# The imidazoline / guanidine receptor site and its role in potassium channel modulation in vascular smooth muscle

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Facuity of Medicine

by

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Year of submission; 1993



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**Chapter 4: Modulation of K-channel function by imidazoline- and guanidine-based ligands; putative involvement of the imidazoline / guanidine receptor site**

**Abstract**

The putative link between the imidazoline / guanidine receptor (IGR) site and K-channel function has been investigated. The affinity of a number of imidazoline- / guanidine-based ligands for the IGR site was examined by measuring [$^3$H]-idazoxan binding to a rat liver homogenate. All agents which displayed a high affinity for this site possessed either an imidazoline or a guanidine moiety within their structure. Cirazoline was the most potent agent at the IGR site followed by RX 801023, idazoxan and guanabenz. In addition, the affinity of a wide range of K-channel modulators for this site was examined and in all cases (except pinacidil) these agents were unable to bind to this site. These results confirm the selectivity of the IGR site for agents containing an imidazoline / guanidine moiety and suggest that this site is not closely associated with a K-channel. The vasorelaxant effects of the $\alpha_2$-adrenoceptor agonist, guanabenz were examined on strips of rat aorta. Guanabenz (100nM-10μM) caused a dose-related relaxation of rat aorta preconstricted by noradrenaline (300nM) that was insensitive to the $\alpha_2$-adrenoceptor antagonist, RX 821002 (1μM) and independent of the endothelium, but was inhibited by TEA (3mM-10mM), 4-AP (1mM-3mM) and idazoxan (10μM). Guanabenz was unable to relax rat aorta preconstricted by either KCl (25mM) or U-46619 (20nM) but caused a dose-dependent rightward shift in the noradrenaline dose-response curve. Thus it appears that the vasorelaxant action of guanabenz is probably due to $\alpha_1$-adrenoceptor antagonism. The ability of TEA, 4-AP and idazoxan to inhibit this relaxation may occur via functional antagonism as these agents displayed contractile effects when applied alone to the tissue.

The effects of the K-channel openers on whole-cell K-currents were examined on freshly isolated rat portal vein cells using the whole-cell configuration of the patch clamp technique. P1060 (1μM), aprikalim (3μM), levcromakalim (1μM) and pinacidil (3μM) all induced a non-inactivating current, $I_{KCO}$ which was inhibited by the sulphonylurea, glibenclamide (1μM). Single channel noise fluctuation analysis of the increase in holding current induced by either P1060 or aprikalim yielded a conductance value for the channel underlying

$I_{KCO}$ of 10.5pS at 0mV. These agents also caused a decrease in the magnitude of the delayed rectifier current, $I_{K(V)}$ which in the case of aprikalim, levcromakalim and pinacidil was partially reversed by glibenclamide. P1060-Induced inhibition of $I_{K(V)}$ was insensitive to glibenclamide. Under conditions in which intracellular ATP was increased, the ability of P1060 to induce $I_{KCO}$ and to inhibit $I_{K(V)}$ was reduced. The effects of levcromakalim were also examined under conditions designed to alter channel phosphorylation and the ability of these agents to cause channel dephosphorylation seems important in their mechanism of action. It appears that the structurally-diverse K-channel openers all exert similar effects on the whole-cell currents of rat portal vein and that the channel underlying $I_{KCO}$ induced by these agents possesses characteristics in common with those of the so-cailed ATP-sensitive K-channel.

The ability of a series of imidazoline- / guanidine-based agents to modulate the function of K-channels in vascular smooth muscle cells under whole-cell voltage-clamp was also examined. Phentolamine (30μM), cirazoline (30μM), antazoline (30μM), clonidine (30μM) and guanabenz (30μM) all inhibited the induction of $I_{KCO}$ by levcromakaiim and, in addition, these agents inhibited $I_{K(V)}$. The possibiiity thus exists that the inhibition of these K-currents arises from the activation of an imidazoline / guanidine receptor site. The ability of these agents to inhibit both $I_{K(V)}$ and $I_{KCO}$ may indicate that these currents are related. These data are therefore consistent with the view that the $K_{ATP}$ channel exists as a substate of the delayed rectifier channel, $K_V$. Clonidine was able to inhibit $I_{K(V)}$ at concentrations lower than those required to block $I_{KCO}$ and in addition, guanethidine inhibited $I_{KCO}$ but was unable to block $I_{K(V)}$. Further experiments are thus required to determine the manner in which agents such as cionidine and guanethidine are able to inhibit these K-currents.

## Preface

The author graduated from The Hatfield Polytechnic in 1990 with the degree of BSc (Honours) in Applied Biology.

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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**Acknowledgements**

I gratefully acknowledge the enthusiastic guidance and supervision of Professor Arthur Weston who has helped make my research in Manchester both interesting and enjoyable. My thanks also to Dr Gillian Edwards for her useful comments and Mr Martin Miller for his technical help.

I acknowledge Dr. Th. Noack for his help in making the single channel conductance calculations and Dr. P. Greengrass for her supervision of the radio-ligand binding studies.

I thank my compatriots of the past three years, Iain and Duncan and all other members of the smooth muscle research group for always making life in Manchester 'eventful'.

Finally, my fondest thanks to my parents without whose unceasing care and interest the past three years would not have been possible.

The work in this thesis was generously funded by Pfizer Central Research and by the Medical Research Council.

**Publications arising from the work described in this thesis**

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## Abbreviations

The following abbreviations have been used routinely in this thesis;

| | |
|---|---|
| 4-aminopyridine | 4-AP |
| adenosine 5'-triphosphate | ATP |
| alpha | $\alpha$ |
| amperes | A |
| analysis of variance | ANOVA |
| ATP-sensitive potassium channel | $K_{ATP}$ |
| calcium ion | $Ca^{++}$ |
| carbon dioxide | $CO_2$ |
| chloride ion | $Cl^-$ |
| clonidine-displacing substance | CDS |
| concentration causing 50% inhibition of binding | $IC_{50}$ |
| curie | Ci |
| current | I |
| current induced by the K-channel openers | $I_{KCO}$ |
| current variance | $\sigma$ |
| cyclic adenosine monophosphate | cAMP |
| degrees centigrade | °C |
| delayed rectifier channel | $K_V$ |
| delayed rectifier current | $I_{K(V)}$ |
| dimethylsulfoxide | DMSO |
| equilibrium dissociation constant | Kd |
| et alia (and others) | *et al.* |
| holding potential | HP |
| hour | h |
| imidazoline / guanidine receptor site | IGR site |
| grammes | g |
| gravity | xg |

| | |
|---|---|
| guanosine triphosphate | GTP |
| inositol 1,4,5-trisphosphate | $IP_3$ |
| kraftbrühe | KB |
| large conductance, calcium-activated K-channel | $BK_{Ca}$ |
| litre | l |
| -log hydrogen ion concentration | pH |
| maximum number of binding sites | Bmax |
| magnesium ion | $Mg^{2+}$ |
| mean current | $\mu$ |
| micro- | μ |
| milli- | m |
| minutes | min |
| molar concentration | M |
| multiple analysis of variance | MANOVA |
| nano- | n |
| non-inactivating current | $I_{NI}$ |
| non-specific binding | NSB |
| number of channels per cell | $N$ |
| ohm | Ω |
| open probability | $Po$ |
| oxygen | $O_2$ |
| phosphatidyl inositol bisphosphate | $PIP_2$ |
| physiological salt solution | PSS |
| pico- | p |
| potassium channel | K-channel |
| potassium ion | $^{42}K^{+}$ |
| protein kinase A | PKA |
| protein kinase C | PKC |
| rubidium ion | $^{86}Rb^{+}$ |
| seconds | s |

| | |
|---|---|
| siemens | S |
| single channel conductance | *g* |
| single channel current | *i* |
| small conductance, calcium-activated K-channel | $SK_{Ca}$ |
| standard error of the mean | s.e.mean |
| tetraethylammonium | TEA |
| tricarboxylic acid | TCA |
| tritiated | [$^3$H] |
| voltage / volts | V |
| voltage-operated calcium channel | VOCC |

# Chapter 1:

# General introduction

## 1.1. The imidazoline / guanidine receptor site

Ligands that activate α-adrenoceptors can be sub-divided into several groups based on their chemical structure. Classically, the catecholamines are regarded as the prototype agonists with noradrenaline and adrenaline representing the endogenous activators of these receptors. In addition, it has been recognised for some time that agents possessing either imidazoline or guanidine moieties, eg cirazoline and clonidine are also potential ligands at this site. Recently, however, it has been reported that these imidazoline / guanidine derivatives also bind to a site distinct from the α-adrenoceptor, namely the imidazoline / guanidine receptor (IGR) site. The endogenous α-adrenoceptor ligands have a very low affinity for the IGR site and, based largely upon this evidence, it has been suggested that this binding site may represent a novel receptor and not a sub-type of the α-adrenoceptor. This binding site has now been characterised in a wide variety of tissues including smooth muscle but as yet no definite functional role has been assigned to it. Evidence is accumulating to suggest that this site may play a role in the modulation of potassium (K) channel function within the cell, this possibility will be addressed more fully in a later section (1.1.9).

### 1.1.1. Nomenclature

Some confusion exists within the literature regarding the correct nomenclature for these receptors. One of the more common designations for this site is the non-adrenergic idazoxan binding site (NAIBS) and is so-called because of the extensive use of [$^3$H]-idazoxan in the characterisation of this site. However, it is unclear whether idazoxan is a high affinity ligand at all of these sites in different tissues. These binding sites have also been described as imidazoline receptors, this nomenclature being given credence by its use at a recent international meeting (Michel & Ernsberger, 1992). However, it is clear that ligands other than imidazolines are also high affinity ligands at this site and thus again this name is potentially misleading. The putative endogenous ligand at this site, clonidine-displacing substance (CDS; see later), has also been named 'endazoline' and thus the receptor sites have been referred to as 'endazolinergic receptors'. This again seems premature as many of these receptors have yet to be demonstrated to be high affinity sites for this ligand.

In this thesis the site will be referred to as the imidazoline / guanidine receptor site or IGR site. The suffix 'receptor site' has been employed because the majority of work examining this site has been performed through the technique of radio-ligand binding and it is uncertain as to whether this represents a 'receptor' in the classical sense (ie. a membrane associated protein capable of transducing extracellular messages into intracelilular events).

### 1.1.2. The IGR site in the central nervous system

An extensive number of binding studies has been conducted on the IGR sites in brain tissue. Specifically, it has demonstrated that a dense population of IGR sites exist at the nucleus reticularis lateralis in the rostral ventrolateral medulla (Tibirica *et al.*, 1991). It is at this site that $\alpha_2$-adrenoceptor agonists such as clonidine are thought to reduce blood pressure via a decrease in sympathetic vasomotor output. Bousquet *et al.* (1984) demonstrated that this centrally-mediated lowering of blood pressure is mediated via receptors that preferentially

bind compounds with an imidazoline structure such as clonidine and idazoxan. It has since been confirmed that the lowering of blood pressure caused by imidazoline-based agents is mediated via sites selective for the imidazoline structure but that the sedative side-effects associated with the $\alpha_2$-adrenoceptor ligands are absent from these compounds (Tibirica *et al.*, 1991). It therefore seems that the sedative side-effects are mediated via $\alpha_2$-adrenoceptor activation whilst the hypotensive effects are mediated via the IGR site (Tibirica *et al.*, 1991). This theory is confirmed by the findings of Gomez and coworkers (1991) who found that rilmenidine, the oxazoline analogue of clonidine, possesses a higher selectivity than clonidine for the imidazoline sites as opposed to $\alpha_2$-adrenoceptors and that rilmenidine also has fewer sedative effects than clonidine. At present, the ability of imidazolines to reduce blood pressure without inducing drowsiness probably represents the most convincing possible functional correlate for the IGR site. There are, however, other central actions of imidazoline derivatives which may also be mediated by the IGR site.

Maiese *et al.* (1992) demonstrated that the imidazoline derivatives idazoxan and rilmenidine both reduce focal ischaemia by a mechanism not associated with either $\alpha_2$-adrenoceptor activation or an increase in local blood flow. Jackson *et al.* (1991) demonstrated that 6,7-benzoidazoxan was anticonvulsant against both electrically- and chemically-induced seizures in mice and rats. In addition, they reported that this agent also has a nanomolar affinity for the IGR site but that the anticonvulsant actions of this compound were not shared by other potent IGR site ligands. It therefore seems unlikely that this anticonvulsant activity represents a functional correlate for the IGR site.

### 1.1.3. The IGR site in the periphery

This site has been studied extensively using radio-ligand binding studies and is now known to exist in several peripheral tissues. The IGR site has been identified in rabbit renal proximal tubule (Coupry *et al.*, 1989), adipocyte membranes (Langin & Lafontan, 1989) and urethral smooth muscle (Yablonsky *et al.*, 1988), rat liver (Zonnenschein *et al.*, 1990), pig kidney membranes (Vigne

*et al.*, 1989), guinea-pig ileum (Wikberg *et al.*, 1991) and human liver (Tesson *et al.*, 1991). Despite the extensive number of binding studies conducted on these tissues, conclusive evidence for a functional role of this site in the periphery has not been forthcoming. However, a number of putative correlates for this site do exist.

Göthert and Moiderings (1991) suggested that presynaptic imidazoline receptors were involved in the inhibition of noradrenaline release from sympathetic neurone terminals. They demonstrated that release of [$^3$H]-noradrenaline from rabbit pulmonary artery and aorta by electrical field-stimulation could be attenuated by imidazoline derivatives following blockade of α-adrenoceptors by phenoxybenzamine. Further investigation of this site by these authors (Moiderings *et al.*, 1991) revealed that this site could be activated by noradrenaline and blocked by rauwolscine, both of which possess low affinity for the IGR site and thus raising the possibility that this site actually represents a so-far undescribed sub-type of α-adrenoceptor.

Limon *et al.* (1992) reported that activation of the IGR site in the human and rabbit kidney caused inhibition of the $Na^+$ / $H^+$ antiporter. This transport system is activated by $\alpha_2$-adrenoceptor agonists and is localised on the basolateral membranes of cells lining the proximal tubule. Inhibition of this antiporter by IGR site agonists could therefore cause a decrease in tubular $Na^+$ reabsorption and a consequent loss of fluid and possible decrease in blood pressure. The balance of IGR site to $\alpha_2$-adrenoceptor activation may be critical in controlling $Na^+$ reabsorption in the kidney.

Regunathan *et al.* (1991a) demonstrated that the imidazoline derivative clonidine increases the uptake of $^{45}Ca^{++}$ into bovine adrenal chromaffin cells. These authors speculated that imidazoline derivatives activate a receptor which may be coupled to, or be part of, a ligand-gated ion channel system via which they either directly or indirectly increase $Ca^{++}$ influx. One possibility is that

activation of this site leads to closure of K-channels involved in the control of the resting membrane potential of the cell. This would cause membrane depolarisation, opening of voltage-operated calcium channels (VOCCs) and exocytosis arising from the concomitant increase in intracellular $Ca^{++}$. This possibility is discussed more thoroughly in a later section (1.1.9).

### 1.1.4. The IGR site in pancreatic β-cells

The sympathetic nervous system serves to decrease insulin release via activation of post-junctional $\alpha_2$-adrenoceptors on the pancreatic β-cell. Schulz and Hasselblatt (1989a) reported that imidazoline derivatives such as phentolamine are able to increase insulin release by a mechanism distinct from their ability to inhibit $\alpha_2$-adrenoceptors. This ability was not confined to agents regarded as α-adrenoceptor antagonists such as phentolamine, but was also shared by other imidazolines such the $H_1$-histamine antagonist, antazoline. In addition, non-imidazoline α-adrenoceptor antagonists such as benextramine, are unable to potentiate insulin release. These authors further demonstrated that clonidine (an imidazoline) causes a decrease in glucose-stimulated insulin release via the activation of $\alpha_2$-adrenoceptors (Schulz & Hasselblatt, 1989b). Following $\alpha_2$-adrenoceptor blockade however, clonidine was able to stimulate the release of insulin. It therefore seems that the ability of these ligands to cause insulin release is distinct from their ability to block α-adrenoceptors and could arise from the possession of an imidazoline moiety within their structure. Since these initial findings, it has been demonstrated by a number of authors that imidazoline derivatives, regardless of their affinity for α-adrenoceptors, are able to block the ATP-dependent K-channel ($K_{ATP}$) in pancreatic β-cells. Plant and Henquin (1990) were the first to demonstrate this phenomenon for phentolamine and a similar mechanism has been reported for antazoline, tolazoline (Dunne, 1991) and efaroxan (Chan *et al.*, 1991). In a further study, Jonas *et al.* (1992) demonstrated that in addition to the ability of alinidine, antazoline, tolazoline and phentolamine to block $K_{ATP}$, these agents also inhibited the efflux of $^{86}Rb^+$ and

potentiated glucose-induced release of insulin from these cells.

The putative imidazoline receptor in the pancreatic β-cell may not directly correspond to either the brain or the kidney IGR site as idazoxan and tolazoline have a high affinity for the brain and kidney sites but are only weakly active at blocking the β-cell $K_{ATP}$. Whether the IGR site is an integral part of the $K_{ATP}$ channel complex or the two are coupled via intracellular second messengers is unclear. However, in a recent review Edwards and Weston (1993) have speculated that the IGR site represents the site of action of agents such as phentolamine and antazoline in blocking $K_{ATP}$ in these cells.

### 1.1.5. Clonidine-displacing substance

Clonidine-displacing substance (CDS) was first isolated by Atlas and Burstein (1984) from rat brain. These authors demonstrated that CDS is able to displace clonidine and yohimbine binding from $\alpha_2$-adrenoceptors. It has since become apparent that CDS will bind to both $\alpha_2$-adrenoceptors and IGR sites and thus it has been suggested that CDS represents an endogenous ligand for the IGR site (Bousquet *et al.*, 1986). This agent is a non-catecholamine, low molecular weight substance (587 daltons) which competes with [$^3$H]-clonidine and [$^3$H]-yohimbine in rat brain and human platelets respectively. CDS has no affinity for either $\alpha_1$- or β-adrenoceptors. It is ninhydrin and fluorescamine sensitive, is not a peptide and is resistant to heat and acid (Atlas & Burstein, 1984). Thus so far, the exact nature of this agent remains undetermined.

CDS, like clonidine, inhibits the twitch response of the electrically-stimulated rat vas deferens, an effect which is partially reversed by phentolamine and yohimbine (Diamant & Atlas, 1986; Atlas *et al.*, 1987). A crude preparation of CDS also induces contraction in the rat gastric fundus (Felsen *et al.*, 1987) and rat aorta (Synetos *et al.*, 1991), a phenomenon which in the aorta is rauwolscine- but not prazosin-sensitive. It therefore appears that CDS is biologically active in smooth muscle preparations and also that this agent does not possess complete selectivity for the IGR site but also displays some affinity for the $\alpha_2$-adrenoceptor

(competition with [$^3$H]-yohimbine binding and inhibition by rauwolscine are indicative of an $\alpha_2$-adrenoceptor agonist as these agents possess relatively low affinities for the iGR site).

Microinjection of CDS into the rostral ventrolateral medulla produces an increase in arterial blood pressure. CDS is also an effective inhibitor of the hypotensive effects of clonidine, a finding which leads to speculation that clonidine and CDS share a common site in the rostral ventrolateral medulla (for review see Atlas, 1991). Whether this locus is an $\alpha_2$-adrenoceptor or an IGR site remains to be determined.

Recently, it has been demonstrated (Regunathan *et al.*, 1991b) that CDS is able to bind to IGR sites on adrenal chromaffin cells and induce the release of catecholamines. CDS-induced release of catecholamines is partially blocked by cobalt, a $Ca^{++}$ channel blocker but the binding is unaffected by the non-hydrolysable analogue of GTP, Gpp(NH)p, suggesting the lack of involvement of a G-protein linked receptor.

Meeley and coworkers (1992) have recently demonstrated that CDS-like activity is not confined to brain extracts but is also found in a wide range of peripheral tissues. These workers tested for CDS activity using both radioimmunoassay (antibodies raised to p-amino clonidine) and a gastric fundus smooth muscle bioassay. in the stomach, the CDS-induced contraction is insensitive to a variety of antagonists (adrenergic, cholinergic, monoaminergic and peptidergic), is completely inhibited by nanomolar concentrations of verapamil but is not blocked by the potent IGR site ligand, idazoxan (Meeley *et al.*, 1992). Whether this indicates that CDS does not exert its gastric effects via a IGR site or that this is insensitive to idazoxan is unclear. However, the lack of idazoxan inhibition does indicate that the effects of CDS in fundus are not mediated via a recognised $\alpha_2$-adrenoceptor. These findings contradict those of Synetos *et al.* (1991) who reported that the contractile effects of CDS on rat aorta could be antagonised by the $\alpha_2$-adrenoceptor antagonist, rauwolscine. Therefore, whereas the contraction of rat aorta is probably mediated via an $\alpha_2$-

adrenoceptor, the effects in the gastric fundus may be evoked via an IGR site.

Meeley *et al.* (1992) also found that CDS-like activity as measured by radioimmunoassay was 12- and 6-fold greater than brain in the adrenal gland and the gastric fundus respectively. Other tissues that display significantly greater CDS activity than brain are heart > small intestine > kidney=liver. The extracts from the gastric fundus, heart and kidney were also found to be fully active in the gastric fundus bioassay. Extracts of the adrenal gland were not, however, characterised in this bioassay as this extract consistently caused a relaxation of this tissue. This relaxation was insensitive to the antagonists described above and was probably due to contamination of the extract by vasoactive intestinal polypeptide (VIP). Finally, Meeley *et al.* (1992) also demonstrated that adrenalectomy caused a profound and significant decrease in the serum levels of CDS, but it is not yet clear at this time whether the CDS originates from either the adrenal cortex or the adrenal medulla. The structural similarity between certain $\alpha_2$-adrenoceptor ligands and IGR site ligands coupled with the co-localisation of catecholamines and CDS within the adrenal gland may indicate that these systems are closely linked in their physiological function.

At a recent meeting, Michel and Ernsberger (1992) reported that some of the earlier CDS preparations from bovine brain may have been contaminated by either GABA or glutamate. This could explain the anomalous hyper- and hypotensive effects of CDS (for review see Atlas, 1991) and highlights the difficulties in interpreting data obtained from crude extracts of unknown composition.

Collectively, the above evidence indicates that CDS is a biologically active moiety with actions seemingly mediated via the IGR site and / or $\alpha_2$-adrenoceptor. However, more research is required to determine the exact nature of this agent and its role in the control of blood pressure.

### 1.1.6. Subtypes of the IGR site

The possibility that the IGR sites in various tissues are not homogeneous is now becoming increasingly evident and Michel and Insel (1989) suggested that they comprise three subtypes. The first of these recognises imidazolines but not guanidines, benzazepine derivatives or amiloride. The second class of sites binds all imidazolines, all guanidines and amiloride while the third subtype is believed to exhibit a high affinity for guanidines and amiloride but only a low affinity for imidazolines.

Wikberg *et al.* (1991) compared the binding of [$^3$H]-idazoxan to IGR sites in guinea-pig cerebral cortex and ileum smooth muscle. They found that the majority of agents had similar binding affinities for the different sites but that the stereo-isomers of the $\alpha_2$-adrenoceptor agonist, medetomidine exhibited some selectivity for the sites in the different tissues. The (+)-enantiomer of medetomidine showed almost equal affinity for the cortical and ileal IGR sites whereas the (-)-enantiomer demonstrated a 26-fold higher affinity for the ileal site than for the cortical site. Because the two sites are both insensitive to clonidine, histamine, imidazole-4-acetic acid and cimetidine, they are not compatible with the classification suggested by Michel and Insel (1989). Nevertheless these authors (Wikberg *et al.*, 1991) suggest a fundamental difference between the two sites and thus have denoted the cortical receptor as type $I_A$ and the receptor found in the ileum smooth muscle as type $I_B$.

Ernsberger and colleagues (1992) have recently divided imidazoline receptors into $I_1$ and $I_2$ subtypes. Under their system, the former type typifies those sites in the rostral ventrolateral medulla which seem to be responsible for the decrease in blood pressure. The $I_2$ receptors may be those involved in the inhibition of the $Na^+/H^+$ antiport system in renal basolateral membranes. The rank order of potency of agents at these two sites characterises moxonidine and efaroxan as potent $I_1$ ligands whilst guanabenz is the most potent $I_2$ ligand.

Recently, Michel and Ernsberger (1992) suggested that imidazoline receptors could be divided into two subtypes based on their affinity for clonidine.

The first subtype, designated $I_1$, are labelled with nanomolar affinity by clonidine derivatives and are sensitive to analogues of GTP. It is these receptors that are involved in the centrally-mediated lowering of blood pressure and also thought to stimulate the release of insulin from pancreatic β-cells via coupling to $K_{ATP}$. By contrast, the $I_2$ imidazoline receptors are labelled with micromolar affinity by clonidine analogues and are insensitive to GTP analogues. These $I_2$ sites are enriched in mitochondrial fractions and it was reported that rabbit and bovine $I_2$ sites have been purified. These receptors are monomeric non-glycosylated proteins with apparent molecular masses of 60-66KDa (Michel & Ernsberger, 1992).

There is now general agreement that a certain degree of heterogeneity does exist within the IGR sites. Once the functional correlates of these receptors have been elucidated a classification using both physiological and pharmacological parameters may be possible. The classification according to Michel and Insel (1989) to date seems to be the most comprehensive, accommodating data obtained from a variety of tissues. For this reason the classification described by these authors will be adopted in this thesis.

### 1.1.7. Putative second messengers linked to the IGR site

Although the function of the IGR site is unclear, extensive research has been directed at determining the cellular consequences that arise as a result of IGR site stimulation. Binding to $\alpha_2$-adrenoceptors can be inhibited by addition of GTP (0.1mM) to the incubation medium (Piletz *et al.*, 1991) and several workers have investigated whether addition of GTP can modulate binding to the IGR site. Remaury and Paris (1992) reported that addition of GTP (0.1μM) and high $Na^+$ (100mM) caused a rightward shift in the displacement curves of [$^3$H]-RX821002 binding to $\alpha_{2D}$-adrenoceptors in RINm5F cells but has no effect on the displacement of [$^3$H]-idazoxan from the IGR site in the same cells.

