<td>54 ± 6 (n=5)</td>
<td>15 ± 7 (n=5)</td>
</tr>
</tbody>
</table>

Paired $t$-tests performed on the figures given in table 5.5.1 showed that the inhibition of the peak and steady-state currents by both drugs was significantly different at the 0.05 level.

It may be that the phenomenon of inactivation is indicative of more than one type of $I_k$ channel being present in SMP neurones, one that inactivates over the sixteen seconds of the voltage step and one that does not. Alternatively, the inactivation may be due to a single channel that is changing from one open channel state of high conductance to another state of lower conductance. If that is so then the transition from one state to the other brings about a change in the pharmacology of the channel; it becomes less sensitive to the blockers shown in table 5.4.1.

In the experiments described in section 5.3, the inhibition of $I_k$ by the four drugs tested must be treated as inhibition of the peak current since only 150 ms voltage steps were used in these experiments. The dose response curve for propafenone in figure 5.3.1 was shallow (a Hill slope of 0.6) suggesting that the drug may have more than one site of action. It is possible that these may correspond to two types of delayed rectifier channel; certainly propafenone is far more active on the peak current than steady-state. This would
favour the idea that there are two or more $I_K$ channels one of which may be responsible for
the inactivation observed over long depolarising steps, and which is more sensitive to
cetiedil and propafenone.

Experiments by Dubois (1981, 1983) and Benoit and Dubois (1986) using frog
node of Ranvier tissue have revealed at least three different types of delayed rectifier. Two
of these channels (components $f_1$ and $f_2$) rapidly activated and slowly inactivated, whilst
the third (component $s$) was slow to activate and did not inactivate after 180 seconds. The
three channels were different in their sensitivities to such agents as mamba toxins,
capsaicin and 4-aminopyridine, and the possibility of channel heterogeneity in the SMP
neurone may be just as great.
Figure 5.4.1: A current trace showing the inactivation of the delayed rectifier current during a sixteen second depolarising voltage step from -50 to 0 mV.
5.5 The voltage-dependence of inactivation of $I_K$.

In the work by Adrian (1970) and Schwarz and Vogel (1971) experiments were performed to determine the extent of inactivation of $I_K$ at a number of different test potentials. These experiments revealed that at potentials of 0 mV and more positive, $I_K$ of frog skeletal muscle inactivates completely, whereas $I_K$ of frog node of Ranvier does not show complete inactivation at potentials of 25 mV and above even with test pulses of 60 seconds.

The voltage dependence of $I_K$ inactivation was studied in SMP neurones by holding the cells at -70 mV to remove any channel inactivation, and stepping to a range of more positive test potentials for sixteen seconds each time. After each step, the cell was held at -70 mV for fourteen seconds to ensure that there was time to remove any inactivation before the next test pulse.

The extent of inactivation was expressed by calculating the residual current 15.8 seconds after the beginning of the step as a percentage of the initial peak current measured after 160 ms. These values were plotted against the membrane potential and showed a gradual increase in the extent of inactivation as the membrane potential was stepped to more positive levels (figure 5.5.1). The inactivation of $I_K$ reached a maximum of eighty percent at potentials more positive than 0 mV.

These results bear some resemblance to those of Schwarz and Vogel who found that $I_K$ of frog node of Ranvier could not be made to inactivate completely at depolarised potentials.

5.6 The mechanism of block of the delayed current.

A number of experiments were performed to look at whether the block of the delayed rectifier was use-dependent. A rapid series of depolarising steps was performed in the presence of blocker; if the level of channel block increased with successive depolarisations this would be taken to indicate use-dependence.
Short voltage steps of 150 ms duration from a holding potential of -60 mV to a test potential of -20 mV were performed every 1.4 seconds. This was done first in control conditions (i.e. in calcium-free saline in the presence of 300 nM tetrodotoxin) and the delayed outward current produced by twenty-five consecutive steps was measured. The steps were stopped and the solution was switched to one containing the channel-blocking drug. After the cell had been exposed to the blocker for two minutes, a further twenty-five short depolarising steps were again performed to see whether the level of block changed. Because these steps were too short to reveal time-dependent inactivation of the current, the data obtained could only be said to apply to the ‘peak’ delayed rectifier current.

Data from two such experiments using cetiedil (5 μM) and clotrimazole (3 μM) are shown in figure 5.6.1. The outward currents in the presence of blocker have been calculated as the percentage inhibition of control. The drugs produced an expected level of block, round about fifty per cent, which did not increase over the course of the protocol.