Similar findings to these were also reported by Piletz and coworkers (1991) who found that $\alpha_2$-adrenoceptor, but not IGR site, binding was inhibited

by GTP in human platelet plasma membranes.

Regunathan and coworkers (1991a) conducted an extensive study into the effects of IGR site ligands on bovine adrenal chromaffin cells. They found that clonidine had no effect on either basal or stimulated cAMP levels but that this agent could significantly increase the production of cGMP an effect not shared by other IGR site ligands. However, clonidine and other IGR site ligands such as rilmenidine and naphazoline were found to increase the influx of $Ca^{++}$ into these cells. These authors (Regunathan *et al.*, 1991b) have also reported that the non-hydrolysable analogue of GTP, Gpp(NH)p has no effect on [$^3$H]-idazoxan binding in adrenal chromaffin cells, and that the release of catecholamines by CDS can be inhibited by the $Ca^{++}$-channel blocker, cobalt.

Zonnenschein *et al.* (1990) also reported that Gpp(NH)p had no effect on [$^3$H]-idazoxan binding in rat liver membranes. However, these workers did demonstrate that 4-AP, $Cs^+$ and $NH_4^+$ all effectively reduced [$^3$H]-idazoxan binding at the IGR site suggesting the possible coupling of IGR sites to K-channels.

The existing data seem to indicate therefore that this IGR site is not a member of the family of receptors coupled to a G-protein. Preliminary evidence, however, does exist to indicate that these receptors modulate ion channels and specifically the function of potassium channels. This possibility will be discussed more thoroughly in a later section (1.1.9).

### 1.1.8. Cellular location of the IGR site

The exact location of the imidazoline receptor within the cell is controversial. Limon *et al.* (1992) have reported that the IGR sites in rabbit and human kidney lie in the basolateral membrane and in the mitochondrial fraction. These authors also reported that the IGR site is at its highest density in the mitochondrial fraction of both the liver and the brain although no function for this site in the mitochondria has been suggested. Tesson and Parini (1991) found

that the major subcellular localisation of the IGR site was associated with the mitochondrial fraction in human and rabbit liver. Zonnenschein *et al.* (1990) showed that [$^3$H]-idazoxan binding to hepatocytes was reversible by washing in a high ionic strength / low pH buffer and thus concluded that IGR sites were localised on the plasma membrane. It may be that the different subtypes of IGR sites have different cellular localisations. According to the recent advances in nomenclature, $I_2$ (ie. imidazoline type 2) receptors are thought to be localised within the mitochondrial fraction whilst $I_1$ receptors are probably associated with the plasmalemma (Michel & Ernsberger, 1992).

### 1.1.9. The IGR site and its putative link with a potassium channel

There is some evidence to suggest that a close link exists between the IGR site and K-channel functioning. Zonnenschein *et al.* (1990) demonstrated that the binding of [$^3$H]-idazoxan to the IGR site in rat liver was reversible by the K-channel blocker 4-AP. More recently, Göthert and Molderings (1991) showed that imidazoline derivatives could decrease the electrically-stimulated release of [$^3$H]-noradrenaline from sympathetic nerve endings. A possible explanation for this is that the imidazoline derivatives increase K-permeability in the neurone terminal with a consequent hyperpolarisation of the cell. No experimental evidence for this hypothesis exists, but North and Surprenant (1985) have demonstrated that presynaptic inhibition of neurotransmitter release by $\alpha_2$-adrenoceptors is mediated by K-channel opening. Zoltay and Cooper (1990) extended these findings to suggest that all pre-synaptic feedback inhibition, regardless of intermediate (biochemical) events, is mediated via the opening of K-channels in the synaptic terminal.

Soares-da-Silva and Villanueva (1990) reported that the $\alpha_2$-adrenoceptor agonist, guanabenz caused a dose-dependent relaxation of rat aorta pre-contracted with noradrenaline or 5-hydroxytryptamine, an effect which was unaffected by yohimbine or propranolol but which was blocked by TEA. These workers suggested that this vasorelaxant property of guanabenz was not

mediated via the $\alpha_2$-adrenoceptor but via the opening of plasmalemmal K-channels. However, in this study guanabenz was unable to relax rat aorta precontracted with low (25mM) $K^+$-rich PSS, a feature of recognised K-channel openers. The structural similarity between agents such as guanabenz and K-channel openers such as pinacidil (see later) was recently highlighted by Edwards and Weston (1990a).

It has been recognised for some time that several imidazoline- / guanidine-derivatives are capable of inhibiting K-channels. The mechano-inhibitory effects of the K-channel openers in smooth muscle (section 1.3.5) are antagonised by a number of imidazoline- and guanidine-derivatives most notably phentolamine (McPherson & Angus, 1989), naphazoline (Grana *et al.*, 1991) and guanethidine (Berry *et al.*, 1992). Dunne (1991) demonstrated that both antazoline and tolazoline are able to block $K_{ATP}$ opened by diazoxide in RINm5F insulin-secreting cells. Interestingly, the IGR site has also been reported to exist in this cell line (Remaury & Paris, 1992). The blockade of $K_{ATP}$ in these insulinoma cells by imidazoline-derivatives causes membrane depolarisation and release of insulin. Analogous to the ability of the imidazolines to increase the release of insulin by blockade of K-channels, may be the ability of CDS to stimulate the release of catecholamines from adrenal chromaffin cells (Regunathan *et al.*, 1991b) via a $Ca^{++}$-sensitive mechanism. The possibility thus exists that CDS actually represents an endogenous K-channel blocker. However, no direct experimental evidence exists at this time in favour of this theory.

## 1.2. Potassium channels

It has been suggested that as many as fourteen major types of potassium channel now exist in different tissue types (Watson & Abbott, 1992). These channels differ in their sensitivity to voltage and $Ca^{++}$, they may be receptor-coupled or metabolically-controlled or they may be modulated by intracellular mediators. In short, a vast array of K-channels has been identified, one indication of the fundamental importance of $K^+$ ion movement in the control of membrane potential in electrically excitable cells. The following sections will give a brief overview of the primary groups of K-channels and their physiological roles with specific reference to their presence and roles in smooth muscle.

### 1.2.1. Voltage-gated potassium channels

#### 1.2.1.1. The delayed rectifier channel

The delayed rectifier channel ($K_V$) was first observed by Hodgkin and Huxley (1952) although it was not until more recently that this channel received its present designation (Conti & Neher, 1980). $K_V$ is present in neurones (Conti & Neher, 1980) and in skeletal muscle (Standen *et al.*, 1985). In smooth muscle, delayed rectifiers are probably present in vascular, intestinal, vas deferens, uterine and tracheal cells (for review see Bolton and Beech, 1992). These channels are activated by depolarisation after a slight time-delay. Current rises sigmoidally upon activation and under constant depolarisation a slow exponential inactivation usually occurs and this can take several seconds to complete. $K_V$ is probably involved in the repolarisation phase of slow electrical waves and agonist-induced contractions.

The delayed rectifier channel in squid axon is larger and has slower activation kinetics when phosphorylated. At -60mV between 30-50% of the delayed rectifier channel population is inactivated in the absence of ATP and this value decreases to 5% after addition of ATP (Perozo & Bezanilla, 1991).

The delayed rectifier in vascular and tracheal smooth muscle is blocked by 4-aminopyridine (4-AP) and activates at potentials positive to -40mV (for

review see Bolton & Beech, 1992). However, the corresponding channel in small intestine and taenia caeci is insensitive to 4-AP and activates at potentials positive to -20mV (Ohya *et al.*, 1986). In addition to 4-AP, $K_V$ can be blocked by $Cs^+$, $Ba^{++}$ and intracellular TEA. $K_V$ in smooth muscle cells is a relatively low conductance channel with a mean single channel conductance of approximately 9pS (Edwards *et al.*, 1993).

### 1.2.1.2. The A-channel

The A-channel ($K_A$), like the delayed rectifier, is activated upon depolarisation but can be distinguished from $K_V$ by the differences in activation and inactivation time-course (Connor & Stevens, 1971). Whereas $K_V$ activates and inactivates slowly, $K_A$ kinetics are much faster and the channel conducts only transiently. In neurones the interaction between $K_V$ and $K_A$ serves to control the rate of neuronal firing (Hille, 1992). $K_A$ is also found in a variety of smooth muscles such as rabbit and rat portal vein and guinea-pig ureter (for review see Bolton & Beech, 1992)

The function of $K_A$ in smooth muscle is uncertain. In microelectrode studies the resting membrane potential in, for example, portal vein cells is approximately -50mV in which case $K_A$ would be permanently inactivated. Recordings made from rat portal vein cells by Hamilton and colleagues (1986) give a resting membrane potential of -60mV. Thus, it is possible that $K_A$ is involved in the regulation of firing of pacemaker cells. 4-AP is a potent blocker of $K_A$ and also serves to increase spike frequency in portal vein thus suggesting that $K_A$ may indeed serve a functional role in the spontaneous myogenic activity of this tissue.

A gene encoding a fast activating, fast inactivating K-channel with the properties of $K_A$ has been cloned from the fruitfly, *Drosophila melanogaster* (Catterall, 1988). The protein encoded by the cloned gene possesses 616 amino-acids and closely resembles the sequence cloned from an electroplax Na-channel. Hydrophobicity analysis of the sequence reveals six or seven

transmembrane spanning segments and this protein closely resembles a single domain of the Na- or Ca-channel structure. Injection of mRNA encoding a single protein into *Xenopus* oocytes is sufficient for functional expression of $K_A$ (Catterall, 1988).

### 1.2.1.3. The inward rectifier

The inward or anomalous rectifier ($K_{IR}$) differs from other voltage-gated K-channels in that its capacity to conduct $K^+$ increases upon hyperpolarisation. The mechanism controlling the voltage-dependence of $K_{IR}$ channels is unknown but the relatively poor conductance of these channels at potentials positive to $E_K$ is attributed to the ability of intracellular $Mg^{++}$ to block these channels (in a voltage-dependent manner) at such potentials. Raising extracellular $K^+$ shifts the range over which these channels rectify because the increased $K^+$ displaces the $Mg^{++}$ from the channel.

$K_{IR}$ channels have been reported in sea urchin eggs, skeletal and cardiac muscle and in neurones (for review see Edwards & Weston, 1990b). In smooth muscle, an inwardly rectifying current ($I_{K(IR)}$) has only been described in submucosal arteriolar smooth muscle (Edwards & Hirst, 1988). The $I_{K(IR)}$ current is activated at potentials negative to -60mV, exhibits no time-dependence and is completely blocked by extracellular $Ba^{++}$. In this tissue, some $I_{K(IR)}$ current flow was detected at resting membrane potentials and therefore could contribute to the control of the resting membrane potential. In general, it seems that $K_{IR}$ is not present in the majority of smooth muscle types and is only restricted to some specialised smooth muscles (Bolton & Beech, 1992). This conclusion, however, may be incorrect and due to intracellular dialysis and consequent reduction of intracellular $Mg^{++}$ during whole-cell voltage-clamp experiments designed to identify the current.

The channels underlying $I_{K(IR)}$ have a conductance of approximately

20pS and are blocked by $Cs^+$, $Ba^{++}$ or intracellular TEA (North, 1989).

### 1.2.2. Calcium-activated K-channels

#### 1.2.2.1. The large conductance, calcium-activated K-channel

The large conductance $Ca^{++}$-activated K-channels ($BK_{Ca}$) require micromolar concentrations of $Ca^{++}$ to open and their sensitivity to $Ca^{++}$ increases with membrane depolarisation. These channels are found in a wide variety of tissues and are most easily distinguished by their large single channel conductance, typically 200-300pS under symmetrical $K^+$ conditions. The functions of $BK_{Ca}$ are becoming clearer due to the availability of toxins to block these channels. In general, $BK_{Ca}$ is involved in the repolarisation phase of action potentials in excitable cells. The increase in intracellular $Ca^{++}$ coupled with membrane depolarisation are conditions under which these channels will open. It has also been suggested that they may be involved in the control of insulin secretion from pancreatic β-cells and of saliva from salivary glands (Blatz & Magleby, 1987).

$Ca^{++}$ increases the open probability of the $BK_{Ca}$ channel. In smooth muscle the $Ca^{++}$ concentration necessary to achieve an open probability of 0.5 at 0mV is approximately 1μM (McManus, 1991). Hill coefficients for the binding of $Ca^{++}$ to the channel seem to indicate that as many as 6 binding sites may be available for the $Ca^{++}$ (McManus, 1991).

$BK_{Ca}$ is blocked by TEA, this agent being more effective from the exterior of the membrane (0.1-1mM) than from the interior (50-100mM). The information available about $BK_{Ca}$ is constantly increasing due to recent availabilities of highly purified toxins that block this channel. Charybdotoxin (ChTX) is a 37 amino-acid polypeptide component of the venom isolated from the scorpion *Leiurus quinquestriatus*. ChTX blocks $BK_{Ca}$ by reversibly binding to the exterior

surface of the channel and in excised patches, blockade is typified by periods of silence followed by periods of bursting activity. It is thought that the periods of activity occur when the molecule of toxin is not bound to the channel (Garcia *et al.*, 1991). In portal veins, ChTX increases the myogenic activity by blocking the repolarisation pathway. Originally, it was thought that ChTX represented a highly selective tool for the examination of $BK_{Ca}$ but further evidence seems to indicate that this is not the only channel blocked by ChTX. Hermann and Erxleben (1987) have demonstrated that ChTX can also block a smaller conductance (35pS; assymetrical $K^+$-gradient) $Ca^{++}$-activated K-channel and Schweitz *et al.* (1989) reported that charybdotoxin also blocks the dendrotoxin-sensitive, delayed rectifier K-channel.

The findings that ChTX does not represent a totally specific tool for the investigation of $BK_{Ca}$ have prompted the search for further, more selective ligands at this site. The venom from the scorpion *Buthus tamulus* was found to contain a peptide subsequently named iberiotoxin (IbTX). IbTX reversibly inhibits $BK_{Ca}$ causing much longer silent periods and seems to be totally selective for $BK_{Ca}$ (Garcia *et al.*, 1991). IbTX is also a 37 amino-acid peptide and hybrid molecules consisting of part ChTX, part IbTX have indicated that the peptide sequence amino-acids 20-37 is the portion dictating the biological activity (Garcia *et al.*, 1991).

### 1.2.2.2. The small conductance, calcium-activated K-channel

Blatz and Magleby (1986) reported the existence of a small conductance (10-14pS), $Ca^{++}$-sensitive K-channel ($SK_{Ca}$) in cultured rat skeletal muscle. The channel is blocked by the neurotoxin, apamin (derived from bee venom), but not by high (5mM) external TEA. $SK_{Ca}$ is highly sensitive to $Ca^{++}$ at the negative potentials associated with after-hyperpolarisation (Blatz & Magleby, 1986). $SK_{Ca}$ also differs from $BK_{Ca}$ in that it exhibits little voltage-dependency, the current at +40mV being 80% of that at -40mV in 0.1μM intracellular $Ca^{++}$ (McManus,

1991). $SK_{Ca}$ channels are far more sensitive to $Ca^{++}$ than $BK_{Ca}$ at negative potentials but at +40mV both $BK_{Ca}$ and $SK_{Ca}$ exhibit similar $Ca^{++}$-sensitivity.

Weir and Weston (1986) demonstrated that the relaxant effects of ATP and noradrenaline, but not those of BRL 34915 or nicorandil were totally inhibited by apamin in guinea-pig taenia-caeci. Fuji and coworkers (1990) reported that apamin increased the frequency of spontaneous action potentials and completely abolished the after-hyperpolarisation in guinea-pig bladder smooth muscle. In addition, Maggi *et al.* (1989) demonstrated that the relaxant effects of bradykinin on carbachol-contracted strips of guinea-pig urinary bladder were blocked by apamin and by a selective $\beta_2$-adrenoceptor antagonist. Therefore, in intact smooth muscle there seems to be good, if indirect, evidence for apamin-sensitive K-channels, presumably reflecting the presence of $SK_{Ca}$ in these tissues. However, evidence for this channel at the whole-cell and single channel level has not been forthcoming. Inoue *et al.* (1985) demonstrated the presence of a small conductance channel (92pS; symmetrical $K^+$) in patches isolated from rabbit portal vein. This channel was sensitive to $Ca^{++}$ and was relatively independent of voltage. However, sensitivity to apamin was not demonstrated. It is therefore unclear whether this channel represents the smooth muscle equivalent of the $SK_{Ca}$ found in skeletal muscle by Blatz and Magleby (1986).

### 1.2.2.3. Other calcium-sensitive K-channels

Some K-channels do not fit well into a classification based on either large or small conductance channels. Blatz and Magleby (1986) reported that in rat skeletal muscle cells there was also a very small conductance channel (4pS) that was more sensitive to $Ca^{++}$ than either $BK_{Ca}$ or $SK_{Ca}$. A $Ca^{++}$-sensitive channel with a conductance of 40pS has been identified in red blood cells which is highly $Ca^{++}$- and voltage-sensitive. Other $Ca^{++}$-sensitive channels which do not fall into the categories of either $BK_{Ca}$ or $SK_{Ca}$ have also been described in

HeLa cells (a cancer cell-line) and snail neurones (for review see Blatz & Magleby, 1987).

### 1.2.3. Receptor-operated K-channels

#### 1.2.3.1. The M-current

The M-current is so-called because it is inactivated upon stimulation of muscarinic receptors by agents such as acetylcholine. This is a voltage- and time-dependent current which is $Ca^{++}$-insensitive. Sims *et al.* (1985) reported the M-current in the stomach muscle from the marine toad *Bufo marinus*. This current is maximally activated at -20mV, inactivated at -70mV, is insensitive to external $Ca^{++}$ and depressed by muscarinic agonists. However, the M-current as described above has not been identified in any *mammalian* smooth muscle types (Bolton & Beech, 1992).

#### 1.2.3.2. Fatty acid-activated K-channels

Ordway *et al.* (1989) reported that arachidonic acid and other fatty acids are able to directly activate K-channels in smooth muscle cells from the stomach of the marine toad *Bufo marinus*. The channel has a conductance of 23pS ($[K^+]_i$ 130mM, $[K^+]_o$ 20mM) and shows no pronounced voltage-dependence. Whether this represents an already-described channel is unclear but the lack of $Ca^{++}$- and nucleotide-sensitivity coupled with the low conductance does not seem to correlate with any other previously described channel.

### 1.2.4. ATP-sensitive K-channels

The ATP-sensitive K-channel ($K_{ATP}$) was first described by Noma (1983) in cardiac myocytes. Noma (1983) found that depleting intracellular ATP either by hypoxia or by metabolic inhibition caused an increase in outward K-current. Since this report, $K_{ATP}$ has been well-characterised in both cardiac myocytes and pancreatic β-cells. In a recent review, Ashcroft and Ashcroft (1990) described

5 different types of K-channel modulated by intracellular concentrations of ATP, which differed in their voltage-dependence, conductance and sensitivity to ATP and calcium. ATP is believed to bind to a modulatory site on the channel complex and it is this association that causes the channel to close. The $K_{ATP}$ channel is thought to be subject to modulation via a number of intracellular mediators.

ATP is able to regulate the opening of $K_{ATP}$ in two distinct ways. Firstly, phosphorylation of the channel is required for opening to occur (Ashcroft, 1988). It is possible that dephosphorylation may be responsible for the loss of activity or 'run-down' of this channel seen in excised membrane patches. Run-down can be prevented or reversed by exposure to MgATP (Findlay & Dunne, 1986), but ATPγS, the thiophosphate resistant to hydrolytic enzymes is unable to prevent run-down of $K_{ATP}$ in pancreatic β-cells (Ohno-Shosaku *et al.*, 1987). In addition, ATP seems also able to cause closure of the channel by a direct interaction with the channel complex in a manner not involving hydrolysis. The non-hydrolysable analogues of ATP, AMP-PNP and AMP-PCP are both effective inhibitors of the $K_{ATP}$ channel and in addition other nucleotide triphosphates and adenosine di- and monophosphates are also able to cause channel closure although all are less potent than ATP itself (for review see Ashcroft, 1988).

The finding that $K_{ATP}$ is responsible for controlling resting membrane potential in pancreatic β-cells prompted an investigation into the method by which agents which cause insulin release might operate. Sturgess *et al.* (1985) demonstrated that the sulphonylureas, tolbutamide and glibenclamide both cause the release of insulin by inhibition of $K_{ATP}$ in these cells. Closure of $K_{ATP}$ causes membrane depolarisation and the consequent influx of $Ca^{++}$ initiates the release of insulin. Trube *et al.* (1986) showed that the $K_{ATP}$ channel found in cardiac muscle is also blocked by the sulphonylureas. This group of agents, together with certain benzoic acid derivatives such as AZ-DF-265, remain to date the only completely selective agents for blocking the $K_{ATP}$ channel.

The physiological role of $K_{ATP}$ in smooth muscle is still the subject of some debate. It could be expected that a K-channel closed as intracellular ATP

concentrations rise would be important in conditions such as hypoxia and ischaemia. For instance, if a vascular bed has inadequate blood supply the tissue will become oxygen deprived and levels of ATP will fall causing $K_{ATP}$ to open. There will be a subsequent hyperpolarisation and decrease in the open probability of the voltage dependent, L-type calcium channels. The tissue will relax, the vascular bed will dilate and adequate blood supply will be restored. The problem with this argument arises when one considers the cellular levels to which ATP concentrations must fall to in order for $K_{ATP}$ to open. Intracellular levels of ATP range between 3-5mM and at these concentrations $K_{ATP}$ is closed (Ashcroft, 1988). At 1mM ATP, 95% of $K_{ATP}$ channels are closed and for half maximal opening ATP levels would have to fall to 10-200μM (Ashcroft, 1988). Thus, for $K_{ATP}$ to open under hypoxic conditions ATP levels would need to fall by approximately 80%.This represents a marked fall and it may be that $K_{ATP}$ is regulated by a more subtle mechanism such as cyclic nucleotides, pH or is even G-protein linked and that regulation by ATP solely does not occur in the *in vivo* situation.

### 1.2.5. Cloned K-channels

The vast majority of molecular characterisation of K-channels has involved the *Shaker* mutants of the fruitfly, *Drosophila melanogaster*. These are voltage-gated channels each comprising 4 almost homologous subunits. Each of these subunits comprises six hydrophobic subunits (S1-S6) which are thought to exist as transmembrane spanning segments. In addition, a further segment (H5) is also tucked into the lipid bilayer from the extracellular side and both N- and C-terminals are intracellular with core regions that are highly conserved. This general topology of the K-channel protein is thought to be uniform for all voltage-gated channels (for review see Pongs, 1992). These voltage-dependent K-channels are inhibited by two types of K-channel blockers. The organic compounds such as TEA , 4-AP and quinine are usually positively charged and most commonly block this channel from the extracellular surface. The peptide blockers such as charybdotoxin, dendrotoxin and mast cell degranulating

peptide comprise the second group of inhibitors and it is thought that the charybdotoxin binding site may overlap or be closely associated with the external TEA binding site. Charybdotoxin in these channels acts as an open channel blocker.

Takumi and coworkers (1988) isolated a rat kidney mRNA which expressed a slowly-activating, voltage-dependent K-channel. The protein consisted of 130 amino-acids with a single putative transmembrane-spanning segment. Expression of the mRNA in *Xenopus* oocytes yielded a K-selective channel blocked by TEA and by $Ba^{++}$.

Ho *et al.* (1993) have recently cloned an ATP-sensitive K-channel from rat kidney outer medulla, the ROMK1 channel. This channel is highly selective for $K^+$ and has a single channel conductance of 48pS when expressed in *Xenopus* oocytes. The channel exhibits little voltage-dependence but does inwardly rectify, a property shared by other $K_{ATP}$ channels. The cDNA sequence predicts a protein of 391 amino-acids and hydropathy analysis of this protein indicates two transmembrane spanning segments and also possession of a putative ATP-binding site as identified by a Walker type A motif. This channel was not blocked by glibenclamide (250μM) or activated by the K-channel openers, but did exhibit rundown in excised patches a phenomenon which was sometimes reversible with MgATP (Ho, personal communication).

## 1.3. The potassium (K-) channel openers

The ability of the benzopyran derivative, cromakalim (± 6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol) to open plasmalemmal K-channels was first recognised by Hamilton and coworkers (1986). Since this time it has been generally accepted that these agents referred to collectively as the K-channel openers, activate K-channels in the plasmalemma causing a consequent hyperpolarisation of the cell and a subsequent decrease in the open probability of the L-type calcium channels. This is manifest in smooth muscle as a relaxation of the tissue itself or, in the

case of spontaneously active tissues, a decrease in myogenic activity. There now exists a variety of synthetic agents which are members of this pharmacological group. These include pinacidil (a cyanoguanidine), RP 49356 (a thioformamide), nicorandil (a pyridine), minoxidil sulphate (a pyrimidine) and diazoxide (a benzothiadiazine; for review see Edwards & Weston, 1990a).

The K-channel openers therefore comprise a structurally-diverse group of compounds which raises the possibility that they do not share an identical mechanism of action. Indeed, it is well-documented that in addition to K-channel opening, nicorandil is also capable of relaxing smooth muscle by activating the soluble guanylate cyclase system in a manner similar to that of the nitrovasodilator group of compounds (Holzmann, 1983). Perhaps more important is the fact that the definitive nature of the K-channel opened by these agents in smooth muscle has not been determined. The sulphonylurea glibenclamide, a well known inhibitor of the ATP-dependent K-channel ($K_{ATP}$) in both cardiac myocytes (Belles *et al.*, 1987) and pancreatic β–cells (Sturgess *et al.*, 1985) antagonises the mechano-inhibitory property of the K-channel openers. This observation raises the possibility that the K-channel openers act via the opening of $K_{ATP}$. Direct evidence for this however, has not been forthcoming.

### 1.3.1. The K-channel openers: chemical diversity

The K-channel openers comprise a group of structurally-dissimilar agents which can be subdivided into seven groups (Edwards & Weston, 1990a). Of these, the benzopyrans, the cyanoguanidines and the thioformamides are of specific relevance to this thesis.

The benzopyrans are typified by the prototype K-channel opener, cromakalim and this group includes a number of different agents such as the Roche compound Ro 31-6930. Cromakalim is a racemate; each component trans enantiomer possesses two chiral carbon atoms. Biological activity of cromakalim has been found to reside largely with the 3S, 4R configuration, levcromakalim (Figure 1.1).

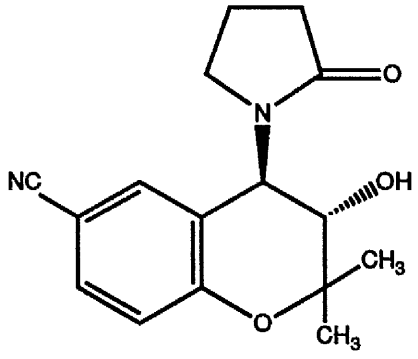
The guanidine-based K-channel openers are typified by agents such as

pinacidil (Figure 1.1). This also contains a chiral carbon atom and again biological activity resides with a single ((R)-pinacidil) enantiomer. More potent cyanoguanidine derivatives such as P1060 (Figure 1.1) are also known.

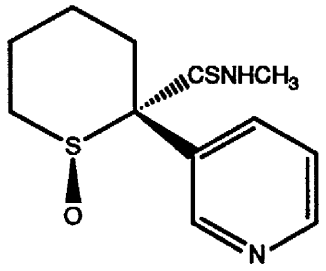
The thioformamides were originally developed as $K^+$ / $H^+$ ATPase inhibitors and comprise a large group of agents. The racemate, RP 49356 and its active enantiomer, aprikalim (RP 52891; Figure 1.1) consist of both a chiral carbon and a chiral sulphur atom.

The pyridine K-channel openers incorporate such agents as nicorandil, known also to activate soluble guanylate cyclase (Holzmann, 1983). The pyrimidines are typified by minoxidil and the benzothiadlazines, by agents such as diazoxide. Finally, attempts to modulate the duration of action of the 1,4-dihydropyridines led to the development of niguldiplne which is able to increase K-currents in smooth muscle cells.

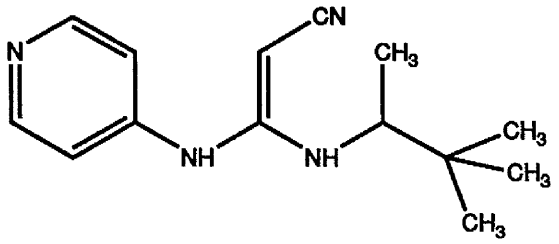
**A. Cromakalim**




**B. Aprikalim**




**C. Pinacidil**




**D. P1060**

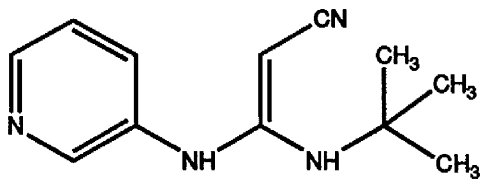



**Figure 1.1**.The structurally-dissimilar K-channel openers. **A**, the benzopyran, cromakalim. **B**, the thioformamide, aprikaiim. **C**, the cyanoguanidine, pinacidil and its potent derivative (**D**) P1060.

As outlined above, the K-channel openers represent a chemically-diverse group of agents and in smooth muscle there is as yet no definitive proof that these agents activate the same channel. The lack of similarity between these compounds has led to speculation that these agents activate different channels or at least activate the same channel by differing mechanisms.

### 1.3.2. The K-channel openers and insulin-secreting cells

The $K_{ATP}$ channel is thought to provide a link between the availability of glucose and the release of insulin from pancreatic β-cells. $K_{ATP}$ is believed to be involved in the control of the resting membrane potential and closes as intracellular ATP ([ATPi]) levels rise, a phenomenon thought to arise as a consequence of an increase in extracellular glucose concentrations. The closure of only a few $K_{ATP}$ channels causes a depolarisation (because of the high membrane resistance) leading to the opening of voltage-operated calcium channels, a consequent influx of $Ca^{++}$ and the release of insulin via stimulation of exocytosis.

Trube *et al.* (1986) demonstrated that the sulphonylurea, tolbutamide has similar effects on this channel to those of high extracellular glucose (5mM) and that these effects are opposed by the hyperglycaemic sulphonamide, diazoxide. The authors concluded that these agents exerted their actions by blocking, and opening $K_{ATP}$ respectively. In addition, they demonstrated that tolbutamide has no effects on any $Ca^{++}$-dependent channels and thus that the sulphonylureas represented a group of agents that selectively blocked the $K_{ATP}$ channel.

Since these initial findings, the modulation of $K_{ATP}$ by intracellular mediators has become the focus of much research. Findlay and Dunne (1986) reported that ATP was actually required to maintain the $K_{ATP}$ channel in a functional state. These workers demonstrated that $K_{ATP}$ channel activity in excised patches disappeared 4-5min following patch excision, a phenomenon referred to as 'run-down'. This run-down can be reversed by briefly exposing the membrane to ATP (1mM) and then returning it to the control solution. Findlay and

Dunne (1986) suggested that the ATP required for reactivation of this channel was used to fuel phosphorylation of the channel which was required for normal channel functioning. These findings were confirmed by Ohno-Shosaku and coworkers (1987) who also demonstrated that non-hydrolysable analogues of ATP were ineffective at reactivating the channel after run-down. Kozlowski *et al.* (1989) reported that the $K_{ATP}$ channels in CR1-G1 cells (an insulin-secreting cell line) required MgATP for activation and that ATP in the absence of $Mg^{++}$ was not sufficient for channel activation. In addition, $Mg^{++}$ was also required for the activation of $K_{ATP}$ by diazoxide. Again, it was speculated that $Mg^{++}$-dependent phosphorylation of $K_{ATP}$ was required for channel activity. Many ion channel types require phosphorylation for normal functioning (Perozo & Bezanilla, 1991) and the data documented above indicate that $K_{ATP}$ requires phosphorylation before activation can occur.

Zünkier *et al.* (1988) reported that ADP, but not other nucleotide diphosphates, increased the sensitivity of the $K_{ATP}$ channel for tolbutamide. They speculated that this may arise from an ability of ADP to reduce the block of the channel by ATP. In the presence of 1mM ADP, the $EC_{50}$ for tolbutamide inhibition of the channel is shifted from 55μM to 4.2μM, but neither AMP, GTP nor GDP improved the sensitivity of the channel to tolbutamide. AMP and GTP alone each had no effect on channel activity whereas GDP almost doubled channel open probability. Schwanstecher *et al.* (1992a) demonstrated that MgADP, MgGDP and MgdADP (2'-deoxyadenosine) all enhanced the ability of diazoxide to open $K_{ATP}$, a property which however was not shared by MgATP. These authors concluded that four binding sites existed on the $K_{ATP}$ complex. These included one for diazoxide, one for the sulphonylureas, one for the inhibitory effects of ATP and ADP and one for the stimulatory effects of the nucleotide diphosphates.

In the presence of MgATP, the K-channel openers will open $K_{ATP}$ and cause membrane hyperpolarisation. However, in the absence of MgATP, many of the K-channel openers actually cause closure of this channel and this may be

caused by accelerated run-down of the channel (Kozlowski *et al.*, 1989).

In addition to being inhibited by the sulphonylureas, the $K_{ATP}$ channel in insulin-secreting cells is also blocked by another group of agents. Schulz and Hasselblatt (1989a) reported that a number of imidazoline derivatives were able to induce insulin release regardless of their ability to inhibit the α-adrenoceptor. Agents containing an imidazoline moiety were first shown to block $K_{ATP}$ in insulin-secreting cells by Plant and Henquin (1990) who demonstrated that the α-adrenoceptor antagonist phentolamine was able to inhibit the actions of diazoxide-induced $^{86}Rb^+$ efflux. Since this time a number of imidazoline-based compounds have been shown to block $K_{ATP}$ in these cells. Antazoline, tolazoline, efaroxan and alinidine are now all known to block $K_{ATP}$ in insulin-secreting cells (Chan *et al.*, 1991; Dunne, 1991; Jonas *et al.*, 1992). Dunne (1991) speculated that an 'imidazoline receptor' may be involved in the blockade of $K_{ATP}$, however this remains to be clarified experimentally.

### 1.3.3. The K-channel openers and cardiac muscle

K-channels that are closed as concentrations of ATP increase were first described by Noma (1983) in cardiac muscle. Noma (1983) reported that when cardiac tissue was exposed to metabolic inhibition by cyanide or to hypoxia there is an increase in outward K-current. The channel carrying this current was insensitive to AMP and relatively insensitive to ADP compared to ATP. The finding that at concentrations in excess of 1mM, ATP was able to inhibit the opening of a voltage-independent K-channel was essentially the first description of a $K_{ATP}$ channel.

Since this initial report it has become evident that in cardiac tissue, $K_{ATP}$ represents the target of the K-channel openers. Belles *et al.* (1987) reported that the activity of this $K_{ATP}$ channel could be depressed by the sulphonylurea, tolbutamide, indicating that this cardiac channel is similar to that described in pancreatic β-cells. Thuringer and Escande (1989) suggested that the ability of RP 49356 to open the cardiac $K_{ATP}$ channel arises from an apparent

competition between RP 49356 and ATP for the site at which ATP binds in order to inhibit channel activity. These workers found that increasing concentrations of ATP (175-700μM) caused a rightward shift in the dose-response curve to RP 49356 in inside-out patches.

Findings similar to those reported in the pancreatic β-cell have also been suggested for the cardiac $K_{ATP}$ channel concerning the reversibility of run-down. Notsu *et al.* (1992) demonstrated that channel run-down could be immediately reversed by addition of cAMP to the intracellular solution of inside-out patches. This reactivation could also be mimicked by addition of protein kinase A (PKA) and channel activity could be depressed by addition of an inhibitor of cAMP-dependent protein kinase. It therefore appears that channel phosphorylation is essential for opening activity and that it is probably dephosphorylation of the channel that is responsible for the run-down observed in membrane patches excised from cardiac muscle.

### 1.3.4. The K-channel openers and the central nervous system

Mourre *et al.* (1989) have recently reported details of the distribution of sulphonylurea binding sites ([$^3$H]-glibenclamide) within the rat brain. The pyramidal and extrapyramidal motor systems were reported to contain the highest density of sulphonylurea receptors particularly in the giobus paliidus and the substantia nigra.

Amoroso *et al.* (1990) reported that $K_{ATP}$ channels within the substantia nigra could be blocked by high glucose concentrations and by the sulphonylureas. Furthermore, the channels could be opened by depletion of ATP and by exposing the tissue to anoxia. These authors were able to demonstrate a direct correlation between the ability of the sulphonylureas to inhibit either anoxia-evoked or ATP depletion-evoked $^{86}Rb^+$ efflux and [$^3$H]-GABA release. A later report from the same group (Schmid-Antomarchi *et al.*, 1990) reported that these observations could be extended to include the K-channel openers. The ability of agents such as cromakalim and pinacidil to induce $^{86}Rb^+$ efflux directly

correlated with the ability of these agents to inhibit the release of [$^3$H]-GABA from slices of the substantia nigra. These authors therefore speculated that the K-channel openers were able to inhibit neurosecretion by activating $K_{ATP}$ in nerve terminals.

Schwanstecher *et al.* (1992b) reported that pinacidil only weakly displaced [$^3$H]-glibenclamide binding from rat cerebral cortex but that this displacement was significantly increased in the presence of MgATP. Furthermore, MgATP alone was also capable of inhibiting [$^3$H]-glibenclamide binding. They concluded that under phosphorylating conditions, i.e. in the presence of MgATP, the binding of agents to the sulphonylurea receptor was severely reduced and thus that a tightly associated protein kinase existed on the $K_{ATP}$ complex. This again provides evidence for the control of $K_{ATP}$ binding by MgATP and specifically via phosphorylation.

Finally, Noack *et al.* (1992c) reported that the anorectic agent, ciclazindol, inhibited the effects of levcromakalim in rat portal vein. These authors suggested that the actions of this compound in the ventromedial hypothalamus could be mediated via the inhibition of $K_{ATP}$. Indeed, this is an area known to be excited by increasing extracellular glucose, an action mimicked by tolbutamide and associated with closure of $K_{ATP}$ (Ashford *et al.*, 1990a,b).

### 1.3.5. The K-channel openers and smooth muscle

Hamilton *et al.* (1986) reported that the novel antihypertensive agent, BRL 34915, abolished the mechanical activity of rat portal vein and caused a consequent hyperpolarisation of the plasmalemma. They also reported that cromakalim increased the efflux of $^{86}Rb^+$, actions which were not shared by the $Ca^{++}$-channel blocker, verapamil. They concluded that BRL 34915 was exerting its effects via the opening of plasmalemmal K-channels, with resultant hyperpolarisation of the tissue and a decrease in the open-probability of the voltage-operated calcium-channels. These authors also reported that larger

concentrations of BRL 34915 were required to produce hyperpolarisation than were necessary for its mechano-inhibitory effects, thus suggesting that an alternative mechanism might be responsible for the relaxant effects of this agent. It is now well-documented that agents such as cromakalim and RP 49356 and specifically their active enantiomeric forms, levcromakalim and aprikalim (RP 52891) respectively, are able to mediate relaxation of a wide variety of both vascular and non-vascular smooth muscle (for review see Weston & Edwards, 1992). In addition, it is also well-recognised that the relaxant effects of these compounds in smooth muscle are selectively inhibited by the sulphonylureas such as glibenclamide. (Murray *et al.*, 1989; Piper *et al.*, 1990; Edwards *et al.*, 1991).

The K-channel openers can be distinguished from other relaxants by their ability to relax smooth muscle contracted with low (20mM) but not high (80mM) $K^+$-rich solution (Weir & Weston, 1986; Piper *et al.*, 1990; Edwards *et al.*, 1991). This is because when precontracted with 80mM $K^+$-rich solution $E_K$ is shifted into the range of activation of voltage-operated calcium channels, and therefore movement of the resting membrane potential towards $E_K$ by the K-channel opener will not result in the closure of voltage-operated calcium channels. However, in the presence of a 20mM $K^+$-rich solution $E_K$ will still be more negative than the threshold for voltage-operated calcium channels activation. Under these conditions, movement of the resting membrane potential towards $E_K$ will therefore still result in the closure of voltage-operated calcium channels and subsequent relaxation.

A similar profile of action exists for all the members of the K-channel opener group regarding their ability to relax and hyperpolarise smooth muscle. Differences do, however, exist between cromakaiim and the K-channel openers diazoxide, nicorandil and minoxidil sulphate. Diazoxide is able to relax aortic strips precontracted with 80mM KCl, a property not generally shared by other K-channel openers (Newgreen *et al.*, 1990). This would seem to indicate that diazoxide possesses other smooth muscle relaxant qualities in addition to K-

channel opening. As well as its K-channel opening properties, nicorandil is able to mediate relaxation in a manner similar to many nitrovasodilators by activation of soluble guanylate cyclase (Holzmann, 1983). Glibenclamide inhibition of cromakalim is apparently competitive and this is similar for all K-channel openers except for minoxidil sulphate where inhibition by glibenclamide is of a non-competitive nature (Newgreen *et al.*, 1990).

This ability of glibenclamide to inhibit selectively the actions of the K-channel openers has always raised the possibility that agents such as levcromakalim mediate their effects via the opening of $K_{ATP}$ channels. However definitive proof of this fact has not been forthcoming and indeed many other channels such as $BK_{Ca}$ (Carl *et al.*, 1992) have been implicated in the mechanism of action of the K-channel openers. This will be discussed more thoroughly in the following section (1.3.5.1.).

Sheppard and Welsh, (1992) have recently reported that an ATP-sensitive chloride channel ($Cl_{ATP}$) was blocked by both the K-channel openers, levcromakalim and diazoxide and by the sulphonylureas, glibenclamide and tolbutamide. A genetic mutation in this chioride channel is thought to be responsible for causing cystic fibrosis and this $Cl_{ATP}$ is thus also referred to as the 'cystic fibrosis transmembrane conductance regulator' (CFTR). This chloride channel is essentially voltage- and $Ca^{++}$-insensitive and is regulated by protein kinase-dependent phosphorylation, all of which features are common to $K_{ATP}$. The primary difference between these two channels is that whereas the $K_{ATP}$ is *closed* by high intracellular ATP levels, $Cl_{ATP}$ is *activated.* If the mode of action of the K-channel openers involves displacement of ATP from its regulatory site on the channel complex, as suggested by Thuringer and Escande (1989) then this would be consistent with the abiiity of the K-channel openers to activate $K_{ATP}$ and to inhibit $Cl_{ATP}$.

In addition to the ability of these agents to open plasmalemmal K-channels these agents have also been implicated in the modulation of intracellular $Ca^{++}$. Bray *et al.* (1991) suggested that the ability of cromakalim to

relax noradrenaline-induced contractions in rabbit aorta arises partially as a result of inhibiting $Ca^{++}$ uptake into and release from $Ca^{++}$ stores. In a more recent study, Chopra *et al.* (1992) reported that the active enantiomer of cromakaiim, ievcromakaiim was able to reduce the loading of $IP_3$-sensitive $Ca^{++}$ stores, an effect which was reversed by giibenclamide.

The ability of agents such as cromakalim to modulate processes other than K-channel opening may indicate an effect on a (relatively) ubiquitous cellular system rather than a selective action on a K-channel alone.

### 1.3.5.1. K-channel openers: the target channel in smooth muscle

The channel activated by the K-channel openers in smooth muscle has for some time been the subject of contention. As previously mentioned, the ability of sulphonylureas to block the mechano-inhibitory effects of these agents has led many authors to speculate that the K-channel openers activate $K_{ATP}$. However, electrophysiological evidence has never fully confirmed this hypothesis.

In cells from the rat and rabbit mesenteric artery, Standen *et al.* (1989) reported that the K-channel openers activated an ATP-dependent K-channel that was inhibited by the sulphonylurea, glibenclamide. However, the unitary conductance of the channel reported by these authors was much larger (135pS; 60mM $[K]_o$, 120mM $[K]_i$) than the $K_{ATP}$ channels in either pancreatic β-cells or in cardiac myocytes activated by these agents. Indeed, the conductance was closer to that of $BK_{Ca}$ and several other authors (Gelband *et al.*, 1990; Hu *et al.*, 1990; Carl *et al.*, 1992) have suggested that $BK_{Ca}$ is the target of the K-channel openers. However, the lack of effect of charybdotoxin on the relaxant effects of these agents (Wickenden *et al.*, 1991) indicates that even if the K-channel openers are able to modulate the opening of $BK_{Ca}$ under special patch clamp conditions, this probably does not represent the means by which these agents exert their mechano-inhibitory properties in whole tissues.

Clapp and Gurney (1992) reported that $K_{ATP}$ channels are involved in the control of resting membrane potential in pulmonary arterial smooth muscle cells.

These channels are opened by levcromakalim, inhibited by glibenclamide (50μM) and closed by ATP. However, Klöckner and Isenberg (1992) reported that the disodium salt of ATP (also used by Clapp and Gurney, 1992 to close their putative $K_{ATP}$) is able to chelate $Ca^{++}$ leading to a decrease in the opening of $BK_{Ca}$. Thus the identity of the channel modulated in the experiments of Clapp and Gurney (1992) may be questionable.

Using whole-cell perforated patches, Silberberg and van Breemen (1992) reported that metabolic inhibition of rabbit mesenteric artery cells with iodoacetic acid and dinitrophenol induced a K-current, similar in nature to that induced by levcromakalim. Both of the reported currents were voltage-insensitive and blocked by the sulphonylurea, glibenclamide. This led the authors to suggest that the same class of channel carried both the levcromakalim-induced current and that associated with metabolic inhibition and that this was dependent on intracellular concentrations of ATP.

Portal veins have been extensively used to determine the target of the K-channel openers but even here discrepancies exist between reports. Russell *et al.* (1992) reported that in cells of the rabbit portal vein, levcromakalim activates a current that possesses characteristics of $K_{ATP}$. This current increased linearly over the physiological range of potentials but displayed slight inward rectification at potentials positive to +30mV. The current was blocked by glibenclamide and by phentolamine, but its ATP-dependency was not demonstrated.

In rat portal vein, Kajioka *et al.* (1990) reported that nicorandil activated a a low conductance (10pS; quasi-physiological $K^+$ gradient), $Ca^{++}$-dependent current. This current possessed a similar unitary conductance to that reported for $K_{ATP}$ in other tissues and, in addition, this current was also activated by lowering intracellular ATP. Okabe *et al.* (1990) reported that cromakalim activated a $Ca^{++}$-dependent K-current and inhibited a $Ca^{++}$-current simultaneously. They concluded that both of these actions contributed to the vasorelaxant actions of these agents. In this study, the ATP-dependency of the channel was not

demonstrated. In both the studies of Kajioka *et al.* (1990) and of Okabe *et al.* (1990), the activation of a $Ca^{++}$-dependent current by nicorandil and cromakalim, respectively was demonstrated. However, in both studies the inclusion of $Na_2ATP$ in the intracellular solution makes interpretation of the data difficult due to the possible chelation of $Ca^{++}$ as mentioned earlier.

Beech and Bolton (1989) found that the rank order of potency for inhibition of the current induced by cromakalim was identical to the rank order of potency for the inhibition of the delayed rectifier ($I_{K(V)}$) current (phencyclidine > quinidine > 4-AP > TEA). The current induced by cromakalim was essentially voltage-independent and associated with relatively low current noise, implying an underlying channel of relatively low conductance. Beech and Bolton (1989) also found that upon activation of the cromakalim-dependent current, $I_{K(V)}$ was simultaneously inhibited. They speculated that cromakalim was able to modulate the voltage-sensor of the delayed-rectifier channel and to convert this voltage-dependent, inactivating current into one which was voltage-independent and non-inactivating. $I_{K(V)}$ has an underlying conductance of 5pS and this is consistent with the low noise associated with the cromakalim-induced current. Therefore, Beech and Bolton (1989) suggested that cromakalim does not directly open a K-channel but alters the voltage-sensitivity of the delayed-rectifier channel allowing it to open at more negative potentials. However, unlike $I_{K(V)}$, the cromakalim-induced current was sensitive to glibenclamide, a feature which these authors concluded was a serious weakness of their 'channel conversion' idea.

Noack *et al.* (1992a) have recently reported that levcromakalim induces a non-inactivating K-current ($I_{KCO}$) in cells isolated from the rat portal vein. This current was blocked by glibenclamide and carried by a channel with an underlying conductance of approximately 17pS (quasi-physiological K-gradient). In addition, these authors also reported that levcromakalim inhibited the delayed rectifier current, $I_{K(V)}$. In a subsequent report, Noack *et al.* (1992d) demonstrated that removal of substrates for the tricarboxylic acid cycle from the intracellular

solution and removal of glucose from the perfusing solution induced a K-current in these cells. These conditions were designed to cause intracellular ATP depletion. The resultant K-current ($I_{met}$) was carried by a channel of conductance between 10-20pS and Inhibited by glibenclamide. Following run-down of $I_{met}$, levcromakalim was unable to induce a K-current. The authors concluded that both $I_{met}$ and $I_{KCO}$ were in fact carried by a single class of channels which seemed to possess all the characteristics of the $K_{ATP}$ described in pancreatic β-cell and cardiac myocytes.

These findings of Noack *et al.* (1992a,d) are essentially very similar to those reported by Beech and Bolton (1989) with any differences essentially lying in the interpretation of the data. Both groups were able to demonstrate that cromakaiim (or levcromakalim) induced a voltage-independent current carried by a low-conductance channel and inhibited by giibenciamide, and the simultaneous inhibition of $I_{K(V)}$. Whether the inhibition of the delayed rectifier is due to 'dephosphorylation' of the channel (Noack *et al.*, 1992d) or to a change in its voltage-sensitivity giving it the appearance of a non-inactivating current (Beech & Bolton, 1989) remains to be determined.

## 1.4. Specific aims

The work described in this thesis examines the possible existence of the so-called imidazoline / guanidine receptor site and specifically the putative role that this site plays in K-channel modulation. This possibility was studied first using radio-ligand binding studies in rat liver and the existence of a functional correlate was investigated in vascular smooth muscle (Chapter 1).

The target channel and mechanism of action of the structurally-dissimilar K-channel openers has long been the subject of some debate. The possibility that these chemically-diverse agents all activate the same channel in smooth muscle and the mechanism by which they act was examined using whole-cell voltage clamp (Chapter 2).

The ability of ligands at the IGR site to modulate K-channel function in pancreatic β-cells is well-established. Whether this phenomenon occurs in smooth muscle and whether this represents a functional correlate for the IGR site is also addressed (Chapter 3).

# Chapter 2:

# The imidazoline / guanidine receptor site; radio-iigand binding studies and a putative functional correlate in vascular smooth muscle

## 2.1. Introduction

Many recent studies on the IGR site have been conducted using radio-ligand binding and it is now well-documented that this site exists in a number of peripheral tissues (Atlas, 1991). These range from kidney and liver to vascular and non-vascular smooth muscle. Of particular interest are the findings of Zonnenschein and coworkers (1990), who implicated a K-channel in the physiological functioning of the IGR site in the rat liver.

### 2.1.1. Work of Zonnenschein *et al.* (1990)

Zonnenschein *et al.* (1990) reported that an homogenate derived from rat liver was totally devoid of $\alpha_2$-adrenoceptors whilst containing a dense population of IGR sites. Many IGR site ligands also interact with $\alpha$-adrenoceptors and thus the presence of a mixed population of $\alpha$-adrenoceptors and IGR sites complicates the analysis of binding data. For this reason, the homogenate described by Zonnenschein *et al.* (1990) represents an ideal preparation for investigating further the characteristics of the IGR site.