Altogether this experiment was performed three times for both cetiedil and clotrimazole, and no evidence of an increase in block was observed with successive depolarising steps. Furthermore, no A. H. P. currents were observed over the twenty-five steps performed in control conditions.

It may be that the block of the delayed rectifier by these compounds is genuinely not use-dependent. Alternatively, the block may be use-dependent, but the kinetics of block were too rapid to enable the onset of action of the blockers to be resolved using the above protocol. The current traces corresponding to the first few voltage steps performed in the presence of blocker, were examined on an expanded time scale and once the current had risen to its maximum level, there was none of the ‘sag’ indicative of the sort of use-dependent block observed with clotrimazole on the two G protein-coupled channels. So either the block of the SMP I_k channels is not use-dependent or it is but with much faster kinetics than those observed with the G protein-coupled conductance. In either case this contrasts with the situation observed with the latter conductance.
Figure 5.5.1: A plot showing the inactivation of the delayed rectifier current at a series of test potentials. Holding potential was $-70$ mV. Performed in calcium-free saline containing 300 nM tetrodotoxin. Each point is the mean of five or six observations.
Figure 5.6.1: Plots showing a series of twenty-five voltage steps performed in the presence of cetiedil and clotrimazole. The degree of channel block is expressed as the percentage inhibition of the delayed rectifier current. See text for protocol.

5 μM cetiedil

3 μM Clotrimazole
Hille, Courtney and Dum (1975) showed the block of sodium channels in frog node of Ranvier by the lipophilic local anaesthetic lidocaine to be dependent on the frequency of opening of the channels. The greater the frequency of depolarising steps, the greater the observed blockade of sodium permeability. This is due to the blocker dissociating from the channel between stimulating pulses; the more rapidly the tissue was stimulated, the greater the level of block. The block was more obviously use-dependent at high stimulation frequencies since there was less time for the dissociation of blocker from channel between depolarisations. The compounds that showed frequency dependence in this way tended to be the more hydrophobic local anaesthetic drugs such as benzocaine and lidocaine. It may be that the stimulation frequency used to study the actions of drugs on the delayed rectifier of SMP neurones (once every 1.4 s) was too slow to resolve use-dependence of block if the drugs were able to dissociate from the K⁺ channel rapidly enough.

5.7 The A current.

In a number of recordings, it was noticed that after a hyperpolarising voltage step (during a current-voltage protocol for instance), a transient outward current arose on returning to the holding potential (figure 5.7.1). The current developed very quickly and decayed within a few tens of milliseconds. This was identified as an A-like current because its time and voltage-dependent characteristics resembled those described by various workers for A currents in other tissues (see Rudy, 1988 for review).

Routinely, the A current was induced by holding the cell at -50 and stepping to -90 mV for 150 ms (figure 5.7.1). Presumably at this potential some inactivation was removed, so that when the cell was returned to -50 mV the current could be observed. The peak current reached 711 ± 140 pA (n=10) in experiments performed in standard bathing solution (containing 2 mM calcium), and it decayed with an average time constant of 25 ± 7 ms (n=10) when fitted to a single exponential.
Figure 5.7.1: The A current in an SMP neurone occurring after a 150 ms voltage step from -50 to -90 mV. Recorded in standard saline containing 2 mM calcium.
The A current was found to be insensitive to cetiedil and clotrimazole (table 5.7.1) at concentrations that produced roughly a fifty per cent block of the delayed rectifier and G protein-coupled conductances in SMP neurones and atrial myocytes, making it one of the few potassium conductances that was unaffected by these drugs. It was also insensitive to tetrodotoxin (300 nM). The effects of removing extracellular calcium on the A current were not studied.

Table 5.7.1. The effects of clotrimazole and cetiedil on the A current of SMP neurones. Experiments were performed in standard bathing solution containing 2 mM calcium.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean percentage inhibition of control ± standard error of the mean</th>
<th>Number of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole, 3μM</td>
<td>4 ± 10</td>
<td>5</td>
</tr>
<tr>
<td>Cetiedil, 5μM</td>
<td>-8 ± 11</td>
<td>5</td>
</tr>
</tbody>
</table>

5.8 The sodium current.

5.8.1 Introduction.

In neuronal tissue, sodium conductances are normally associated with the upstroke of an action potential; like the potassium currents covered earlier, sodium conductances are also activated on depolarisation. Sodium channels are very widespread occurring in neurones, skeletal muscle and many endocrine tissues, but they are not as diverse as K⁺ channels. They typically activate and inactivate rapidly on depolarisation in excitable tissue giving transient inward currents after which potassium conductances such as the delayed
rectifier occur repolarising the membrane potential. This has the effect of removing sodium channel inactivation, enabling the channels to open again transiently when the membrane is next depolarised.