Zonnenschein *et al.* (1990) used displacement of [$^3$H]-idazoxan binding in this homogenate to determine the structural requirements for binding. The rank order of potency described by these authors for the IGR site in the liver suggests that only ligands which possess either an imidazoline or guanidine moiety are likely to have a high affinity for this site. Imidazolines such as cirazoline and naphazoline and guanidines such as guanabenz and guanoxan all exhibited $K_i$ values in the low nanomolar range. In contrast, adrenaline and noradrenaline demonstrated only low affinity ($K_i = 6x10^{-5}M$) for the IGR site. This is indicative of the lack of $\alpha_2$-adrenoceptors in this preparation and also the low affinity that catecholamines exhibit for the IGR site. Zonnenschein *et al.* (1990) reported that in addition to the imidazoline / guanidine ligands, the K-channel blocker 4-AP was also able to displace bound [$^3$H]-idazoxan ($IC_{50}=3.4x10^{-4}M$). Inclusion of the monovalent cations, $NH_4^+$ and $Cs^+$, both of which interfere with K-channel

gating, also decreased [$^3$H]-idazoxan binding at the IGR site. Other K-channel blockers tested were TEA ($10^{-4}$M) which had a relatively low affinity for this site and charybdotoxin ($10^{-5}$M) which did not affect [$^3$H]-idazoxan binding.

A number of authors (North & Surprenant, 1985; Zoltay & Cooper, 1990) have suggested that $\alpha_2$-adrenoceptor-mediated pre-junctional inhibition of neurotransmitter release may be mediated via the activation of K-channels in the neurone terminal. Zonnenschein *et al.* (1990) speculated that the common pharmacological features between $\alpha_2$-adrenoceptors and IGR sites may extend to a common biochemical trigger, ie. that both are linked to K-channel modulation.

It is now becoming apparent that ligands with a high affinity for the IGR site are capable of modulating K-channel function. This is especially apparent in the pancreatic β-cell where imidazolines such as antazoline and tolazoline inhibit the opening of the ATP-dependent K-channel ($K_{ATP}$; Jonas *et al.*, 1992).

### 2.1.2. Work of Soares-da-Silva and Villaneuva (1990)

Soares-da-Silva and Villaneuva (1990) reported that the $\alpha_2$-adrenoceptor agonist, guanabenz was able to relax strips of rat aorta pre-contracted with noradrenaline and 5-hydroxytryptamine (5HT) by a mechanism distinct from its ability to activate $\alpha_2$-adrenoceptors. The lack of involvement of adrenoceptors was demonstrated by performing experiments in the presence of either yohimbine ($\alpha_2$-adrenoceptor antagonist) or propranolol (β-adrenoceptor antagonist). In addition, the relaxant effect of guanabenz was partially inhibited by the K-channel blocker, TEA. These authors concluded that the guanabenz-induced relaxation of this tissue could be attributed to K-channel opening. The structural similarity between agents such as guanabenz and the cyanoguanidine K-channel openers (eg. pinacidil) has recently been highlighted (Edwards & Weston, 1990a) and it may be that the structural similarities between these agents are consistent with a common mechanism of action.

However, Soares-da-Silva and Villaneuva (1990) reported that

guanabenz was unable to relax tissues pre-contracted with $K^+$-rich PSS ($10^{-2}$M). K-channel openers are known to relax rat aorta pre-contracted with $2x10^{-2}$M KCl (Newgreen *et al.*, 1990) and it is therefore possible that the guanabenz-induced relaxation of rat aorta is not mediated via the opening of K-channels but exerted by interference with intracellular $Ca^{++}$ handling. This may explain why guanabenz can relax noradrenaline pre-contracted tissue (dependent on both intra- and extracellular $Ca^{++}$; Bolton, 1985) but not $K^+$-contracted tissues (dependent only on extracellular $Ca^{++}$).

Guanidinium derivatives have been reported to block the $Na^+$ / $H^+$ exchange in cardiac tissue (Frelin *et al.*, 1986). The most potent of these derivatives, guanachlor, bares close structural resemblance to guanabenz (Figure 2.1) and is also known to be a potent IGR site ligand (Vigne *et al.*, 1989). Thus, it is possible that guanabenz causes relaxation of the rat aorta via inhibition of the $Na^+$ / $H^+$ exchange system, a feature of the vasorelaxant properties of amiloride.

The vasorelaxant action of guanabenz is evidently not mediated via adrenoceptor activation and thus it may be mediated via activation of an IGR site. At present, however, it is unclear whether K-channel opening, modulation of intracellular $Ca^{++}$ handling or inhibition of the $Na^+$ / $H^+$ pump is directly responsible for the vasorelaxant mechanism of guanabenz.

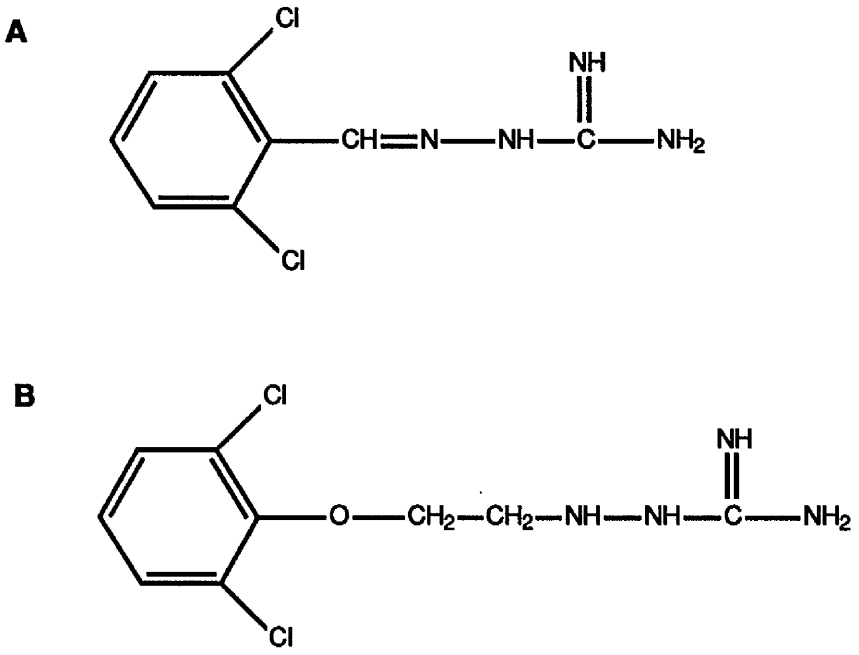



**Figure 2.1**

Structures of (A) guanabenz; [(2,6-dichlorobenzylidene)amino]guanidine and (B) guanachlor; [[2-(2,6-dichlorophenoxy)ethyl]-amino]guanidine. Note the similarities between the two molecules in respect of their guanidine and dichlorobenzene substituents.

### 2.1.3. Aims of the present study

The current investigation was designed to determine whether a direct link exists between the IGR site and K-channel function. The ability of known K-channel modulators to displace [$^3$H]-idazoxan binding at the IGR site was therefore investigated using radio-ligand binding studies. The homogenate described by Zonnenschein *et al.* (1990) was used because of its reported lack of $\alpha_2$-adrenoceptors and also because of the preliminary findings linking this site with K-channel modulation. The second part of this study addresses the possibility that the guanabenz-induced relaxation of the rat aorta represents a functional correlate for the IGR site.

## 2.2. Methods

### 2.2.1. Radio-ligand binding studies

#### 2.2.1.1. Preparation of rat crude liver membrane fraction (Zonnenschein *et al.*, 1990)

Adult, male Sprague Dawley rats were stunned by a blow to the head and killed by cervical dislocation. Livers were dissected directly onto ice and stored at -70°C until required.

Livers were allowed to warm slowly to 4°C and then finely minced using scissors. The resulting fragments were suspended in 0.25M sucrose with $10^{-4}$M phenylmethylsulphonylfluoride, (PMSF), added as an inhibitor of serine proteases, to a volume equivalent to 10ml/g of liver. The tissue was disrupted using a motor-driven teflon pestle (15 strokes at 1500rpm) and the suspension was centrifuged at 600xg and 4°C for 10min. The pellet, which consisted of nuclei and other large debris was discarded and the supernatant was then re-centrifuged at 12000xg and 4°C for 30min. The resulting supernatant was discarded and the pellet, resuspended in $5x10^{-2}$M Tris/HCl, pH=7.4, to a final protein concentration of 10mg/ml and then stored in aliquots of 10ml at -70°C.

#### 2.2.1.2. Preparation of rat brain homogenate

Frozen rat brains obtained from Charles River were homogenised in 10ml/g of ice-cold Tris/HCl buffer pH=7.4, at 5°C using a polytron, setting 5, for 20s. The homogenates were pooled and then centrifuged for 20min at 50000xg in a Sorvall centrifuge. After spinning, the supernatant was discarded and the pellet resuspended in fresh ice-cold buffer (10ml/g), using the polytron (setting 5). The suspension was re-centrifuged at 50000xg for 20min and the supernatant discarded. The final pellet was homogenised using the polytron (setting 5 for 20s) in 10ml/g of ice-cold Tris/HCl ($5x10^{-2}$M) . The homogenate was then stored in aliquots at -70°C until required.

### 2.2.1.3. Protein determination

Protein analysis was conducted using a Sigma Diagnostics Total Protein kit and Sigma Protein standards.

### 2.2.1.4. Binding of [$^3$H]-idazoxan to rat liver membranes

#### 2.2.1.4.1. Displacement analysis

Tissue aliquots were thawed to 4°C and made up to a final protein concentration of 1mg/ml in Tris/HCl ($5x10^{-2}M$) + $MgCl_2$ ($2x10^{-3}M$), pH=7.4. Assays were performed in 96-well microtitre blocks and dilutions were conducted using a Tecan diluting robot (RSP 5402).

The protein homogenate was dispersed for approximately 30sec prior to incubation to ensure total homogeneity of the preparation. 400µl of protein homogenate was incubated with [$^3$H]-idazoxan ($4x10^{-9}M$) and the appropriate concentration of displacing agent at a final volume of 500µl for 40min at room temperature. Non-specific binding was defined in the presence of cold idazoxan ($10^{-5}M$). Unbound ligand was separated from the bound fraction using a Brandel cell harvester (MB48R from Semat) by vacuum filtration through GF/B filters. These filters were pre-soaked for a minimum of 30min in polyethyleneimine (PEI; 0.5%) in Tris/HCl ($5x10^{-2}M$) + $MgCl_2$ ($2x10^{-3}M$) to reduce any non-specific binding to the fibres in the filter. The tissue was then washed four times with approximately 2ml Tris/HCl ($5x10^{-2}M$) + $MgCl_2$ ($2x10^{-3}M$), pH=7.4 to remove the unbound ligand. The filters were removed to vials and following addition of approximately 4ml of Ecoscint A, radioactivity was determined using liquid scintillation spectroscopy.

#### 2.2.1.4.2. Saturation analysis

Tissue samples were prepared as outlined for displacement analysis and then incubated in the presence of increasing concentrations of [$^3$H]-idazoxan ($10^{-10}$-4x$10^{-8}$M) for 40min at room temperature. All incubations were performed in duplicate and non-specific binding for each [$^3$H]-idazoxan concentration was defined in the presence of either cold idazoxan ($10^{-5}$M) or cirazoline ($10^{-5}$M). Experiments were terminated and radioactivity levels evaluated in a manner identical to that outlined for the displacement analysis. Calculation of precise concentration of ligand present and ligand bound were made on-line via an IBM PC and saturation isotherms computer-fitted to the data.

### 2.2.1.5. [$^3$H]-idazoxan binding to the rat brain homogenate

The protocol was identical to that performed for [$^3$H]-idazoxan binding in the rat liver with the following amendments.

1. The final protein concentration in the assay was 0.5mg/ml.
2. Tissue and ligand were incubated together for 60min at room temperature.

Saturation analysis was also performed using the rat brain homogenate in a manner identical to that described for [$^3$H]-idazoxan saturation analysis in the liver. Again, non-specific binding was defined in the presence of either idazoxan ($10^{-5}$M) or cirazoline ($10^{-5}$M).

### 2.2.1.6. [$^3$H]-glibenclamide binding to the rat liver homogenate

The protocol was identical to that performed for [$^3$H]-idazoxan binding in the rat liver with the following amendments.

1. The buffer used throughout the assay was Tris/HCl ($5x10^{-2}M$), pH=7.4 containing the following

| | | |
|---|---|---|
| KCl | - | $5x10^{-3}M$ |
| $CaCl_2$ | - | $2.5x10^{-3}M$ |
| $MgCl_2$ | - | $1.2x10^{-3}M$ |
| NaCl | - | $136x10^{-3}M$ |

2. The final [$^3$H]-glibenclamide concentration in the assay was $10^{-9}M$.

3. The non-specific binding was defined in the presence of cold glibenclamide ($10^{-5}M$).

4. Filters were presoaked for at least 2h in PEI (0.5%) and Tris/HCl ($5x10^{-2}M$) together with the supplements described in ammendent 1.

### 2.2.1.7. [$^3$H]-glibenclamide binding to the rat brain homogenate

The protocol was identical to that performed for [$^3$H]-idazoxan binding in the rat liver with the following amendments.

1. The buffer used throughout the assay was Tris/HCl ($5x10^{-2}M$), pH=7.4 containing the following.

| | | |
|---|---|---|
| KCl | - | $5x10^{-3}M$ |
| $CaCl_2$ | - | $2.5x10^{-3}M$ |
| $MgCl_2$ | - | $1.2x10^{-3}M$ |
| NaCl | - | $136x10^{-3}M$ |

2. The final [$^3$H]-glibenclamide concentration in the assay was $10^{-9}M$.

3. The final protein concentration in the assay was 0.5mg/ml.

4. The non-specific binding was defined in the presence of cold glibenciamide ($10^{-5}M$).

5. Tissue and ligand were incubated together for 60min at room temperature.

## 2.2.2. $^{42}K^+$-efflux experiments

### 2.2.2.1. Tissue preparation

Male Sprague Dawley rats were killed by stunning and cervical dislocation. The thoracic aorta was removed into physiological salt solution (PSS) and cleaned of all extraneous connective tissue. Tissues were then opened along their longitudinal axis and the endothelium removed by gentle rubbing with a cotton wool bud soaked in PSS. Tissues were mounted on needles and the ends of the needles bent to secure the tissues. The needles were then mounted on a perspex gasing manifold and the manifold placed over a rack of vials containing PSS such that each tissue was submerged in an individual vial. Tissues were continually aerated with 95% $O_2$ / 5% $CO_2$.

### 2.2.2.2. Experimental protocol

Tissues were allowed to equilibrate for 30min in normal PSS and then transferred to vials containing $^{42}K^+$ ($1.57\mu Ciml^{-1}$) for 180min. $^{42}K^+$ was then allowed to efflux from the tissues into 3ml aliquots of PSS during 4min collection periods. After 8 such periods, tissues were exposed to PSS containing levcromakalim ($5x10^{-6}M$), to PSS containing guanabenz ($10^{-5}M$) and RX 821002 ($10^{-6}M$) or to the appropriate vehicle control for the next five collection periods. For the last four collection periods, the tubes contained PSS alone. The tissues were then blotted and their $^{42}K^+$ content was determined together with that in the collecting tubes using a Packard gamma counter. Correction for decay due to the short half-life were made automatically.

### 2.2.3. Tension recording experiments

#### 2.2.3.1. Preparation of tissue

Male Sprague Dawley rats (250-350g) were killed by stunning and cervical dislocation. A portion of the thoracic aorta was removed and cut into strips each approximately 0.5cm in length. The resulting preparations, with endothelium intact, were then mounted for isometric recording, under a resting tension of 1g, in a tissue bath containing Krebs physiological salt solution (PSS) at 37°C and aerated with 95% $O_2$ / 5% $CO_2$. After a 45min equilibration period the tension was readjusted to 1g and the preparations were left for a further 15min. Changes in tension were recorded on a Maclab chart system (W.P. Instruments); the integral of the tension : time curve over a 4min period was used to quantify mechanical changes.

#### 2.2.3.2. Guanabenz-induced relaxation of rat aorta

In preliminary experiments, the degree of relaxation observed with guanabenz relaxation / response protocols increased with each subsequent cumulative protocol. To counteract this, tissues were first exposed to two priming doses of guanabenz ($10^{-5}$M) for 15min in order to minimise these changes in tissue sensitivity. These priming doses were separated by a 30min washing period. Tissues were then allowed to recover for 1h before commencement of dose-response experiments.

Tissues were contracted with noradrenaline ($3x10^{-7}$M; an approximate $EC_{90}$), U-46619 ($2x10^{-8}$M; an approximate $EC_{80}$) or $K^+$-substituted PSS ($2.5x10^{-2}$M; $K^+$ isosmotically substituted for $Na^+$) and 12min was allowed for the development of a full response. Thereafter guanabenz ($10^{-7}$M-$10^{-5}$M) was added cumulatively with a 5min dosing interval between successive concentration increments. The final 4min of this dosing interval was used for calculation of the drug effect using the integral of the tension. Tissues were allowed to recover for 1h between dose-response protocols; exposure to

modifying agents was made during the last 30min of each 1h period and in the continuing presence of the noradrenaline and guanabenz. Appropriate time- and vehicle-matched control experiments were conducted in the absence of modifying agent. In certain experiments, levcromakalim ($10^{-8}$M-$10^{-6}$M) was used to assess whether a known K-channel opener could cause vasorelaxation under the conditions of the experiment. In other experiments, RX 821002 ($10^{-6}$M) was added to the PSS in order to eliminate $\alpha_2$-adrenoceptor-mediated effects. In spite of the initial priming exposure to guanabenz (see above), the relaxant effect of guanabenz increased somewhat with time. For this, reason dose-response curves in the presence of guanabenz were compared with those obtained from the appropriate time- and vehicle-matched control performed on contiguous sections of tissue.

In certain experiments the role of the endothelium in the guanabenz-induced relaxation was investigated. The endothelium was destroyed by gentle rubbing with a cotton wool bud soaked in PSS. In these experiments the integrity of the endothelium was assessed via acetylcholine-induced relaxation. The endothelium was deemed sufficiently destroyed if acetylcholine ($10^{-6}$M) exerted no relaxant effect upon noradrenaline pre-contracted tissue.

### 2.2.3.3. Noradrenaline and KCl dose-response curves

The effects of modulators on the noradrenaline- or KCl-induced contracture of the rat aorta were investigated as follows. Tissues were mounted as for the guanabenz relaxation studies and tension equilibrated to 1g in a similar manner. Dose-response curves to either noradrenaline ($10^{-9}$M-$10^{-4}$M) or KCl ($5\times10^{-3}$M - $8\times10^{-2}$M) were constructed and then repeated in the presence of modifying agents. 1h was allowed for recovery between subsequent dose-response protocols, the modifying agent being added to the bath for the final 30 min of this recovery period. Appropriate time- and vehicle-controlled experiments were conducted on contiguous sections of tissues.

### 2.2.4. Drugs and solutions

*Binding studies*

[$^3$H]-idazoxan (41-42 Ci/mMol) was purchased from Amersham, England. [$^3$H]-glibenclamide (50.9 Ci/mMol) was purchased from Du Pont. TEA, diazoxide, (-)-noradrenaline, (±)-noradrenaline, (-)-adrenaline, (±)-adrenaline, apamin, 4-aminopyridine, amiloride, guanabenz and glibenclamide were obtained from Sigma. RX 821002, yohimbine, urapidil, idazoxan and dendrotoxin were purchased from RBI. UK-14,304, clonidine, pinacidil, P1060, levcromakalim, guanethidine, glipizide, aprikalim, RX 801023 and ciclazindol were all obtained from Pfizer compound control, Pfizer Central Research, Sandwich, Kent. Rauwoiscine was obtained from Roth and charybdotoxin from Natural Science Products, Utah. Tedisamil was obtained from Kali Chemie Pharma GMBH and cirazoline from Synthelabo. All agents were dissolved in either ethanol or distilled water and then diluted to the required concentration in Tris/HCl.

*Tension recording studies*

The PSS used had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.55, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The K-rich (20mM) PSS had a composition identical to that for normal PSS but with the following modifications:- NaCl 100.3mM and KCl 19mM.

Noradrenaline, guanabenz, glibenclamide, idazoxan, TEA, 4-AP, prazosin and U-46619 were obtained from Sigma. RX 821002 and RX 801023 were gifts from Reckitt and Colman. Levcromakalim was obtained from Pfizer Central Research, Kent. Noradrenaline (10mM) was made up in N/10 HCl with ascorbate added (1% v/v) to maintain the stability in solution. Guanabenz (10mM), levcromakalim (10mM), U-46619 (1mM) and glibenclamide (5mM) were made up to stock concentrations in ethanol. RX 801023 (1mM), RX 821002 (1mM), 4-AP (1M), idazoxan (1mM), prazosin (1mM) and TEA (1M) were readily soluble in distilled water.

*$^{42}K^+$-efflux experiments*

The physiological salt solution was of identical composition to the 'normal' PSS described for tension recording studies. Guanabenz was obtained from Sigma, RX 821002 from Reckitt and Colman and levcromakalim was obtained from Pfizer Central Research, Kent. $^{42}K^+$ ($K_2CO_3$) was obtained from the University of Manchester reactor, Risley. Agents were dissolved in the same vehicle as described in the tension recording studies.

### 2.2.5. Data analysis

*Binding studies*

Scatchard analysis of saturation isotherms was performed on-iine and Bmax and Kd values were obtained from the line of best fit. Bmax values were calculated from the intercept of the line with the abscissa and Kd values as the reciprocal of the slope of the line (see Bennett & Yamamura, 1985). $IC_{50}$ values are computer-derived from the best fitted sigmoidal function. Each agent was performed with a minimum of 3 replicates and the $IC_{50}$ in Tables 2 and 3 are -log M concentrations $\pm$ sem.

*Tension recording studies*

The relaxation of tissues by guanabenz and levcromakalim is expressed as a percentage of the tension of the tissue immediately prior to addition of the first concentration of relaxant. Statistical analysis of the results was performed using a paired Student's t-test (Statworks). For clarity, only those differences occurring with a probability level of $(p)<0.05$ are indicated.

*$^{42}K^+$-efflux experiments*

The efflux data were expressed in terms of the rate coefficient (fractional loss of $^{42}K^+$ from the tissue standardised for a 1min period) expressed as a percentage.

$$\text{Rate coefficient (\%min)} = \frac{^{42}K^+ \text{ lost during collection period}}{\text{mean tissue } ^{42}K^+ \text{ content during this period}} \times \frac{100}{\text{time}}$$

Efflux rate coefficients were determined from the raw counts using a Basic computer programme 'Efflux 1'.

Statistical analysis of the results was performed using a paired Student's t-test.

## 2.3. Results

### 2.3.1. Radio-ligand binding studies

#### 2.3.1.1. [$^3$H]-idazoxan saturation binding analysis

Saturation analysis of [$^3$H]-idazoxan binding was performed on data obtained from rat liver and rat brain homogenates. Non-specific binding (NSB) in each case was defined either by idazoxan ($10^{-5}$M; Figures 2.2 & 2.4) or by cirazoline ($10^{-5}$M; Figures 2.3 & 2.5). Scatchard analysis of the data (performed according to Bennett & Yamamura, 1985) yields values for the Bmax and the dissociation constant, Kd (Figures 2.2, 2.3, 2.4 & 2.5; Table 2.1). The Bmax is defined as the maximum number of specific receptor sites. Bmax values obtained from the liver homogenate are similar for cirazoline and idazoxan NSB. In the brain homogenate however, the Bmax values defined by cirazoline and idazoxan NSB differed (Table 2.1). The dissociation constant (Kd) is a ratio of the binding equilibrium reactions forward and reverse rate constants. This value provides a measure of the affinity of the drug for a particular receptor. The Kd is calculated as the reciprocal of the slope of the line. In the liver assays, the Kd values for idazoxan and cirazoline which were used to define NSB do not appear to differ. However, the Kd values for these agents to define NSB in the brain preparation do appear to be different. Bmax and Kd values were calculated for each preparation and these are summarised in Table 2.1.

#### 2.3.1.2. [$^3$H]-idazoxan displacement analysis

Compounds were tested for their ability to displace [$^3$H]-idazoxan binding to the IGR site in the rat liver homogenate. These were predominantly agents which modulate $\alpha$-adrenoceptor function although compounds with other pharmacological profiles such as guanethidine, amiloride and pinacidil were also investigated. A rank-order of potency was constructed, based on the $IC_{50}$ values of the compounds which demonstrated a relatively high affinity for the IGR

site. The $IC_{50}$ is defined as the concentration of drug required to cause a 50% inhibition of the specific [$^3$H]-idazoxan binding. In these experiments, specific binding (NSB defined by idazoxan, $10^{-5}$M) represented more than 80% of total binding. The $IC_{50}$ values of agents exhibiting a relatively high affinity for the IGR site and their structures are shown in Tables 2.2 and 2.3.

Cirazoline was the most potent of the compounds tested with an $IC_{50}$ of $5x10^{-9}$M. Other agents with $IC_{50}$ values in the low nanomolar range were idazoxan itself, guanabenz and UK-14,304 (Tables 2.2 & 2.3). RX 801023 is a derivative of the $\alpha_2$-adrenoceptor antagonist, RX 821002 and is reported to possess a thirty-fold selectivity for the IGR site as opposed to the $\alpha_2$-adrenoceptor (Mallard *et al.*, 1991). RX 801023 potently displaces [$^3$H]-idazoxan from the IGR site in the liver with an $IC_{50}$ of $1.2x10^{-8}$M. (Table 2.2). Other agents active at this site but with $IC_{50}$ values approximately one to two orders of magnitude higher are clonidine, guanethidine, pinacidil and amiloride (Tables 2.2 & 2.3).

In addition to those compounds which exhibited a relatively high affinity for the IGR site, a number of agents were tested and found to exhibit low affinities for this site (Table 2.4). Inactivity was defined as those agents possessing an $IC_{50}$ for the IGR site exceeding $10^{-5}$M. These inactive compounds include such agents as prazosin and phentolamine as well as the catecholamines, adrenaline and noradrenaline. The catecholamines were tested both as the negative (active) enantiomer and also as the racemic mixture. No difference in affinity for the site was observed between these two forms in either case. Ethanol vehicle treatment alone had no effect on the [$^3$H]-idazoxan binding.