The majority of sodium channels are sensitive to the toxin of certain tetraodontiform fish, tetrodotoxin in nanomolar concentrations. This has enabled the purification of the sodium channel protein; the α subunit is a large molecule with numerous sugar and carboxylic acid residues and has the functional properties of the channel when studied in expression systems; the roles of the smaller subunits associated with sodium channels remain unclear. As with calcium channels, the major sodium channel subunit is largely composed of a sequence of three to four hundred amino acids that is repeated four times, the ‘internal repeat’, a feature not found in the structures of potassium channel proteins. Each internal repeat sequence is believed to span the membrane six times, with one such segment, the S4 region being rich in positively charged amino acids and probably acting as the voltage sensor. The ion pore is likely to be formed by the four internal repeats arranged in radial symmetry to form a central ion conducting portal.

5.8.2 The sodium current of SMP neurones.

The sodium current of SMP neurones was observed during experiments in which depolarising voltage steps were used to study the delayed rectifier (figure 5.2.1). In later experiments tetrodotoxin (TTX) was used to inhibit the current; figure 5.8.1 shows the abolition of the inward sodium current by 300 nM TTX.

The mean sodium current occurring at the beginning of a step from -60 to -20 mV reached a peak value of \(-1.84 \pm 0.26 \, \text{nA}\) which became \(-1.52 \pm 0.28 \, (n=16)\) on removal of extracellular calcium, a fall of 17.2 per cent. This modest reduction of inward current may be attributable to the loss of inward calcium currents also occurring during depolarisation. The sodium current lasted 2-3 ms in calcium-free solution.

The finding made during experiments studying delayed rectifier pharmacology, was that the sodium current was also sensitive to the four blockers clotrimazole, cetiedil, propafenone and UCL 1495 in calcium-free saline solution. Figure 5.8.2 shows the dose-
Figure 5.8.1: Currents obtained from an SMP neurone held at -60 mV and stepped to -20 mV. A. Performed in normal saline containing 2 mM calcium. B. Performed in calcium-free saline containing 300 nM tetrodotoxin. Both the outward current and the initial transient inward current are diminished or abolished.
Figure 5.8.2: Percentage blockade of peak inward sodium conductance by the four test compounds in guinea-pig submucous plexus neurones.

Cetiedil (○) and UCL 1495 (●)
response curves obtained for the block of the peak sodium current and represents data from only those recordings in which the current recovered after washing out the blocker. The data points for cetiedil and propafenone were fitted with Hill equations without constraining the maximum blockade to one hundred per cent, since the drugs produced a blockade of the control sodium conductance that was actually greater than one hundred per cent. The reason for this is that at top concentrations that totally abolished the inward current, a small transient outward current was unmasked at the point where the sodium current had been maximal. It may have been some A current and it is illustrated in figure 5.8.3. The magnitude of this transient was about ten per cent of the control peak sodium current.

The IC_{50}s and Hill slopes obtained from these fits are summarised in table 5.8.1. The data points for clotrimazole could not be fitted with a Hill equation because the maximum level of block was ill-defined from the figures obtained with this drug. The IC_{50} for clotrimazole was estimated by eye to be around 4 μM.

Table 5.8.1. Calculated IC_{50}s and Hill slopes with standard errors for the blockade of the sodium current in submucous plexus neurones. * The IC_{50} for clotrimazole has been estimated by eye.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (μM) ± sem</th>
<th>Hill Slope ± sem</th>
<th>Maximum Percentage Inhibition of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetiedil</td>
<td>3.3 ± 1.6</td>
<td>1.9 ± 0.1</td>
<td>111</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>4 *</td>
<td>-</td>
<td>114</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.0 ± 0.8</td>
<td>1.9 ± 0.4</td>
<td>107</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>&lt; 5 μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.8.3: Sodium currents recorded during a step from $-60$ to $-20$ mV.
A. Four currents, control and in the presence of 2, 5 and 10 μM cetiedil.
B. The current recorded in 10 μM cetiedil shown in A. on expanded current scale.
The sodium current is abolished leaving a small transient outward current behind, which is marked with an arrow.
The dose-inhibition curves for cetiedil, clotrimazole and propafenone were steep, and their potencies were very similar. The finding that the drugs were also effective sodium channel blockers was generally unexpected except in the case of propafenone which has been reported to have class 1 antidysrhythmic activity (Dukes and Vaughan Williams, 1984).