### 2.3.1.3. Interaction of K-channel modulators with the IGR site

A series of compounds known to modulate the opening of K-channels was tested for their ability to displace [$^3$H]-idazoxan binding in an attempt to determine whether a close link exists between the IGR site and a K-channel. All K-channel modulators tested, with the exception of pinacidil were unable to displace [$^3$H]-idazoxan from the IGR site. TEA and 4-AP were tested to a concentration of $10^{-3}$M in the assay and were found to have no effect on the binding of [$^3$H]-Idazoxan at these concentrations. Charybdotoxin, dendrotoxin (up to $10^{-7}$M) and apamin (up to $10^{-6}$M) were also found to be ineffective at these concentrations. Pinacidil displaced the binding of [$^3$H]-idazoxan from the IGR site with an $IC_{50}$ of $4.1x10^{-6}$M (Table 2.3). P1060, a potent analogue of pinacidil in terms of K-channel opening properties, had no effect on binding when tested up to a concentration of $10^{-4}$M (Table 2.4).

### 2.3.1.4. [$^3$H]-glibenclamide binding in the rat liver homogenate

[$^3$H]-glibenclamide binding studies were performed using the rat liver homogenate. The agents identified as potent IGR site ligands were tested for their ability to displace [$^3$H]-glibenclamide binding to assess whether glibenclamide-sensitive (ATP-sensitive) K-channels ($K_{ATP}$) were modulated by IGR site ligands.

A population of glibenclamide-sensitive sites was identified within the liver but displacement analysis was made difficult because of the small size of this population. This problem was compounded by the large proportion of non-specific glibenclamide binding which represented upwards of 60% of total binding and was, in some cases as high as 85-90% of the total. As a result of this, analysis of the data obtained was impossible and so no results were obtained from this study.

### 2.3.1.5. [$^3$H]-glibenclamide binding in the rat brain homogenate

A study of [$^3$H]-glibenclamide binding was performed using rat brain homogenate. This is a preparation in which a population of glibenclamide-sensitive sites is known to exist (Angel & Bidet, 1991) and so the interaction of IGR site ligands with glibenclamide binding sites could be investigated more easily. Identification of a population of glibenclamide binding sites within the rat brain that are modulated by IGR site ligands would provide preliminary evidence for the involvement of a K-channel ($K_{ATP}$) in the physiological functioning of the IGR site.

The compounds identified as potent IGR site ligands in the liver were tested for their ability to displace [$^3$H]-glibenclamide binding (Table 2.5). Glibenclamide and glipizide (a sulphonylurea derivative) both displaced [$^3$H]-glibenclamide binding with $IC_{50}$ values of $2x10^{-9}$M and $10^{-8}$M respectively. None of the imidazoline- or guanidine-based ligands was able to displace the [$^3$H]-glibenclamide binding (Table 2.5).

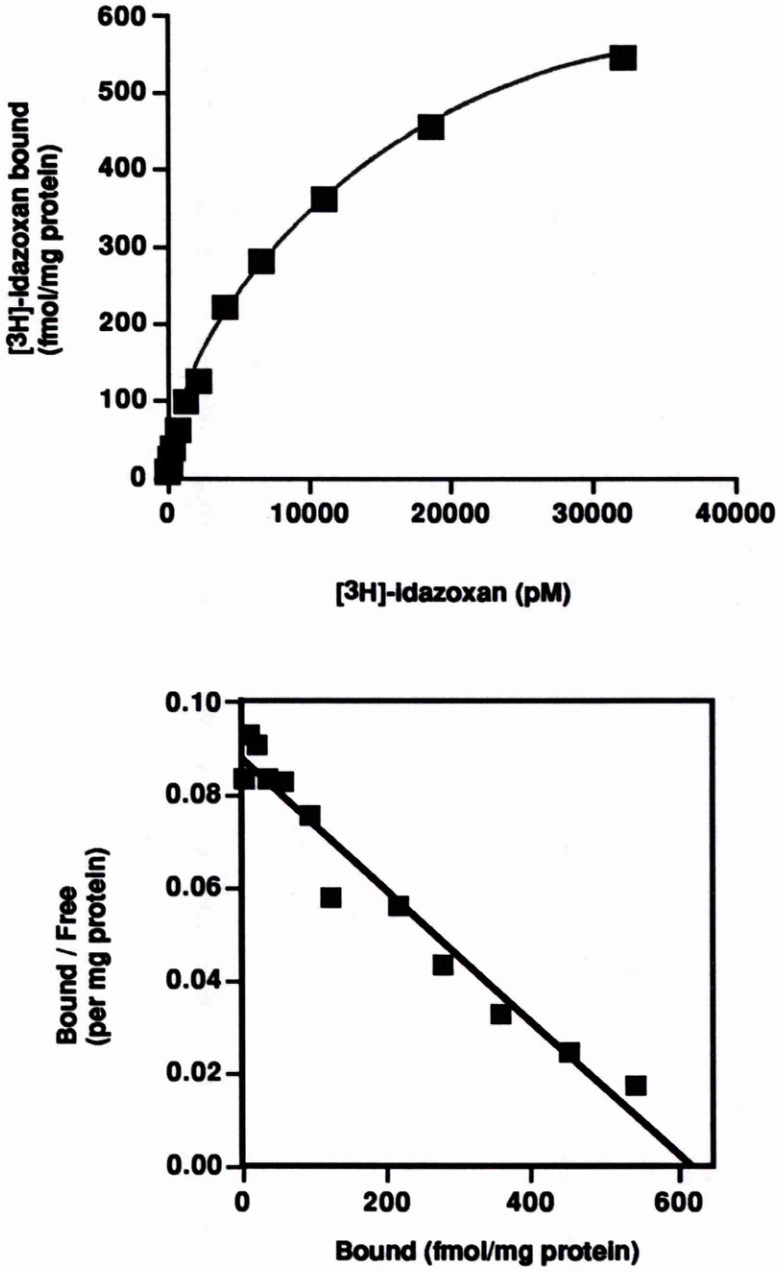



**Figure 2.2.** Saturation analysis of [$^3$H]-idazoxan binding in the rat liver homogenate. Upper panel: Specific binding of [$^3$H]-idazoxan. Non-specific binding was defined as that binding in the presence of idazoxan ($10^{-5}$M) and represented 5-10% of total binding. Lower panel: Scatchard plot for [$^3$H]-idazoxan specific binding. Bmax and Kd values were calculated with a computer curve-fitting programme and are summarised in Table 2.1.

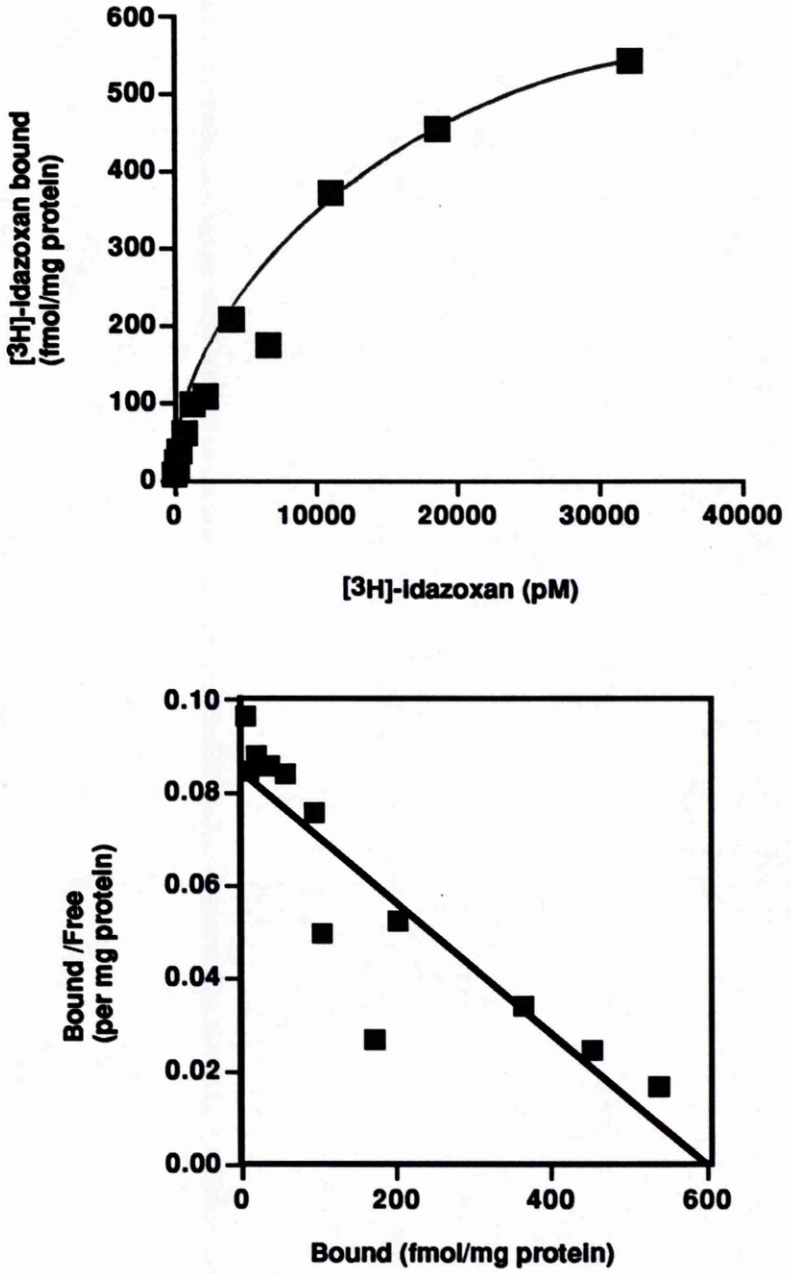



**Figure 2.3.** Saturation analysis of [$^3$H]-idazoxan binding in the rat liver homogenate. Upper panel: Specific binding of [$^3$H]-idazoxan. Non-specific binding was defined as that binding in the presence of cirazoline ($10^{-5}$M) and represented 5-10% of total binding. Lower panel: Scatchard plot for [$^3$H]-idazoxan specific binding. Bmax and Kd values were calculated with a computer curve-fitting programme and are summarised in Table 2.1.

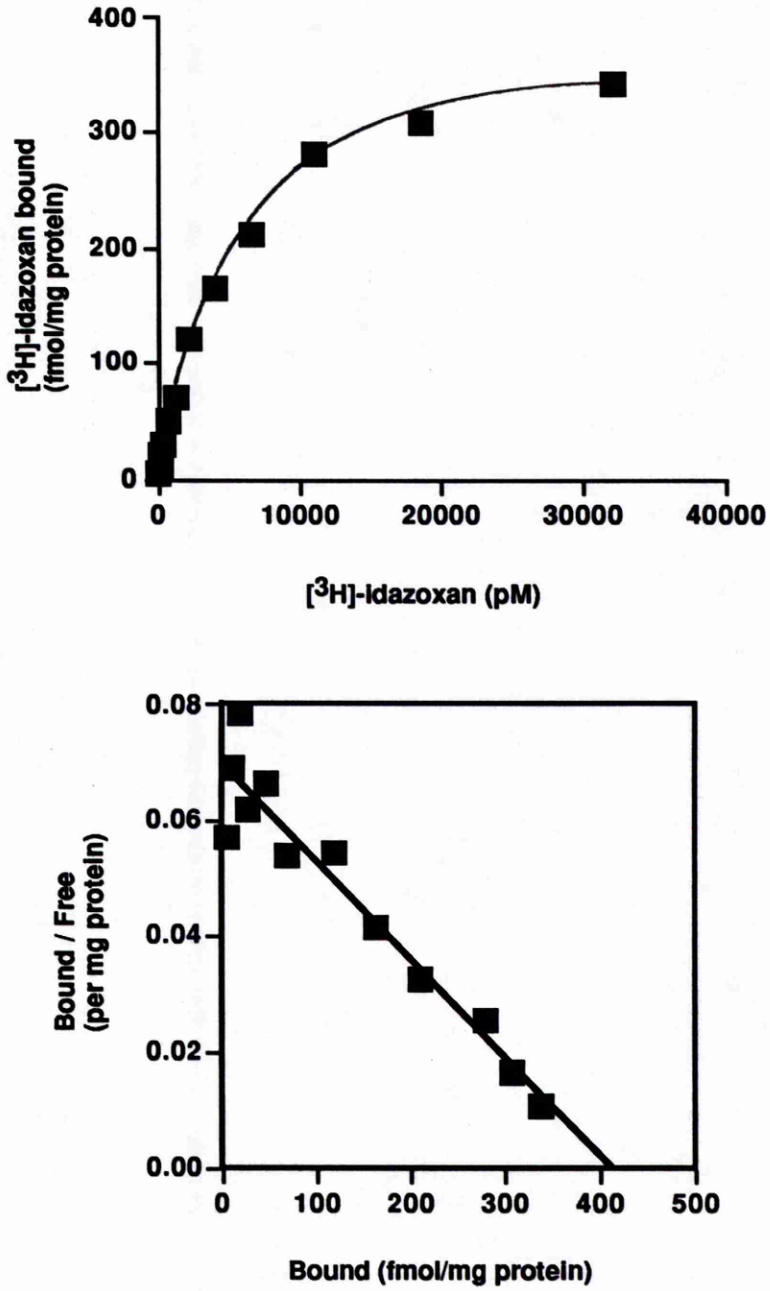



**Figure 2.4.** Saturation analysis of [$^3$H]-idazoxan binding in the rat brain homogenate. Upper panel: Specific binding of [$^3$H]-idazoxan. Non-specific binding was defined as that binding in the presence of idazoxan ($10^{-5}$M) and represented 10-15% of total binding. Lower panel: Scatchard plot for [$^3$H]-idazoxan specific binding. Bmax and Kd values were calculated with a computer curve-fitting programme and are summarised in Table 2.1.

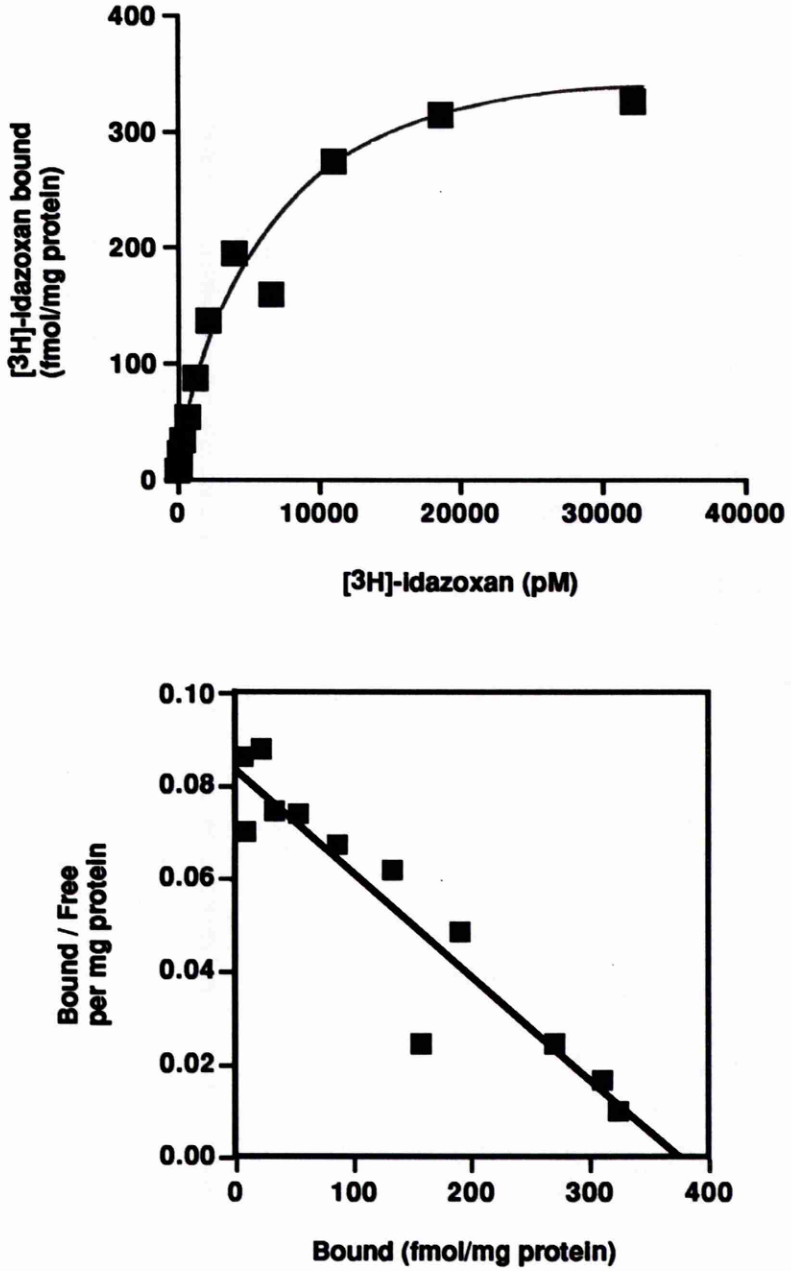



**Figure 2.5.** Saturation analysis of [$^3$H]-idazoxan binding in the rat brain homogenate. Upper panel: Specific binding of [$^3$H]-idazoxan. Non-specific binding was defined as that binding in the presence of cirazoline ($10^{-5}$M) and represented 10-20% of total binding. Lower panel: Scatchard plot for [$^3$H]-idazoxan specific binding. Bmax and Kd values were calculated with a computer curve-fitting programme and are summarised in Table 2.1.

| Preparation | Non-Specific Binding | $B_{max}$ (fmol/mg protein) | Kd (nM) |
|---|---|---|---|
| Liver | Idazoxan | 619 | 7055 |
| | Cirazoline | 600 | 7128 |
| Brain | Idazoxan | 413 | 5916 |
| | Cirazoline | 376 | 4509 |

**Table 2.1.** Summary of the Bmax and Kd values calculated by Scatchard analysis of the saturation isotherms. Bmax and Kd values were calculated for specific binding according to Bennett & Yamamura, (1985) with NSB defined by both idazoxan and cirazoline in liver and brain homogenates.

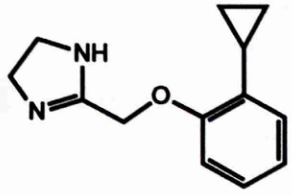
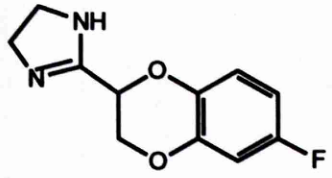
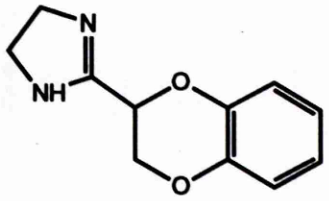
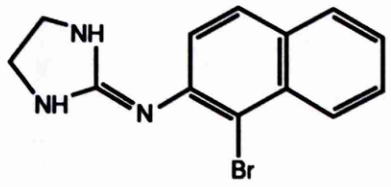
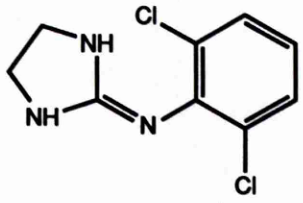
| Substance | Structure | -Log $IC_{50}$ ± s.e.m |
|---|---|---|
| **CIRAZOLINE** |  | **8.34 ± 0.116** |
| **RX 801023** |  | **7.93 ± 0.06** |
| **IDAZOXAN** |  | **7.87 ± 0.06** |
| **UK-14,304** |  | **6.35 ± 0.01** |
| **CLONIDINE** |  | **5.38 ± 0.06** |

**Table 2.2.** Displacement analysis of [$^3$H]-idazoxan binding in the rat liver homogenate by agents containing an imidazoline moiety. $IC_{50}$ values are given as -log M concentrations. Values are the mean of between 3 and 6 observations ± the s.e.mean.

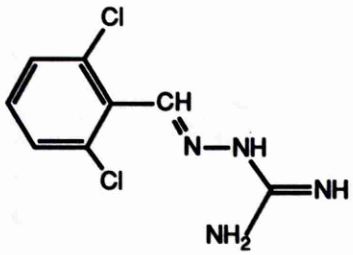
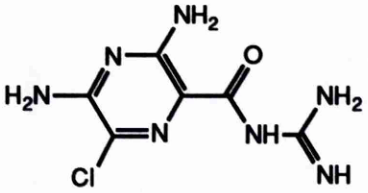
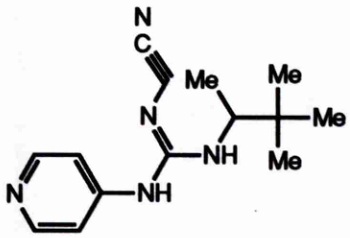
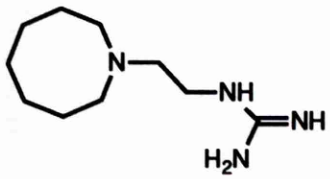
| Substance | Structure | -Log $IC_{50}$ ± s.e.m |
|---|---|---|
| **GUANABENZ** |  | **7.66 ± 0.04** |
| **AMILORIDE** |  | **5.98 ± 0.1** |
| **PINACIDIL** |  | **5.39 ± 0.12** |
| **GUANETHIDINE** |  | **5.31 ± 0.13** |

**Table 2.3.** Displacement analysis of [$^3$H]-idazoxan binding in the rat liver homogenate by agents containing a guanidine moiety. $IC_{50}$ values are given as -log M concentrations. Values are the mean of between 3 and 6 observations ± the s.e.mean.

| α-adrenoceptor ligands | potassium channel modulating ligands |
|---|---|
| phentolamine | tetraethylammonium |
| RX 821002 | 4-aminopyridine |
| yohimbine | levcromakalim |
| rauwolscine | P1060 |
| urapidil | aprikalim |
| prazosin | glibenclamide |
| adrenaline | glipizide |
| noradrenaline | diazoxide |
| | ciclazindol |
| | tedisamil |
| | apamin |
| | charybdotoxin |
| | dendrotoxin |

**Table 2.4.** This table illustrates those agents unable to displace [$^3$H]-idazoxan binding from the IGR site in the rat liver homogenate. Agents exhibiting $IC_{50}$ values in excess of $10^{-5}$M were defined as inactive, with the following exceptions. Dendrotoxin and charybdotoxin were assayed to a highest concentration of $10^{-7}$M and apamin to a highest concentration of $10^{-6}$M and no displacement was observed at these concentrations. TEA and 4-AP were assayed up to a highest concentration of $10^{-3}$M and again, no displacement of the [$^3$H]-idazoxan was observed. Adrenaline and noradrenaline were tested both as racemates and as the individual enantiomers and found to be inactive in both cases.

| Displacing compound | $IC_{50}$ (- Log M) |
|---|---|
| glibenclamide | 8.69 (0.12) |
| glipizide | 8.04 (0.16) |
| idazoxan | >5 |
| cirazoline | >5 |
| UK-14,304 | >5 |
| guanabenz | >5 |

**Table 2.5.** This table illustrates the inability of potent IGR site ligands to displace [$^3$H]-glibenclamide binding in the rat brain homogenate. Non-specific binding in these experiments was defined in the presence of glibenclamide ($10^{-5}$M). Inactivity is defined as $IC_{50}$ values in excess of $10^{-5}$M. $IC_{50}$ values are expressed as -log M values and the figures in parentheses represent the s.e.mean.

### 2.3.2. $^{42}K^+$-efflux experiments

Basal efflux levels (1.24 ± 0.04%/min; 20-28min) were unaffected by the presence of ethanol vehicle treatment (1.19 ± 0.01%/min; 32-40min). Application of either levcromakalim ($5x10^{-6}M$) or guanabenz ($10^{-5}M$) and RX 821002 ($10^{-6}M$) caused a significant increase in the rate of $^{42}K^+$ efflux from the tissues when compared to vehicle-treated tissues (guanabenz: 32-40min, $p<0.05$; levcromakalim: 32-48min, $p<0.05$: Student's paired t-test, n=6 in both cases; Figure 2.6.).

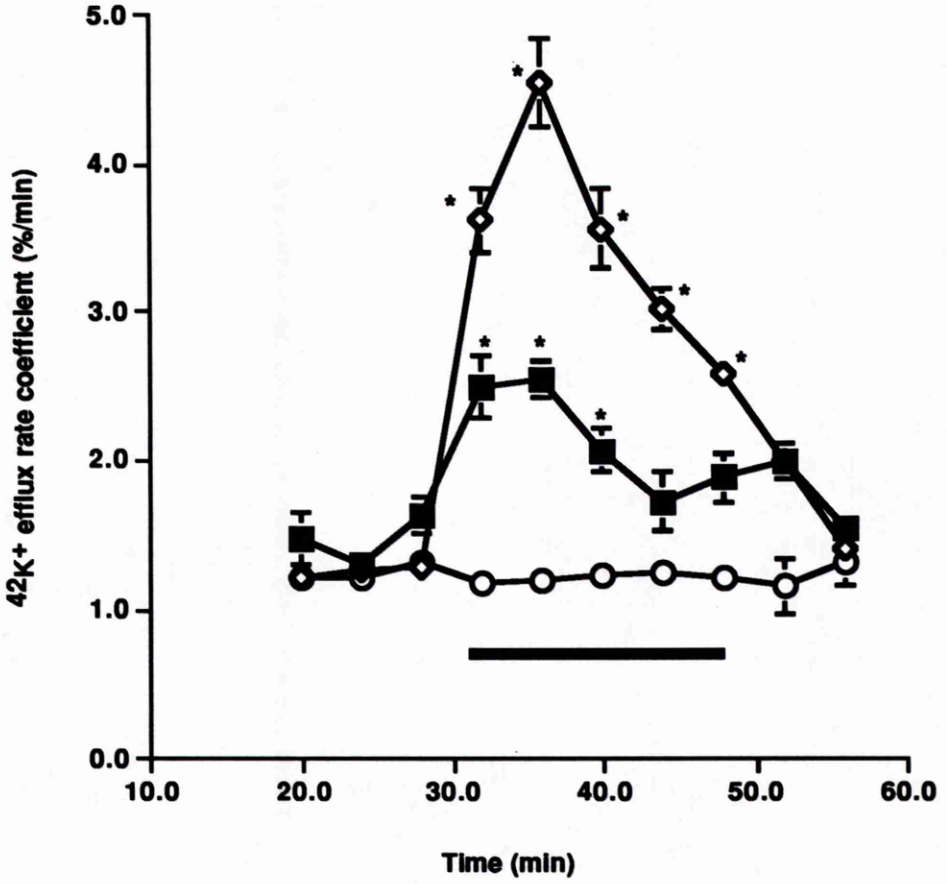



**Figure 2.6.** Guanabenz- and levcromakalim-induced $^{42}K^+$-efflux from strips of rat aorta. (○) vehicle; (◊) levcromakalim ($5x10^{-6}$M); (■) guanabenz ($10^{-5}$M) and RX 821002 ($10^{-6}$M). Drugs were applied for the period denoted by the horizontal bar. In the case of guanabenz, RX 821002 was present in the PSS throughout the duration of the experiment. * indicate significant increases in the efflux rate coefficient compared to vehicle controls (Student's t-test; $p<0.05$). Points are the mean of 6 observations and vertical bars denote the s.e.mean.