5.9 The mechanism of block of the sodium current.

Experiments were also performed to examine the possible use-dependence of the block of the sodium current by clotrimazole and cetiedil using a similar protocol to that described in section 5.7 for the study of use-dependent block of $I_K$. In calcium-free saline, the cell was stepped from -60 to -20 mV for 50 ms every 1.4 seconds, twenty-five times. The cell was then bathed in saline solution containing either 5 μM cetiedil or 3 μM clotrimazole (i.e., concentrations expected to produce roughly a fifty per cent block of the current) for two minutes. The same protocol was performed once again to see if the inward sodium currents elicited by depolarisation reduced with successive steps. The inward currents obtained in the presence of blocker were compared to those obtained in control saline, which were all of similar magnitudes and showed no time-dependent diminution. Calcium-free saline was again used partly to avoid contamination by simultaneously-occurring inward calcium currents, and partly to reduce the outward $K^+$ currents that also occur during depolarisation.

Of four cells tested, three showed a gradual onset of block in the presence of 5 μM cetiedil. The first few steps of the protocol induced sodium currents that were already partially blocked (twenty-five per cent inhibition of control) and the level of block increased to a steady value of sixty per cent over the next fifteen steps. These experiments then, suggested that cetiedil may block the sodium current partly by a use-dependent mechanism and partly by a non-use-dependent mechanism.

Of five cells tested with 3 μM clotrimazole, only two showed characteristics of open-channel block. In these recordings, however, the initial level of block was ten per cent inhibition of control before increasing to a steady level of fifty per cent over the first
twenty steps. In all other recordings the level of block remained unchanged (at around fifty per cent) during the course of the twenty-five step protocol, and in all cases the block of the sodium current reversed appreciably on removal of the drug. No other obvious differences existed between those cells in which the block was use-dependent and those in which it was not.

Figure 5.9.1 summarises the results from only those experiments in which use-dependence was apparent. The inward currents are normalised as the percentage inhibition of the control current elicited in calcium-free saline solution. The cetiedil data is the mean of three recordings, and the clotrimazole data the mean of two. The extent of inhibition of sodium current is clearly seen to increase.

Figure 5.9.2 shows two sample current traces obtained using the twenty-five-step protocol from the same neurone; the first is a control in which the inward currents are all of similar magnitudes, the second is from a recording obtained in the presence of 5 μM cetiedil, in which open channel block was apparent, and shows the gradual reduction in the size of successive inward currents.
Figure 5.9.1: Blockade of the sodium current by 5 μM cetiedil (○) and 3 μM clotrimazole (□). Each point corresponds to an inward current occurring during a depolarising voltage step and is expressed as the percentage inhibition of control current. The cetiedil points are the mean of three recordings, the clotrimazole points the mean of two. Cells were bathed in blocker for two minutes before the steps began.
Figure 5.9.2: Apparent use-dependence of the block of the sodium current by 5 μM cetiedil. A: twenty-five currents recorded in calcium-free saline and B: twenty-five currents recorded two minutes after exposure to cetiedil. (Cell was stepped from -60 to -20 mV). Capacity transients have been *blanked* from the traces.
5.10 The effects of clotrimazole on the action potential.

After finding that the sodium and potassium currents of SMP neurones were sensitive to the blockers clotrimazole and cetiedil, a few experiments using intracellular recording techniques were performed to investigate the effects of these blockers on the action potential.

As in earlier experiments with microelectrodes, the electrode filling solution was molar potassium citrate at pH 7.2. Depolarising current pulses were used to induce action potentials, and the cells were bathed in standard saline solution containing 2 mM calcium. Figure 5.10.1 shows the effects of 10 μM clotrimazole on the SMP neurone action potential, evoked with a 30 pA depolarising current pulse. The rate of rise of spike potential is clearly reduced in the presence of clotrimazole. This may in part be due to the blockade of the sodium conductance responsible for the rapid phase of depolarisation, but clotrimazole has caused the cell to depolarise by about 6 mV from its control resting potential of around -57 mV and this may cause some inactivation of sodium channels which would also account for the slope of the action potential being attenuated.