### 2.3.3. Tension recording studies

Guanabenz ($10^{-7}$-$10^{-5}$M) caused a dose-dependent relaxation of strips of rat aorta pre-contracted with noradrenaline ($3x10^{-7}$M; an approximate $EC_{90}$, Figure 2.7). The sensitivity of the tissue to guanabenz appeared to increase upon reconstruction of the dose-response protocols and thus the degree of relaxation became more pronounced with repeated dose-response curves (Figure 2.7). In an attempt to counteract this problem, tissues were pretreated with two priming doses of guanabenz ($10^{-5}$M) for 15min separated by a 30min washing period (every 10min) prior to the commencement of relaxation protocols. Following these priming doses, there was still some increase in the degree of relaxation between the first and second curves but subsequent curves were superimposable (Figure 2.7). Second and third relaxation curves were thus used to examine the nature of this relaxation with vehicle effects being examined on contiguous sections of tissue. In this manner, two concentrations of antagonist were examined and in all cases the first relaxation response protocol in test and control (time and vehicle) tissues were superimposable (not shown). The use of contiguous tissues as controls eliminated any influence that changes in tissue sensitivity might incur between repeated dose-response protocols.

The $\alpha_2$-adrenoceptor antagonist, RX 821002 had no effect on the guanabenz-mediated relaxation of rat aorta ($10^{-6}$M; Figure 2.8). In addition, neither the selective IGR site ligand, RX 801023 ($10^{-5}$M; Figure 2.8) nor the $K_{ATP}$-inhibitor, glibenclamide ($10^{-5}$M & $10^{-6}$M; Figure 2.9), was able to inhibit the guanabenz-induced relaxation. RX 801023 did however slightly decrease the magnitude of the noradrenaline contraction. The imidazoline-based $\alpha_2$-adrenoceptor antagonist, idazoxan ($10^{-6}$M) also had no effect on the guanabenz-induced relaxation (Figure 10). However, when the concentration of idazoxan was increased ($10^{-5}$M), a significant inhibition of the guanabenz-induced relaxation was observed (Figure 10; $p<0.05$, paired Student's t-test). The

non-selective K-channel blockers TEA ($3x10^{-3}M$ & $10^{-2}M$; Figure 2.11) and 4-AP ($10^{-3}M$ & $3x10^{-3}M$; Figure 2.12) both significantly inhibited the guanabenz-induced relaxation of rat aorta in a dose-dependent manner. Those antagonists found to inhibit the guanabenz-induced relaxation (idazoxan, TEA and 4-AP) also had significant effects, both on the resting tone of the tissue and on the spasmogenic effect of noradrenaline. These are documented below.

In four out six tissues, application of idazoxan ($10^{-5}M$) induced a slight contractile response upon which were imposed rhythmical contractions (Figure 2.13). These varied in amplitude (0.19 ± 0.02g) and frequency (6 ± 1.2 / 10min) and were of relatively short duration (16.7 ± 2.0s). Idazoxan, ($10^{-5}M$) significantly decreased the noradrenaline contractile response (control 3.28 ± 0.20g; +idazoxan ($10^{-5}M$) 1.21 ± 0.21g, n=6; $p<0.05$).

Application of TEA ($10^{-2}M$) to the rat aorta caused a contractile response in 4 out of 6 tissues (0.6 ± 0.26g) and this was accompanied by a gradual development of rhythmical activity in three of these tissues (amplitude 0.36 ± 0.03g; frequency 15.0 ± 1.0 / 3min; Figure 2.13). TEA ($10^{-2}M$) also caused a significant increase in the noradrenaline contraction (control 3.43 ± 0.31g; + TEA ($10^{-2}M$) 4.39 ± 0.25g n=6, $p<0.05$). TEA ($3x10^{-3}M$) did not affect the resting tone of the tissue but caused a slight potentiation of the noradrenaline response (control 3.94 ± 0.42g; + TEA ($3x10^{-3}M$) 4.52 ± 0.25g, n=6).

4-AP ($10^{-3}M$ & $3x10^{-3}M$) had no effect on the resting tone of the tissues. 4-AP ($10^{-3}M$) also had no significant effect on the noradrenaline spasm (control 3.84 ± 0.28g; + 4-AP ($10^{-3}M$) 3.94 ± 0.21g n=6). However, in the presence of 4-AP ($3x10^{-3}M$), the noradrenaline contractile response was significantly increased (control 3.61 ± 0.2g; + 4-AP ($3x10^{-3}M$) 4.37 ± 0.23g n=6, $p<0.05$).

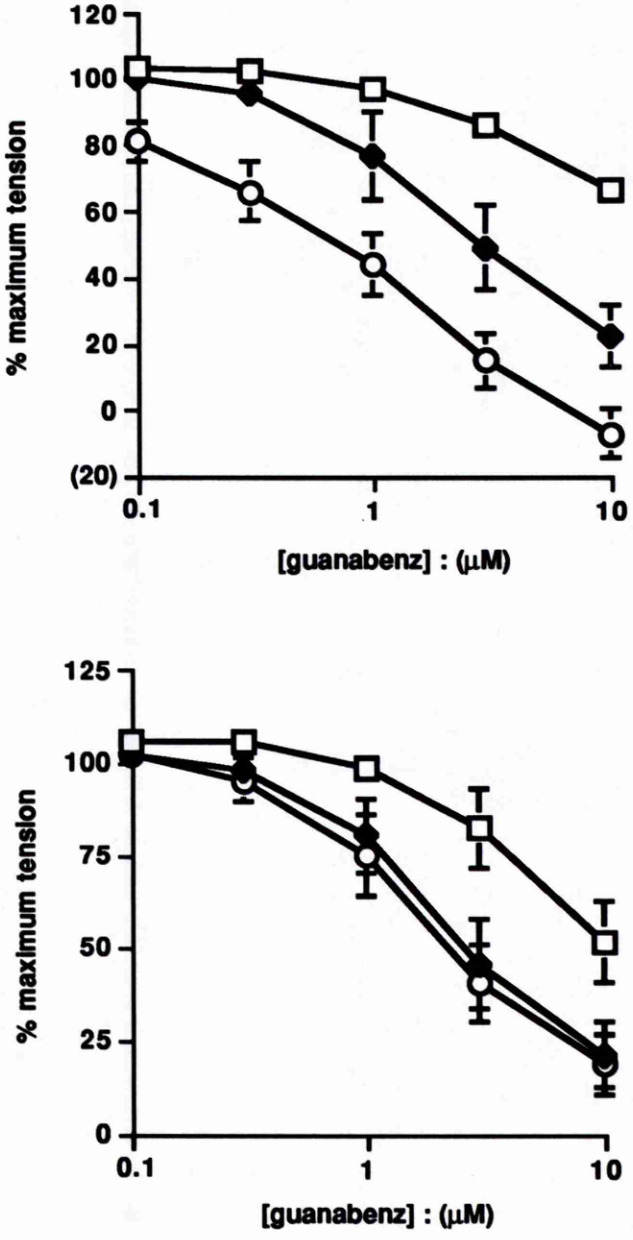



**Figure 2.7.** Guanabenz-induced relaxation of rat aortic strips pre-contracted with noradrenaline ($3x10^{-7}$M). In both panels, curves represent consecutive relaxation-response protocols (❑, first; ◆, second; ❍, third). The upper panel illustrates the increase in sensitivity of the tissue to repeated cumulative applications of guanabenz. The lower panel illustrates the effect of two priming doses of guanabenz ($10^{-5}$M) prior to commencement of the relaxation-response protocols. Points are the mean of 6 observations and vertical bars represent the s.e.mean.

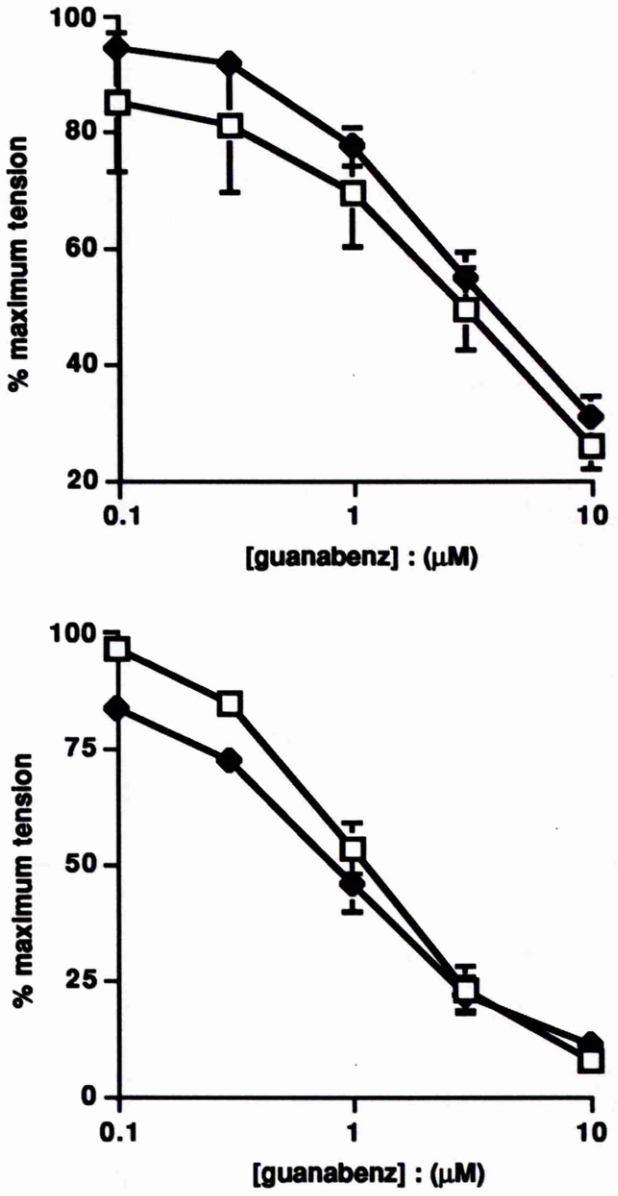



**Figure 2.8.** The effect of RX 821002 and RX 801023 on the guanabenz-induced relaxation of rat aorta pre-contracted with noradrenaline ($3x10^{-7}$M). Neither RX 821002 (◆; $10^{-6}$M, upper panel) nor RX 801023 (◆; $10^{-5}$M, lower panel) had any effect on the guanabenz-induced relaxation when compared to the relaxation caused by guanabenz in the absence of any modifying agent (❑). Points are the mean of 6 observations and vertical bars represent the s.e.mean.

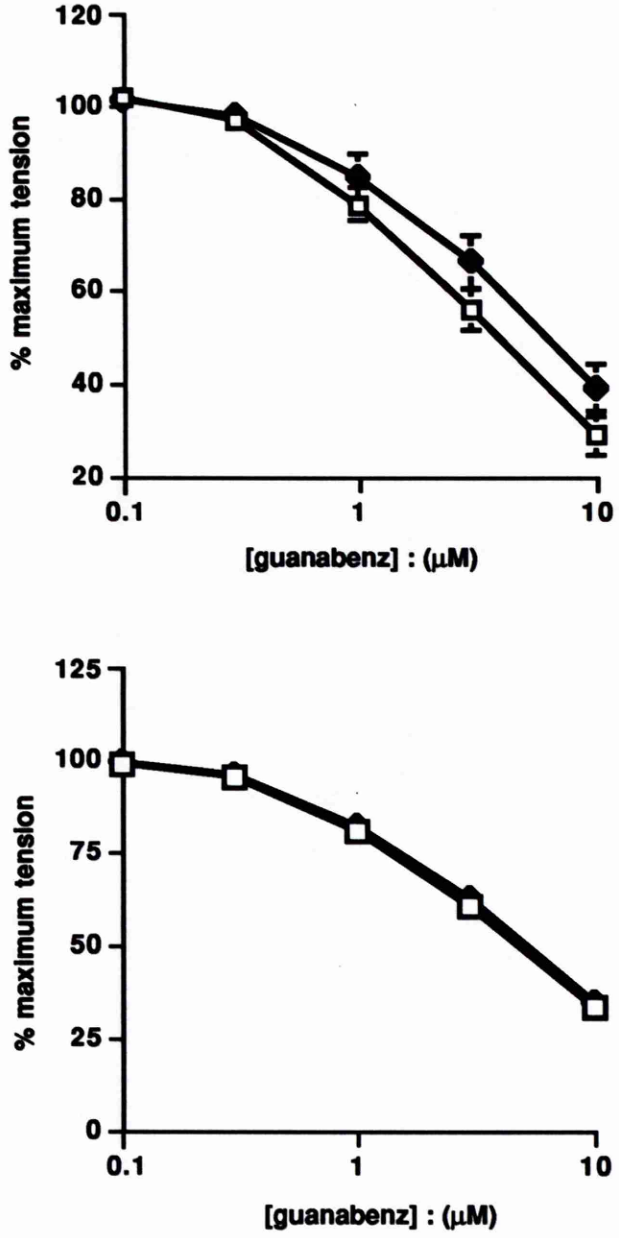



**Figure 2.9.** Effect of glibenclamide ($10^{-6}$M & $10^{-5}$M) on the guanabenz-induced relaxation of rat aorta pre-contracted with noradrenaline ($3x10^{-7}$M). Glibenclamide (◆;$10^{-6}$M, upper panel & $10^{-5}$M lower panel) had no effect on the guanabenz-induced relaxation when compared to the relaxation caused by guanabenz in the absence of any modifying agent (❑). Points are the mean of 6 observations and vertical bars represent the s.e.mean.

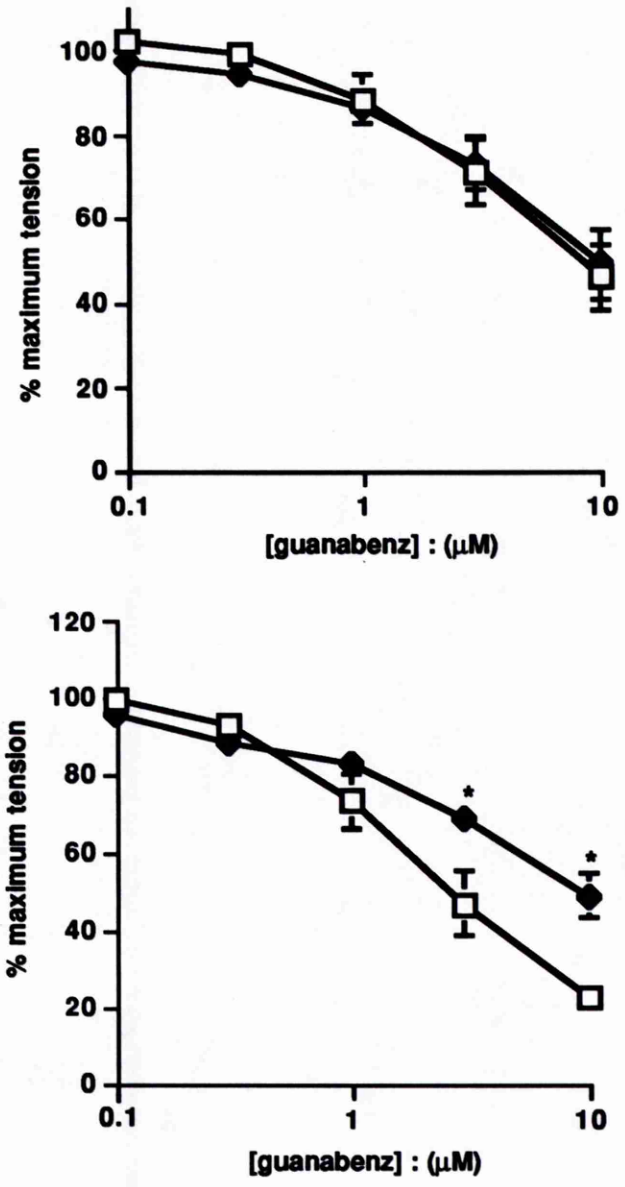



**Figure 2.10.** The effect of idazoxan on the guanabenz-induced relaxation of rat aorta pre-contracted with noradrenaline ($3x10^{-7}$M). Idazoxan (◆; $10^{-6}$M, upper panel) had no effect on the guanabenz-induced relaxation when compared to the relaxation caused by guanabenz in the absence of any modifying agent (❑). However, idazoxan (◆; $10^{-5}$M, lower panel) caused a significant inhibition of the guanabenz-induced relaxation when compared to control values (❑). Points are the mean of 6 observations and vertical bars represent the s.e.mean. * indicates significant inhibition of the guanabenz-induced relaxation when compared to corresponding control values (Student's paired t-test, $p<0.05$).

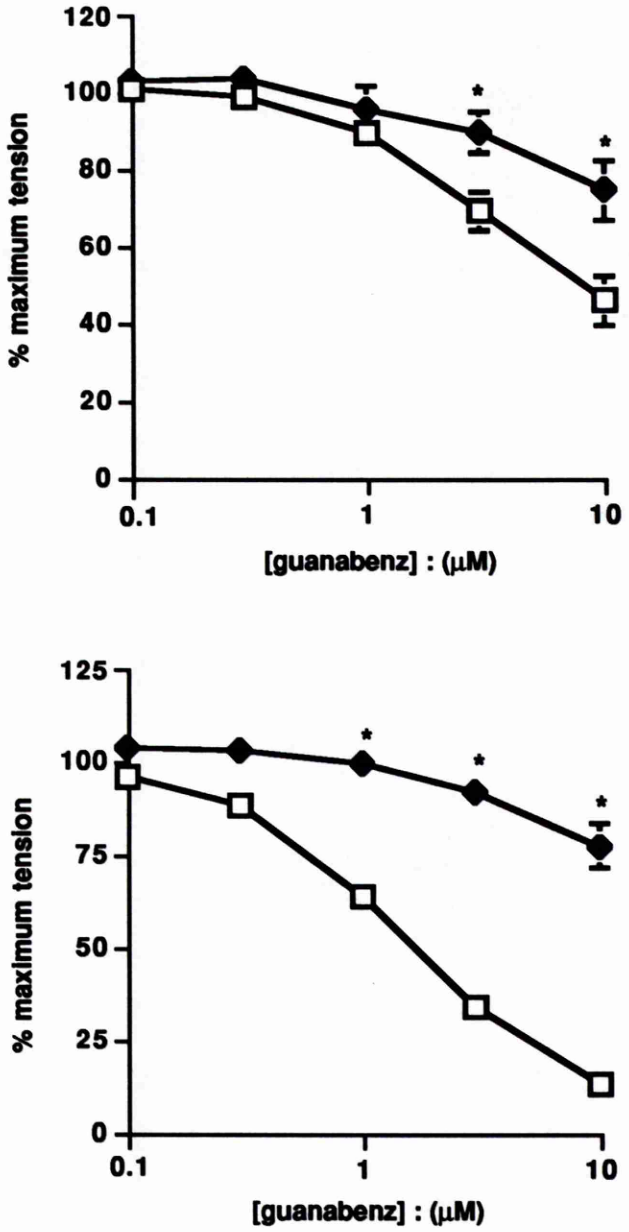



**Figure 2.11.** The effect of TEA on the guanabenz-induced relaxation of rat aorta pre-contracted with noradrenaline ($3x10^{-7}M$). TEA (◆; $3x10^{-3}M$, upper panel & $10^{-2}M$, lower panel) caused a dose-related inhibition of the guanabenz-induced relaxation of rat aorta compared to controls (❑). Points are the mean of 6 observations and vertical bars represent the s.e.mean. * indicates significant inhibition of the guanabenz-induced relaxation when compared to the corresponding control values (Student's paired t-test, $p<0.05$).

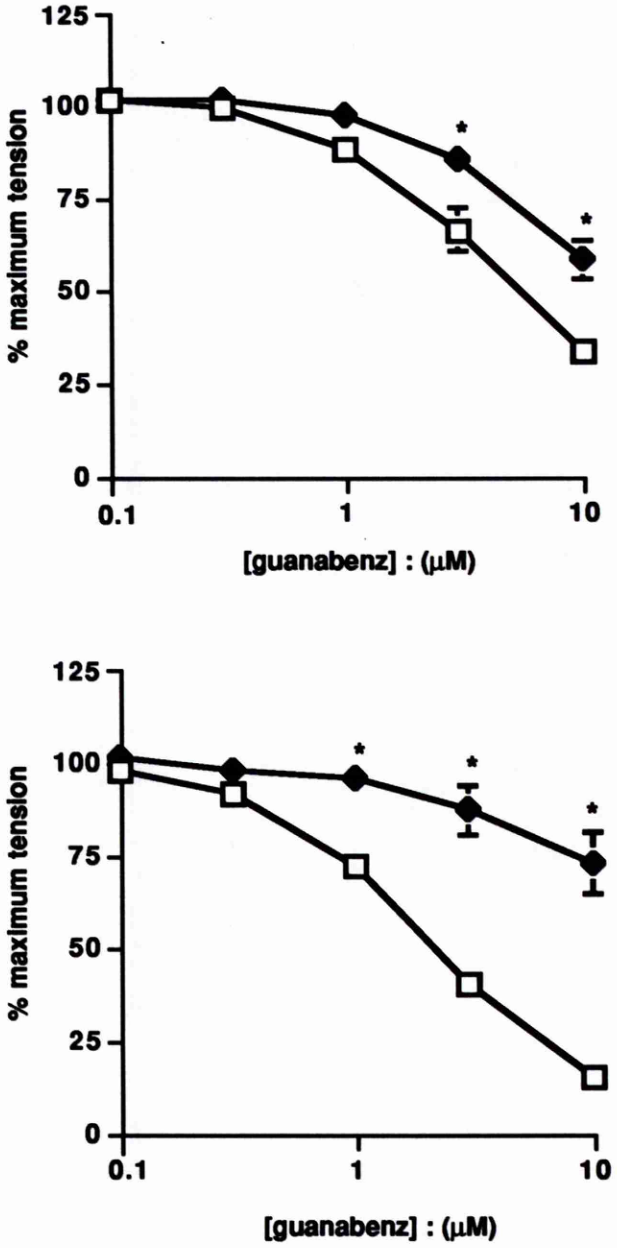



**Figure 2.12.** The effect of 4-AP on the guanabenz-induced relaxation of rat aorta pre-contracted with noradrenaline ($3x10^{-7}M$). 4-AP (◆; $10^{-3}M$, upper panel & $3x10^{-3}M$, lower panel) caused a dose-related inhibition of the guanabenz-induced relaxation of rat aorta compared to controls (❑). Points are the mean of 6 observations and vertical bars represent the s.e.mean. * indicates significant inhibition of the guanabenz-induced relaxation when compared to the corresponding control values (Student's paired t-test, $p<0.05$).

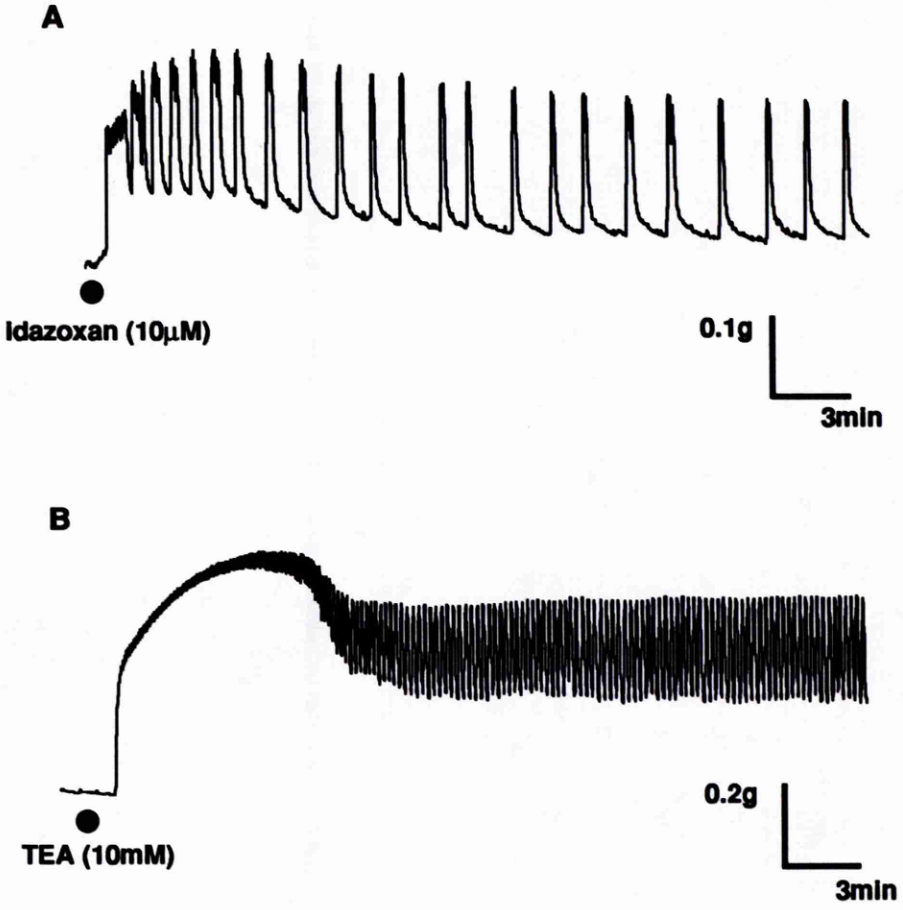



**Figure 2.13.** Effect of idazoxan ($10^{-5}$M; upper panel) and TEA ($10^{-2}$M; lower panel) when applied alone to rat aorta. Both agents caused an increase in the tone of the tissue accompanied by a development of spontaneous rhythmical activity. Both the magnitude of contraction and frequency of rhythmical contraction was greater for TEA than for idazoxan.

The role played by the endothelium in the guanabenz-induced relaxant response was investigated using both endothelium-intact and endothelium-denuded tissues. The functional integrity of the endothelium was assessed using acetylcholine-induced relaxation of tissues pre-contracted with noradrenaline ($3x10^{-7}M$). Acetylcholine ($10^{-6}M$) caused relaxation of the noradrenaiine spasm only in those tissues in which the functional integrity of the endothelium remained intact (Figure 2.14). Tissues treated with vehicle displayed no relaxant response, regardless of the integrity of the endothelium. Guanabenz caused a dose-dependent reiaxation in tissues both with intact endothelium and in those tissues in which the endothelium had been removed. The magnitude of the relaxation was slightly greater in those tissues with an intact endothelium at intermediate guanabenz concentrations, but the final degree of relaxation was the same in both groups of tissues (Figure 2.14).

The ability of guanabenz to relax tissues pre-contracted with spasmogens other than noradrenaline was next examined. Strips of rat aorta with endothelium intact were contracted with the thromboxane-$A_2$ analogue, U-46619 ($2x10^{-8}M$). In these tissues, guanabenz was unable to induce any relaxation in the presence of RX 821002 ($10^{-6}M$; Figure 2.15). Conversely, the K-channel opener, levcromakalim ($10^{-8}M$-$10^{-6}M$) caused a dose-dependent relaxation of tissue pre-contracted with U-46619 (Figure 2.15), albeit with a lower potency than that seen against other spasmogens eg. KCl (see Figure 2.16).

The ability of guanabenz to relax tissues pre-contracted with $K^+$-substituted PSS ($2.5x10^{-2}M$) was also examined. In these tissues, levcromakalim caused a full, dose-related relaxation whereas guanabenz failed to cause any relaxation in these tissues (Figure 2.16). Indeed, guanabenz appeared to prevent any fade in these tissues as compared with controls ie. guanabenz appeared to induce a contractile response. For this reason the relaxant properties of guanabenz on KCl-contracted tissues were investigated in the

presence of RX 821002 ($10^{-6}$M) and prazosin ($10^{-7}$M) to inhibit any α-adrenoceptor-mediated contractile responses. Under these conditions, guanabenz was still unable to induce any relaxation of the tissue but the contractile effects observed previously were also absent (Figure 2.16). It therefore appears that guanabenz is unable to cause relaxation of rat aorta contracted with either U-46619 or KCl.

The effect of guanabenz on noradrenaline dose-response curves was investigated to determine whether guanabenz mediates its relaxant response via antagonism of the noradrenaline contraction directly. Guanabenz ($10^{-6}$M-$10^{-4}$M) caused a dose-related rightward shift in the noradrenaline dose-response curve (Figure 2.17). Lower concentrations of guanabenz ($10^{-6}$M & $10^{-5}$M) caused a shift to the right with no apparent decrease in the maximum attainable response. However, higher concentrations of guanabenz ($10^{-4}$M) also caused a depression in the maximum response to noradrenaline (Figure 2.17).

The effect of guanabenz ($10^{-4}$M) and levcromakalim ($10^{-7}$M) on KCl-induced contracture was also investigated. Levcromakalim caused a decrease in the response to low concentrations of KCl but was without effect on higher concentrations (Figure 2.18). Guanabenz ($10^{-4}$M) however caused a leftward shift in the KCl-induced contracture with an increase in the maximum attainable response (Figure 2.18).

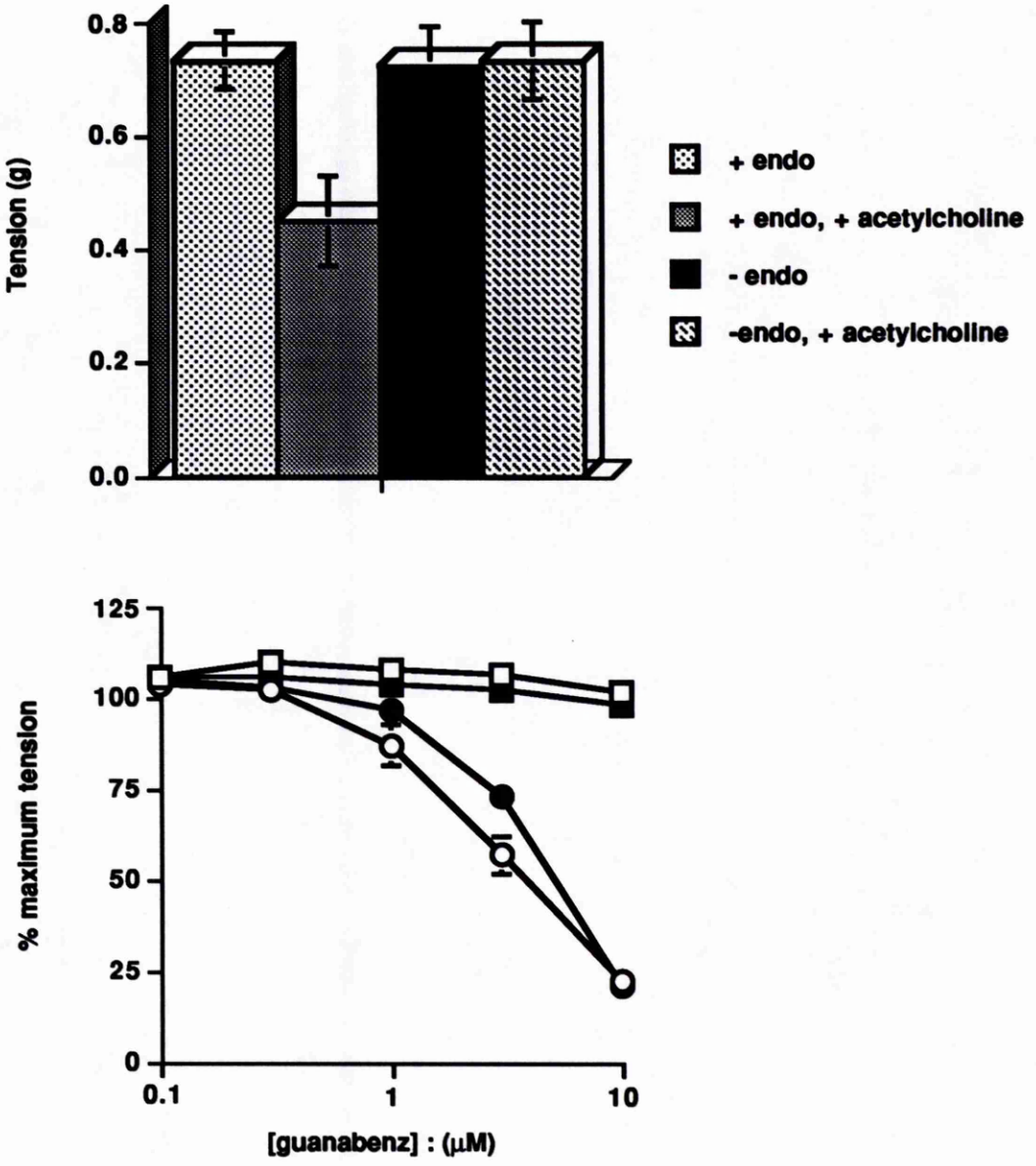



**Figure 2.14.** The effect of destroying the endothelium on the guanabenz-induced relaxant response in rat aorta pre-contracted with noradrenaline ($3x10^{-7}$M). Upper panel: endothelium integrity was assessed by the ability of acetylcholine ($10^{-6}$M) to cause relaxation of the tissues when contracted with noradrenaline. Only those tissues with intact endothelium displayed any relaxation to acetylcholine. Lower panel: guanabenz (○, +endothelium; ●, -endothelium) caused a dose-related relaxation of the rat aorta regardless of the integrity of the endothelium compared to control values (□, +endothelium; ■, -endothelium). Points are the mean of 6 observations and vertical bars represent the s.e.mean.

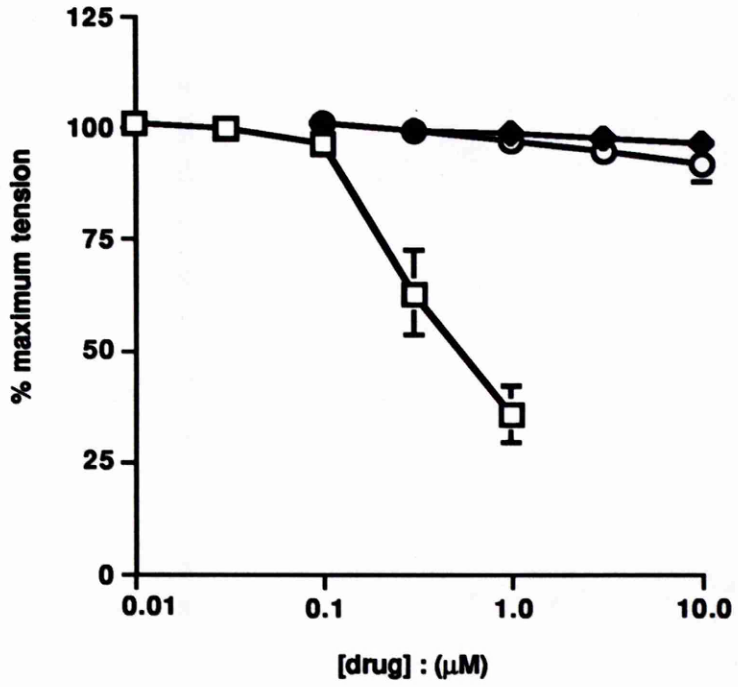



**Figure 2.15.** Effect of guanabenz and levcromakalim on rat aorta pre-contracted with U-46619 ($2x10^{-8}$M). Guanabenz (◆; $10^{-7}$M-$10^{-5}$M) had no relaxant effect on tissues pre-contrated with U-46619 in the presence of RX 821002 ($10^{-6}$M) when compared to control tissues (○). Levcromakalim (□; $10^{-8}$M-$10^{-6}$M) caused a concentration-dependent relaxation of these tissues. Points are the mean of 4 observations and vertical bars represent the s.e.mean.

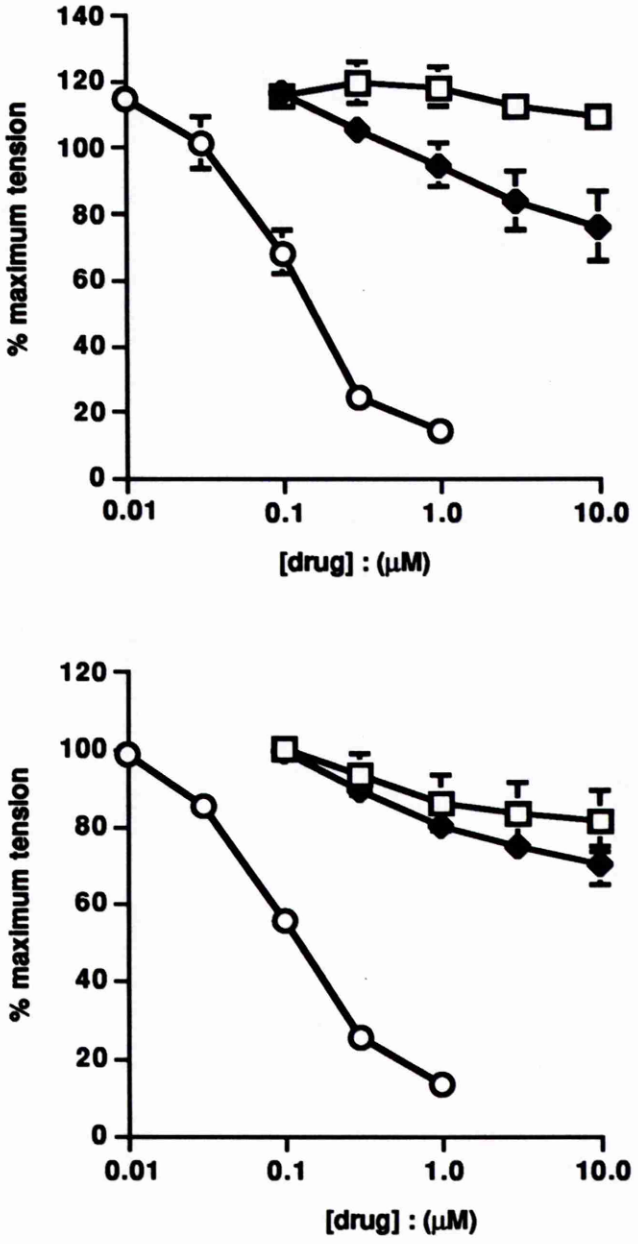



**Figure 2.16.** Effect of guanabenz and levcromakalim on rat aorta pre-contracted with KCl ($2.5x10^{-2}M$). Upper panel: The effect of guanabenz (□; $10^{-7}M$-$10^{-5}M$), and levcromakalim (○; $10^{-8}M$-$10^{-6}M$) on rat aorta pre-contracted with KCl when compared to vehicle controls (◆). Lower panel: The effect of guanabenz (□; $10^{-7}M$-$10^{-5}M$), in the presence of both prazosin ($10^{-7}M$) and RX 821002 ($10^{-6}M$), and levcromakalim (○; $10^{-8}M$-$10^{-6}M$) on rat aorta pre-contracted with KCl when compared to vehicle controls (◆). Points are the mean of 4 observations and vertical bars represent the s.e.mean.

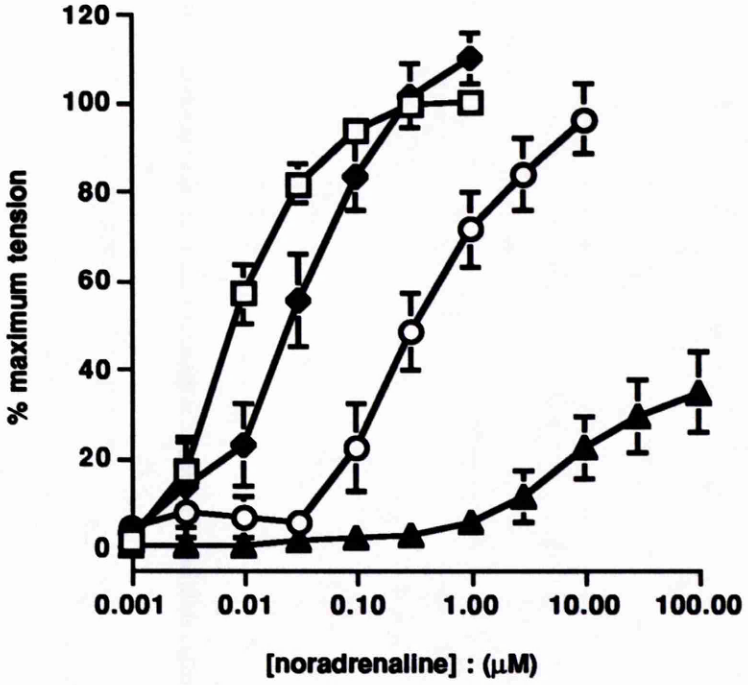



**Figure 2.17.** The effect of guanabenz ($10^{-6}$M-$10^{-4}$M) on the noradrenaline dose-response curve in rat aorta. Guanabenz (◆ ,$10^{-6}$M; ○ ,$10^{-5}$M; ▲,$10^{-4}$M) caused a dose-dependent rightward shift of the noradrenaline dose-response curve with a decrease in the maximum response at high concentrations (▲,$10^{-4}$M) when compared to control values (□). Data are expressed as a percentage of the tension induced by noradrenaline ($10^{-6}$M) in the absence of guanabenz. Points are the mean of 6 observations with the vertical bars representing the s.e.mean.

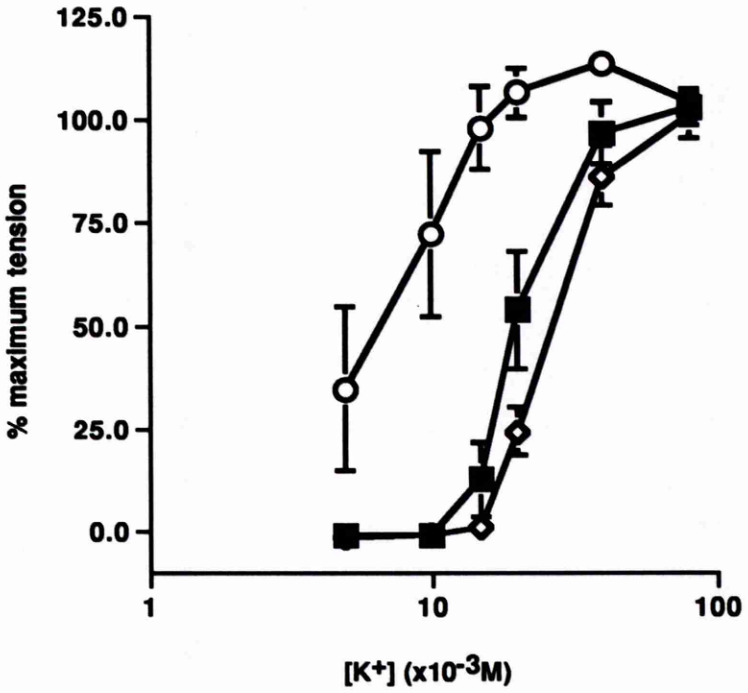



**Figure 2.18**. Effect of guanabenz and levcromakalim on the $K^+$-induced contracture of rat aorta. Levcromakalim ($10^{-7}$M; ◊) caused a decrease in the magnitude of the contraction at low $K^+$ concentrations but was without effect at higher concentrations. Guanabenz ($10^{-4}$M; ○) caused a leftward shift in the $K^+$ dose-response curve with an increase in the maximum attainable response when compared to control values (■). Data are expressed as a percentage of the tension induced by 80mM KCl in the absence of any modifying agent. Points are the mean of 4 observations with the vertical bars representing the s.e.mean.

## 2.4. Discussion

### 2.4.1. Radio-ligand binding studies

The aim of the present study was to identify any possible link between high affinity binding at the IGR site in rat liver and K-channel modulation using the technique of radio-ligand binding. This study therefore was to determine whether a common site existed for the action of a K-channel modulator and for an IGR site ligand.

#### 2.4.1.1. Saturation analysis

Saturation analysis of [$^3$H]-idazoxan binding was conducted both in the rat liver homogenate and in the rat brain homogenate using both idazoxan and cirazoline to define the non-specific binding. These experiments were performed in the different preparations in an attempt to highlight the influence that a mixed population of $\alpha_2$-adrenoceptors and IGR sites would have upon analysis of data when compared to a preparation reported to contain only the IGR site. The use of idazoxan to define non-specific binding alone would give no indication of the presence of any $\alpha_2$-adrenoceptors as this ligand could displace the [$^3$H]-ligand from both $\alpha_2$-adrenoceptors and IGR site. To determine whether only an IGR site population was present in the rat liver, cirazoline was also employed to determine NSB. This agent would only be expected to displace [$^3$H]-idazoxan from the IGR sites and not from any $\alpha_2$-adrenoceptors as it is itself an $\alpha_1$-adrenoceptor agonist. Identical results obtained from the use of cirazoline and idazoxan to define NSB would therefore be indicative of a lack of $\alpha_2$-adrenoceptors. Conversely, in the brain homogenate where a mixed population of $\alpha_2$-adrenoceptors and IGR sites is reported to exist, one would expect to observe fundamental differences between the use of idazoxan and cirazoline to define NSB. Specifically, the use of cirazoline to define NSB would be expected to yield a lower % specific binding (and thus a lower Bmax) compared to idazoxan. This would arise from the inability of cirazoline to displace the [$^3$H]-

ligand from the $\alpha_2$-adrenoceptor and such receptors would therefore have the appearance of non-specific sites.

Saturation binding isotherms were therefore constructed for [$^3$H]-idazoxan binding in the rat liver homogenate and the rat brain homogenate. In both cases NSB was defined in the presence of idazoxan and cirazoline and Kd and Bmax values were evaluated by Scatchard analysis (Bennett & Yamamura, 1985).

The dissociation constant (Kd) is a measure of the affinity of a drug for a receptor and is derived from the reciprocal of the slope of the Scatchard plot. Kd values of similar size imply that the binding dynamics of the populations involved are the same and are thus suggestive that the populations are of the same receptor type. The Bmax value is a measure of the absolute number of specific binding sites (ie. concentration of ligand (fmol) bound to each mg of protein).

The Bmax values obtained from the liver homogenate using either idazoxan or cirazoline to define NSB were very similar and it thus appears that both agents define receptor populations of the same magnitude. In addition, the Kd values for [$^3$H]-idazoxan binding were similar when using either idazoxan or cirazoline to define NSB. This similarity in Kd values suggests similar binding kinetics for each of the defined groups (ie. when using idazoxan or cirazoline to define NSB) and therefore indicates that both agents define the same receptor population. When Bmax and Kd values are considered together it is clear that the receptor populations defined by idazoxan and cirazoline NSB in the rat liver homogenate are in fact the same with virtually identical Bmax and Kd values. Therefore, the NSB defined by cold idazoxan does not over-estimate the IGR site population (as would be the case if $\alpha_2$-adrenoceptors were present) and these data confirm the lack of $\alpha_2$-adrenoceptors in the rat liver preparation (Zonnenschein *et al.*, 1990). It thus appears viable to use cold idazoxan to define [$^3$H]-idazoxan NSB on a routine basis in the rat liver homogenate.

These experiments were complemented by performing the same studies in a homogenate derived from rat brain again using both idazoxan and cirazoline to define NSB. This is a preparation in which a considerable population of $\alpha_2$-

adrenoceptors is known to exist (Burt, 1985). Scatchard analysis was again used to calculate Bmax and Kd values. The differences in the obtained Bmax values are indicative of differences in the size of the receptor population defined by idazoxan NSB and cirazoline NSB. The Bmax value calculated from cirazoline NSB is lower than that defined by idazoxan NSB. The apparent inability of cirazoline to displace [$^3$H]-idazoxan from the $\alpha_2$-adrenoceptor results in a proportionately larger value for NSB and hence lower values for specific binding.

In addition to the observed differences between the Bmax values, differences were also observed in the Kd values. The Kd is a measure of the affinity of a drug for a receptor and thus it may be interpreted that differences between these values indicate different receptor populations or possibly contamination of one of the groups by more than a single receptor population. The differences in Kd values therefore are consistent with the view that differences exist in the receptor populations defined by idazoxan and cirazoline in the brain homogenate.

Although the differences seen in both Bmax and Kd values may not be as pronounced as expected in the brain homogenate they do indicate that idazoxan defines a larger population of receptors ($\alpha_2$-adrenoceptors plus IGR site) compared to cirazoline (IGR site alone). The magnitude of the differences between the two populations seen in the brain preparation was not as marked as expected. This is possibly indicative of some cirazoline binding to $\alpha_2$-adrenoceptors at the high concentrations required to define NSB. This interpretation is supported by the findings of Mottram and Saggar (1985) who demonstrated $\alpha_2$-adrenoceptor antagonism by cirazoline in the guinea-pig ileum.

### 2.4.1.2. Displacement analysis

A series of agents was then tested for their ability to displace [$^3$H]-idazoxan bound to the IGR site in the rat liver and a rank order of potency was constructed for the compounds demonstrating high affinity for this site. Although they mainly comprised recognised α-adrenoceptor ligands, this group also contained pinacidil, guanethidine, RX 801023 and amiloride. All of these agents possess either a guanidine or an imidazoline moiety within their structure and their ability to bind to the IGR site appears to be dependent on this property. The most potent imidazolines were cirazoline > RX 801023 > idazoxan. Both cirazoline and idazoxan are known to be high affinity ligands at the IGR site (Yablonsky *et al.*, 1988; Zonnenschein *et al.*, 1990). The differences between RX 801023 and its structural analogue RX 821002 are discussed below. The guanidine-based compounds generally showed lower affinity than the imidazolines for this site with the exception of guanabenz. This agent was the most potent of the guanidines and the third most potent ligand identified with an $IC_{50}$ value of $2.1 \times 10^{-8}$M. Amiloride, a diuretic agent known to decrease $K^+$ loss and inhibit the $Na^+ / H^+$ exchange system also exhibited a relatively high affinity for this site. The rank order of potency for guanidines at this site was guanabenz > amiloride > pinacidil > guanethidine. The possible reasons for the ability of the K-channel opener, pinacidil to displace [$^3$H]-idazoxan binding are discussed later. The adrenergic neurone blocking agent, guanethidine was also found to be a relatively high affinity ligand at this site. The mode of action of this class of drugs is unknown but guanethidine has recently been shown to block the actions of the K-channel openers in smooth muscle (Berry *et al.*, 1992).

In general, therefore, the findings of the present study are in agreement with those of other binding studies at the IGR site, indicating that the presence of either an imidazoline or a guanidine moiety within the structure is a prerequisite for binding at this site. However, certain agents demonstrated no affinity for this site despite the possession of these structural traits (eg RX 821002 and

phentolamine, both imidazolines). It therefore appears that the simple possession of either an imidazoline or a guanidine moiety within the structure of a molecule is not sufficient to satisfy the structural constraints required for high affinity binding at this site. This is discussed further in a later section (2.4.1.3) with specific reference to the cyanoguanidine, pinacidil and its close structural analogue, P1060.