The action potential duration seems to be increased as well, possibly due to the blockade of outward currents such as the delayed rectifier. Evidence from voltage clamp recordings suggests that calcium-activated potassium conductances will also be active at depolarised potentials and may contribute to repolarisation, but what actions clotrimazole may have on these currents was not studied. The effects of clotrimazole readily reversed after bathing the cell for five minutes in drug-free saline, and the action potential resembled that obtained before exposure of the cell to drug.
Figure 5.10.1: Action potentials recorded from an SMP neurone using a 30 pA depolarising current pulse. A: Control, B: In the presence of 10 μM clotrimazole, and C: Recovery five minutes after washing out clotrimazole.
5.11 Concluding remarks.

5.11.1 Currents found in SMP neurones and their pharmacologies.

Experiments described at the beginning of this chapter reveal that SMP neurones have a slow calcium-activated AHP current and a tonically-active outward current that is sensitive to the removal of extracellular calcium and may well contribute to resting potential (see Akasu and Tokimasa, 1989). It may be that the two conductances are carried by the same K⁺ channels. Akasu and Tokimasa showed the AHP current in SMP cells to be insensitive to apamin. A similar current in myenteric neurones was also insensitive to apamin, but was found to be sensitive to iberiotoxin and charybdotoxin by Kunze et al., 1994. The group also found that iberiotoxin and charybdotoxin caused a fall in resting potential and an increase in input resistance during intracellular recordings, suggesting that an equivalent current may indeed contribute to resting potential as well in myenteric neurones.

Removal of extracellular calcium abolished calcium-activated K⁺ currents enabling the delayed rectifier current to be studied in isolation. The current was shown to inactivate incompletely during prolonged voltage steps, and was sensitive to some of the blockers used in the study of the G protein-coupled conductances. In experiments designed to study the peak (non-inactivated) delayed rectifier, the order of potency of blockers was clotrimazole (IC₅₀=2.7 μM), > cetiedil (IC₅₀=5.0 μM) > UCL 1495 (IC₅₀>5 μM) > propafenone (IC₅₀=11.3 μM). Whilst the Hill slopes for clotrimazole and cetiedil were close to unity, that of propafenone was only 0.6 possibly implying heterogeneity of the channels subserving Iₖ in SMP neurones. It was also found that both propafenone and cetiedil were more active on the peak than the steady-state Iₖ, and the possibility exists that it may be a composite current made up of inactivating and non-inactivating components having differing sensitivities to the blockers tested on them. As mentioned earlier, this is known to be the case with Iₖ of frog node of Ranvier (Dubois 1981, 1983; Benoit and Dubois, 1986). The extent of inactivation of Iₖ was also shown to be dependent on membrane potential, becoming more pronounced at more positive potentials,
without ever becoming complete. This phenomenon has also been described in frog node of Ranvier (Schwarz and Vogel, 1971), whilst in frog skeletal muscle inactivation does become complete at sufficiently positive test potentials (Adrian, 1970).

Experiments were performed to study whether the block of $I_k$ by clotrimazole and cetiedil was use-dependent. At concentrations expected to produce a fifty per cent inhibition of the current, there was no evidence to suggest that use-dependence was a feature of their mechanism of block over a succession of depolarising steps. As pointed out however, such considerations as rapid kinetics of block and drug dissociation between voltage steps mean that possible use-dependence cannot be ruled out. Nonetheless, it was found that whilst both drugs did produce the expected level of block at their test concentrations there was no evidence from any recording for use-dependence even when individual current traces were examined on expanded time scales (see section 5.6).

Cetiedil and clotrimazole, at concentrations expected to inhibit the G protein-coupled conductances by about fifty per cent, had no effect on the A current of SMP neurones. It was thus the only conductance studied in the present work to be insensitive to these drugs, although of course there may have been some effect at higher concentrations. This may be a little surprising given the similarity between the delayed rectifier and A channel proteins as predicted by the Shaker gene and its relatives; but even less expected was the sensitivity of the sodium current to clotrimazole, cetiedil, and UCL 1495 (propafenone has been reported to have class I antidysrhythmic activity (Dukes and Vaughan Williams, 1984) so a sodium channel blocking action by this drug in SMP neurones was not so surprising).

All four drugs seemed to act on the sodium current with very similar potencies, with Hill slopes considerably greater than unity suggesting complex blocker/ion channel interactions. When neurones were bathed in IC$_{50}$ concentrations of cetiedil or clotrimazole for two minutes, and sodium currents subsequently induced by depolarisation, the currents were inhibited to varying extents at the beginning of the depolarising voltage steps, indicating that some non-use dependent block had occurred. In some cells successive currents clearly diminished in magnitude once the steps began, suggesting use-dependent block, but in others this did not occur. Thus the true mechanism of action was not fully
clear for either drug. It may have been that in some cells, the non-use-dependent block was considerable and subsequent use-dependent block difficult to observe. However, the data presented in figures 5.9.1 and 5.9.2 were selected from cells in which there was an unmistakable use-dependent component to the block. It was also found, in keeping with the sodium channel blocking actions of clotrimazole, that 10 μM of the drug reversibly reduced the rate of rise of the action potential of SMP neurones in intracellular recordings.

5.11.2 The pharmacology of some of the ion currents of SMP neurones.

Although compounds have been found to block the G protein-coupled conductances of SMP neurones and atrial myocytes, they are clearly not selective blockers of these currents. In the SMP neurones alone, they have actions at the delayed rectifier and tetrodotoxin-sensitive sodium current, as well as the K⁺ conductances of other tissues (see section 4.7.3) Table 5.11.2.1 summarises the actions of four of the most active compounds as described earlier in the present study. In all cases, they are active in the low micromolar range. However, there are K⁺ conductances which are insensitive to cetiedil and clotrimazole in this concentration range, such as the A current of SMP neurones and the apamin-sensitive AHP current of rat superior cervical ganglion cells (P. Dunn, Pers. comm.). There is also some evidence to suggest that their mechanisms of block differ as well; clotrimazole but not cetiedil produces a use-dependent block of the G protein-coupled conductances—in the case of the rabbit erythrocyte K(Ca) conductance, the reverse is true. Also, the present study did not find either cetiedil or clotrimazole to block I_K in a use-dependent manner, although there was some equivocal evidence suggesting that both drugs were use-dependent in their block of the sodium current of SMP neurones.
Table 5.11.2.1: The actions of blockers on various currents of guinea submucous plexus neurones (SMP) and the G protein-coupled (I_G) conductance of rat atrial myocytes (AM). The figures shown are IC_50s ± standard errors and were obtained from both intracellular recordings (agonists shown) and patch clamp experiments. * IC_50 of clotrimazole on the sodium current estimated by eye.

<table>
<thead>
<tr>
<th>Drug</th>
<th>SMP; I_Na</th>
<th>SMP; I_K</th>
<th>SMP; I_G</th>
<th>AM; I_G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_50 (µM) ± sem</td>
<td>IC_50 (µM) ± sem</td>
<td>IC_50 (µM) ± sem</td>
<td>IC_50 (µM) ± sem</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>4 ± 1</td>
<td>2.7 ± 0.2</td>
<td>1.74 ± 0.1</td>
<td>0.9 ± 0.01</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>3.3 ± 1.6</td>
<td>5.0 ± 0.6</td>
<td>5.87 ± 0.4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Propafenone</td>
<td>3.0 ± 0.8</td>
<td>11.3 ± 2.4</td>
<td>3.53 ± 0.1</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>&lt;5 µM</td>
<td>&gt;5 µM</td>
<td></td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Thus some fairly potent blockers of two G protein-coupled conductances have been found in the present work, although the task of finding selective blockers of such channels is an ongoing one. Clotrimazole one of the most potent blockers of both conductances, is nonetheless poorly selective, being active at I_K, I_K(Ca) and I_Na. There is evidence, covered in chapter 4, to suggest that the drug may be acting in its uncharged form on the G protein-coupled channels; certainly analogues of clotrimazole may yet be developed in an effort to find more selective compounds just as cetiedil analogues have been found to have greater selectivities at some of the channels discussed in section 4.7.3. Two such analogues, UCL 1495 and UCL 1269 showed selectivity between the SMP and atrial myocyte G protein-coupled conductances. Propafenone, a class III antidysrhythmic agent which was also active on these currents, may well have selectivity for certain channels contributing to the I_K current of SMP neurones. Thus channel-selective compounds are beginning to emerge, and the task of developing more selective pharmacological tools that may have future value in the identification and classification of
$K^+$ channels is an ongoing one. This may even extend to the development of novel therapeutic interventions as well.
REFERENCES


APPENDIX 1

The structures of the compounds tested on the G protein-coupled conductances of rat atrial myocytes and guinea-pig submucous plexus neurones.

Cetiedil

UCL 1269
Fluconazole

Propafenone

1,3-di-o-tolylguanidine (DTG)

(+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine (3PPP)