RX 801023 is a structural analogue of the $\alpha_2$-adrenoceptor antagonist, RX 821002 but possesses a higher affinity for the IGR site than for the $\alpha_2$-adrenoceptor (Mallard *et al.*, 1991). This was confirmed in the present study in which RX 801023 was found to be a high affinity ligand ($IC_{50}$ = $1.2x10^{-8}$M), whereas RX 821002 had no affinity for this site ($IC_{50}$>$10^{-5}$M). It thus appears that compound selectivity for this site can be achieved despite the structural similarities between the ligands at the IGR site and the $\alpha$-adrenoceptor. This therefore indicates that the IGR site probably does not represent a subtype of $\alpha_2$-adrenoceptor. This view is given further credence by the finding that the endogenous catecholamine adrenoceptor agonists, adrenaline and noradrenaline possess no affinity for the IGR site, either as racemic mixtures or in their active enantiomeric forms. This represents the most compelling piece of evidence in favour of a distinction between adrenoceptor and IGR sites. It is in agreement with the findings of Zonnenschein *et al.* (1990) who also drew a similar conclusion. Finally, the observation that many other adrenoceptor ligands, notably yohimbine, rauwolscine, prazosin and urapidil, were without effect on the [$^3$H]-idazoxan binding in the rat liver also indicates fundamental differences between the IGR site and the $\alpha$-adrenoceptor.

Clonidine-displacing substance (CDS) is a putative endogenous ligand at the IGR site and it displaces [$^3$H]-idazoxan binding at IGR sites in the medulla oblongata (see Atlas, 1991). In addition, CDS is able to displace ligands from the $\alpha_2$-adrenoceptor as seen from the displacement of [$^3$H]-rauwolscine binding (Atlas *et al.*, 1987) by this agent. CDS may therefore represent a common

endogenous link between the two receptor types.

Based on the rank order of potency for ligands at the IGR site it is possible to determine, at least partially, the IGR site subtype in the rat liver. If the classification of Michel and Insel (1989) is considered, the results indicate that subtype 2 is the type found in the rat liver. This conclusion is based on the fact that imidazoline and guanidine compounds demonstrate a high affinity for this site and in addition that amiloride is also relatively potent. The high affinity of guanabenz and amiloride discounts the possibility that the IGR site in the liver corresponds to subtype 1. Furthermore, the high affinity binding demonstrated by the imidazolines excludes the possibility that the IGR site within the liver corresponds to sub-type 3 (Michel & Insel, 1989).

### 2.4.1.3. IGR site modulation by the K-channel modulators

The physiological functioning of the IGR site is an area about which very little is known but evidence is accumulating to suggest that this receptor may be closely coupled to a K-channel. The majority of data in support of this view are based on non-functional analysis such as the ligand binding performed by Zonnenschein *et al.* (1990). These authors demonstrated that 4-AP displaces the binding of [$^3$H]-idazoxan from the IGR site and also that ions such as $Cs^+$ and $NH_4^+$ (known to interfere with K-channel gating) reduce the affinity of the [$^3$H]-ligand for this site.

It was therefore decided to investigate the possible link between the IGR site in the rat liver and K-channels. The problem was approached from the view-point that if an agent known to modulate K-channel function could displace [$^3$H]-idazoxan binding, this would suggest a close association between a K-channel and the IGR site. This experimental approach, however, would probably only demonstrate the presence of a K-channel that was part of, or closely associated with, the idazoxan binding site. If the putative K-channel was located distal to the physiological functioning of the IGR site it would not be evident from these experiments.

A number of agents known to modulate the activity of a variety of K-channels was assessed for their affinity for the [$^3$H]-idazoxan binding site. These compounds ranged from the relatively non-selective agents such as 4-AP and TEA to the sulphonylurea, glibenclamide which is known to inhibit $K_{ATP}$ in pancreatic β-cells and in cardiac muscle. In addition, the highly selective toxins charybdotoxin, dendrotoxin and apamin which block $BK_{Ca}$, the fast activating neuronal K-channel and $SK_{Ca}$, respectively, were also tested. With the exception of pinacidil (a cyanoguanidine), none of the K-channel modulating agents tested had any effect on the binding of [$^3$H]-idazoxan.

The inactivity of 4-AP contradicts the results of Zonnenschein *et al.* (1990) who reported that 4-AP displaced [$^3$H]-idazoxan with an $IC_{50}$ of $3.4x10^{-4}$M. However, there seems to be some discrepancy in the report of Zonnenschein *et al.* (1990) between the text and the data. The text reports an $IC_{50}$ value of $3.4x10^{-4}$M, whilst the data presented indicates an $IC_{50}$ of at least $10^{-3}$M. In the present study, both TEA and 4-AP were tested to a final concentration of $10^{-3}$M and no displacement of [$^3$H]-idazoxan was observed. Although high concentrations of these compounds are required to exert their pharmacological effects, the non-specific effects of these drugs will increase as the drug concentration increases. Similarly, it was reported by Drukarch *et al.* (1989) that both TEA and 4-AP were able to displace a number of neurotransmitters, including clonidine (an imidazoline with a relatively high affinity for the IGR site) from their binding sites at concentrations of TEA and 4-AP required to block K-channels. It is consequently difficult to interpret accurately the results of Zonnenschein *et al.* (1990) as a specific Interaction of 4-AP with the IGR site.

Pinacidil displaces [$^3$H]-Idazoxan binding from the IGR site with an $IC_{50}$ of $4.1x10^{-6}$M. It seems likely that the ability of pinacidil to displace [$^3$H]-idazoxan from the IGR site is independent of its ability to open K-channels. This observation is based upon the finding of the present study that the pinacidil

derivative, P1060, an agent more potent than pinacidil in terms of K-channel opening, was devoid of any affinity for the IGR site.

When the structures of these two closely related compounds are studied, they appear to be only marginally different. The 3-pyridyl derivative, P1060 contains an extra ethyl group that is absent from the 4-pyridyl derivative, pinacidil (see Introduction Figure 1.1). It therefore seems that simply the possession of a guanidine group is not sufficient to satisfy the structural criteria required for binding at this site. Similarly with the imidazolines, both RX 821002 and phentolamine are imidazoline-based ligands but both lack any affinity for the IGR site. Examination of the structures of these compounds may allow the further determination of the structural requirements for IGR site ligands beyond simply the possession of an imidazoline or guanidine group.

A population of K-channels within the rat liver sensitive to IGR site ligands would implicate a role for the K-channel in the physiological functioning of the receptor. [$^3$H]-glibenclamide binding was therefore investigated in the rat liver. This agent was selected as the ligand because of the ability of several imidazoline compounds such as naphazoline and tolazoline to inhibit the actions of the K-channel openers, a feature also common to glibenclamide. Although a population of glibenclamide-specific sites was present in the rat liver, the absolute amount of the specific binding compared to NSB was very small. Such a result made displacement analysis of this data impossible and thus no conclusions could be drawn.

[$^3$H]-glibenclamide binding was therefore carried out using the rat brain homogenate. This is a preparation in which a considerable population of glibenclamide binding sites are known to exist (Angel & Bidet, 1991) and is routinely used as a screen for compounds active at the sulphonylurea receptor. Agents exhibiting a high affinity for the IGR site were therefore tested for their abiiity to displace the [$^3$H]-glibenclamide binding from the rat brain homogenate. If any of the IGR site ligands were able to displace the [$^3$H]-glibenclamide

binding, the evidence would once again suggest an IGR site with an intrinsic (or at least closely related) K-channel.

Both glibenclamide and glipizide (a sulphonylurea derivative) potently displaced the [$^3$H]-glibenclamide. In contrast, none of the IGR site ligands tested was abie to displace the binding of [$^3$H]-glibenclamide ($IC_{50}$ values >$10^{-5}$M).

It thus appears that there is no direct association between the IGR site and a K-channel ie. the IGR site probably does not gate an intrinsic K-channel. Much more likely is the possibility that the modulation of a K-channel exists distal to IGR site activation via a second messenger (or other) link. However, care must be taken when interpreting these data. It is well-recognised that the functional effects of the K-channel openers are potently inhibited by the sulphonylureas, but agents such as cromakalim are unable to displace [$^3$H]-glibenclamide binding in cerebral cortical membranes (Angel & Bidet, 1991).

The lack of ability of the K-channel modulators to displace [$^3$H]-idazoxan binding therefore does not necessarily imply that these two sites are not functionally linked or even unconnected. The current data suggest that the [$^3$H]-idazoxan binding site in the rat liver is not a K-channel itself but it remains to be established whether the binding site exists on a closely associated sub-unit or is located proximal to the functioning of a K-channel.

### 2.4.1.4. Conclusions from binding studies

An IGR site has been identified within the rat liver homogenate and the ligands active at this site classified into a rank order of potency. The compounds within this potency order meet the general structural criteria of possession of either an imidazoline or guanidine moiety although it seems other chemical criteria must be satisfied as well. The potency order can also be used to classify this IGR site further. The IGR site within the rat liver homogenate corresponds to sub-type 2 described by Michel and Insel (1989). Due to the lack of effect of the K-channel modulating agents (with the exception of pinacidil), it is concluded that

this IGR site does not gate an intrinsic K-channel. This is supported by the data obtained from the [$^3$H]-glibenclamide binding in the rat brain which indicates that the IGR site ligands do not possess a high affinity for the glibenclamide-binding site.

### 2.4.2. The vasorelaxant action of guanabenz

#### 2.4.2.1. $^{42}K^+$-efflux experiments

Guanabenz produced a small but significant increase in the efflux of $^{42}K^+$ from rat aortic strips in the presence of the $\alpha_2$-adrenoceptor antagonist, RX 821002. Levcromakalim also produced a significant increase in $^{42}K^+$ efflux, which was approximately 200% greater than the magnitude of the efflux induced by guanabenz. The presence of RX 821002 in these experiments discounts the possibility that the increase in $^{42}K^+$ efflux caused by guanabenz arises from activation of excitatory post junctional $\alpha_2$-adrenoceptors on the vascular smooth muscle leading to an increase in intracellular $Ca^{++}$ levels and a subsequent opening of $BK_{Ca}$. It thus appears that guanabenz is capable of increasing plasmalemmal K-permeability by a mechanism distinct from $\alpha_2$-adrenoceptor activation.

#### 2.4.2.2. Tension recording studies

In rat aorta pre-contracted with noradrenaline, guanabenz caused a dose-dependent relaxation which appeared to increase in magnitude with repeated dose-response protocols. This difficulty was overcome by exposing the tissue to two priming doses of guanabenz ($10^{-5}$M for 15min each) which eliminated the increase in sensitivity observed between the second and third dose-response curves. No attempt was made to determine the reasons for the increase in sensitivity of the tissue, although certain possibilities exist. It is possible that a desensitisation of the noradrenaline receptors may lead to progressively weaker

contractile responses and that this is manifest as progressively increasing relaxations to guanabenz. However, the dose of spasmogen used in all cases (approximate $EC_{90}$) is unlikely to be sufficient to cause such a desensitising response and there was no decrease in the magnitude of the induced contraction between subsequent protocols. It is also possible that an up-regulation in the guanabenz 'receptors' occurred causing a gradual increase in the observed response. This would explain why exposure to a large priming dose decreased the observed changes in tissue sensitivity.

The effects of a number of antagonists on the guanabenz-induced relaxation was first investigated. The relaxation was resistant to the $\alpha_2$-adrenoceptor antagonist, RX 821002 and to the reputed selective IGR site ligand, RX 801023. In addition, the $K_{ATP}$ inhibitor, glibenclamide was also ineffective at blocking the guanabenz-induced relaxation. The inability of RX 821002 to inhibit this relaxant response confirms the report of Soares-da-Silva and Villaneuva (1990) who indicated that the guanabenz-induced relaxation was independent of $\alpha_2$-adrenoceptor activation. The observation that RX 801023 was also ineffective at blocking the guanabenz-induced relaxation suggests that the IGR site is also not involved in this response. However, it is well-documented that several putative subtypes for this site exist (Michel & Insel, 1989) and at present there is no reason to suggest that the IGR site found in the rat liver, at which RX 801023 is a high affinity ligand, corresponds to the site in vascular smooth muscle. Indeed, Wikberg *et al.* (1991) reported that the IGR site in smooth muscle represented a separate subtype to the IGR site in neuronal tissue. In addition, no evidence exists to implicate RX 801023 as an IGR site antagonist. Finally, the inability of glibenclamide to inhibit the relaxation suggests that the guanabenz-induced response is not mediated via the opening of $K_{ATP}$ channels and that guanabenz does not operate in a manner similar to the cyanoguanidine K-channel openers, such as pinacidil despite the similarities in the structures of these agents.

Several agents were, however, able to inhibit the guanabenz-induced relaxation. Idazoxan, the imidazoline based $\alpha_2$-adrenoceptor antagonist is a

high affinity ligand at many of the IGR sites so far identified. This agent was ineffective at inhibiting the guanabenz-induced relaxant response at relatively low concentrations but when the concentration was increased, a small but significant inhibition of the relaxation was observed. The K-channel blockers TEA and 4-AP both caused a concentration-dependent inhibition of the guanabenz-induced relaxation of rat aorta. This may therefore indicate that the guanabenz-induced relaxation is mediated via the opening of plasmalemmal K-channels.

Because of the non-selective K-channel blocking nature of both 4-AP and TEA, it is possible that the observed inhibition arises from functional antagonism of guanabenz and not from any specific effect per se. 4-AP and TEA either caused contractile responses alone in this tissue or potentiated the noradrenaline spasm. These observations could indeed indicate a non-specific or functional antagonism, the decrease in relaxation arising from an increase in the contractile stimulus to the tissue. K-channel blockers may cause a depolarisation and lead to a decrease in relaxant activity, regardless of whether this relaxation is mediated via the opening of a K-channel or not, especially if the blocked K-channels are normally open at the resting membrane potential.

At the upper end of the tested concentration range, idazoxan caused a contraction of the tissue which was accompanied by a development of rhythmical activity (for further discussion of the mechanisms underlying this contractile response see Chapter 5). This contractile response in itself may be indicative of functional antagonism for reasons similar to those outlined for 4-AP and TEA. However, idazoxan also caused a significant decrease in the noradrenaline response which might potentiate any relaxant response because of a weaker contractile stimulus. The ability of idazoxan to decrease the noradrenaline contractile response may be indicative of $\alpha_1$-adrenoceptor antagonism at the high concentrations used.

The role of the endothelium in the guanabenz-induced relaxation was investigated using both endothelium-intact and endothelium-denuded tissues. Endothelium integrity was assessed by examining the acetylcholine-mediated relaxation of noradrenaline pre-contracted tissues. This is a process known to be

mediated via the release of endothelium-derived relaxing factor (EDRF) which activates the soluble guanylate cyclase enzyme system. In those tissues with an intact endothelium, acetylcholine caused a significant decrease in the tone of the tissue whereas those tissues denuded of endothelium were unaffected by the acetylcholine. The guanabenz-induced relaxation of rat aorta was unaffected by removal of the endothelium. A slight increase in the relaxation in endothelium-intact tissues was observed at intermediate guanabenz concentrations although this was not significantly different from that seen in endothelium-denuded tissues. It therefore appears that the endothelium does not play a major role in the guanabenz-induced relaxation of rat aorta.

The ability of guanabenz to relax tissues pre-contracted by spasmogens other than noradrenaline was next examined. U-46619, is a thromboxane $A_2$-analogue known to be a potent constrictor of this tissue. Guanabenz failed to cause any relaxation of tissues contracted with U-46619. Conversely, the K-channel opener, levcromakalim, caused a dose-dependent relaxation of this tissue. However, levcromakalim was less potent than observed when tested against other spasmogens (eg. KCl see later) and it has since become apparent that U-46619 possesses K-channel blocking properties (Scornik & Toro, 1992). In addition, the majority of $Ca^{++}$ for the contraction induced by U-46619 is obtained from intracellular stores (see later). Therefore, the K-channel opener will probably not be as efficacious as with a spasmogen that employs extracellular $Ca^{++}$ via membrane depolarisation. It is thus possible that both of the above reasons may contribute to the observed decrease in potency of levcromakalim in tissues pre-contracted with U-46619.

Contraction of rat aorta with raised $K^+$ ($2.5x10^{-2}M$) is solely dependent on the influx of extracellular $Ca^{++}$, and provided that the $K^+$ concentration is not raised too high (see Introduction, section 1.3.5), this contraction is relaxed by K-channel openers such as levcromakalim. Guanabenz failed to relax tissues precontracted with $K^+$ ($2.5x10^{-2}M$) whereas levcromakalim caused a complete

relaxation in these conditions. Indeed, guanabenz seemed to exert a contractile effect on the tissue when compared to the time-dependent fall in tension in vehicle-control tissues. The ability of guanabenz to relax tissues pre-contracted with $K^+$ ($2.5x10^{-2}M$) was therefore also evaluated in the presence of RX 821002 ($\alpha_2$-adrenoceptor antagonist) and prazosin ($\alpha_1$-adrenoceptor antagonist) to block any guanabenz-induced contractile effects mediated via $\alpha$-adrenoceptors. Under these conditions, guanabenz was still unable to mediate any relaxation of this tissue and the contractile effects were also absent. It therefore appears that guanabenz is only able to mediate relaxation of tissues pre-contracted with noradrenaline and that this process is not associated with membrane hyperpolarisation, specifically K-channel opening.

The finding that guanabenz potentiated the contractile response to KCl whereas known K-channel openers such as levcromakalim have the opposite effect is also indicative of the lack of K-channel opening properties of this compound. Levcromakalim decreased the contractile response at low KCl concentrations (where $E_K$ lies outside the threshold for activation of voltage-operated calcium channels; VOCCs). As the KCl concentration is increased and $E_K$ becomes progressively more positive the effect of the K-channel openers is decreased until $E_K$ lies below the threshold for VOCC activation at which point the K-channel openers have no effect. The leftward shift of the KCl dose-response curve caused by guanabenz is thus indicative of a depolarising effect of this compound. Lower concentrations of extracellular $K^+$ were required to cause activation of VOCCs and contraction. This effect may be caused by $\alpha_2$-adrenoceptor activation but would also be observed with agents that cause blockade of K-channels. These possibilities are discussed more thoroughly in Chapter 5.

Guanabenz therefore exerts its relaxant effect via a specific action on the noradrenaline contraction and the effects of guanabenz on the noradrenaline dose-response curve were thus investigated. Guanabenz caused a dose-dependent rightward shift in the dose-response curve to noradrenaline, in a

manner similar to that of an $\alpha_1$-adrenoceptor antagonist. At lower concentrations this antagonism seemed to be of a competitive nature but when higher concentrations were used the antagonism was non-competitive with an observed decrease in the maximum attainable response. It thus appears that the guanabenz-induced relaxation of rat aorta is mediated via antagonism of the noradrenaline response and that the agent is specifically acting as an $\alpha_1$-adrenoceptor antagonist. These results are in agreement with the findings of Bognar and Enero (1984) who reported that guanabenz antagonised the phenylephrine-induced increase in perfusion pressure in the rat mesenteric artery, but when applied alone had no effect. At similar concentrations to those used in the current study, these authors concluded that guanabenz possesses $\alpha_1$-adrenoceptor antagonist properties. In addition, Takeuchi *et al.* (1987) also reported that guanabenz could antagonise the effects of both noradrenaline and phenylephrine in the rat vas deferens and in the rabbit aorta.

The ability of guanabenz to cause an increase in the efflux of $^{42}K^+$ may arise from an increase in intracellular $Ca^{++}$ induced by $\alpha$-adrenoceptor activation. It is possible either that the antagonism of the $\alpha_2$-adrenoceptors by RX 821002 was not complete or that guanbenz is also able to stimulate the $\alpha_1$–adrenoceptor. In the latter case this would imply that guanabenz, when used at sufficiently large concentrations possesses partial agonist properties at the $\alpha_1$-adrenoceptor.

It is unlikely that guanabenz exerts its relaxant action via interfering with intracellular $Ca^{++}$ handling or interference with the $Na^+ / H^+$ exchange system. If guanabenz caused relaxation through interference with intracellular $Ca^{++}$ handling it should relax tissues pre-contracted with U-46619. U-46619 mediated contraction involves the stimulation of thromboxane-$A_2$ receptors which are G-protein linked to the activation of phospholipase C. This would result in the formation of $IP_3$ and release of intracellular $Ca^{++}$ causing contraction of the tissue (Nakahata *et al.*, 1989). If guanabenz caused relaxation through inhibition

of the $Na^+$ / $H^+$ exchange system, relaxation of high $K^+$ pre-contracted tissues would be expected in a manner similar to that seen with amiloride (Soares-da-Silva & Villaneuva, 1990). Furthermore, the inability of guanabenz to cause the relaxation of raised $K^+$ pre-contracted tissues rules out the possibility that guanabenz causes relaxation via either K-channel opening or blockade of VOCCs. Treatment of smooth muscle with high $K^+$ causes contraction, the $Ca^{++}$ for which is obtained entirely via membrane depolarisation and the opening of VOCCs. The inability of guanabenz to relax tissues pre-contracted with $K^+$ indicates a lack of inhibition of the VOCC. In addition, the ability of the K-channel opener, levcromakalim to relax these tissues suggests that $E_K$ has not been shifted below the threshold for VOCC opening by the increase in extracellular $K^+$ (see Introduction) and thus that a K-channel opener would be able to relax these tissues. In this respect guanabenz falled and thus is not a K-channel opening agent.

Soares-da-Silva and Villaneuva (1990) reported that guanabenz was also capable of relaxing tissues pre-contracted with 5HT. In the present study, 5HT was found to be a very poor spasmogen (not shown) and thus the relaxant activity of guanabenz against this spasmogen was not evaluated.

### 2.4.2.3. Conclusions from the efflux and tension recording studies

The findings of the present study indicate the guanabenz-induced relaxation of the rat aorta is not mediated via the opening of plasmalemmal K-channels and consequent hyperpolarisation of the cell. The observation that guanabenz was only vasorelaxant in those tissues contracted with noradrenaline is indicative of a direct interaction between the two agents. It is difficult to reconcile the involvement of any other mechanism in this response other than a direct antagonism of the noradrenaline response at the $\alpha_1$-adrenoceptor.

Guanabenz has differing affinity for the different subtypes of $\alpha_1$-adrenoceptor ($IC_{50}$ values ($x10^{-7}M$): $\alpha_{1A}$ = 8.21; $\alpha_{1B}$ = 14.82; $\alpha_{1C}$ = 3.61; P Greengrass, personal communication). The subtype of $\alpha_1$-adrenoceptor in the rat aorta has not been satisfactorily classified but in a recent report, Oriowo and Ruffolo (1992) reported that the $\alpha_1$-adrenoceptors in the rat aorta cannot simply be classified as either $\alpha_{1A}$ or $\alpha_{1B}$. Whatever the subtype, guanabenz has a relatively high affinity for these sites compared to other $\alpha_2$-ligands such as UK-14,304 ($IC_{50}$ values for all $\alpha_1$-adrenoceptor subtypes $>1.5x10^{-6}M$). It is therefore likely that guanabenz relaxes this tissue via $\alpha_1$-adrenoceptor antagonism.

### 2.4.3. General discussion

The findings described in the first chapter indicate that the IGR site found in the liver is not closely associated with a K-channel and that in vascular smooth muscle the potent IGR site ligand, guanabenz, does not cause vasorelaxation via K-channel opening. These results confirm the presence of an IGR site within the periphery but do not indicate a role for K-channels in the functioning of this site. This, however, does not conclusively show that the IGR site is not associated with K-channel function. The limitations of the ligand binding technique mean that any functional link between the IGR site and a K-channel (eg. via a second messenger) will not be apparent from these studies. The inability of guanabenz to induce K-channel opening seems to indicate that this agent does not operate in a manner similar to pinacidil (despite the structural similarities), but does not rule out the possibility that guanabenz may induce K-channel closure as suggested by the potentiation of the KCl response (see Chapter 5).

# Chapter 3:

## The K-channel openers: their effects on whole-cell K-currents and the importance of channel phosphorylation in their mechanism of action

## 3.1. Introduction

### 3.1.1. Target channel of the K-channel openers

The target channel of the K-channel openers is the subject of some controversy. Standen *et al.* (1989) reported that cromakalim induced a K-current in cells isolated from the rat mesenteric artery that was carried by a large conductance channel (135pS: $[K]_o$ 60mM; $[K]_i$ 140mM). These authors reported that this channel was closed as intracellular ATP concentrations were increased and concluded that the K-channel openers act via the opening of an ATP-dependent K-channel ($K_{ATP}$). This hypothesis is strengthened by observations that sulphonylureas such as glibenclamide which are known to block $K_{ATP}$ channels in pancreatic β-cells and cardiac myocytes (Trube *et al.*, 1986; Findlay, 1992), inhibit the actions of the K-channel openers. However, the $K_{ATP}$ channels in cardiac myocytes and the β-cell generally exhibit unitary conductances of between 20-40pS under a quasi-physiological K-gradient. The conductance reported by Standen *et al.* (1989) correlates more closely with that of $BK_{Ca}$ than that of any recognised glibenclamide-sensitive $K_{ATP}$ channel. Modulation of $BK_{Ca}$ by the K-channel openers has also been suggested by a number of other groups (Hu *et al.*, 1990; Carl *et al.*, 1992) but the lack of effect of charybdotoxin on the K-channel opener-induced relaxation of smooth muscle (Wickenden *et al.*, 1991) indicates that opening of $BK_{Ca}$ is most unlikely to play a pivotal role in the vasorelaxant effects of these agents in whole tissues.

Silberberg and van Breemen (1992) briefly reported that metabolic inhibition of cells isolated from rabbit mesenteric artery (resulting in presumed depletion of intracellular ATP) induced a voltage-independent current that was similar to that induced by levcromakalim. Both currents were carried by a channel of relatively low conductance. These authors further suggested that both of these currents were carried by the same class of underlying channel, namely a low conductance, voltage-independent ATP-sensitive K-channel. Several other groups have also reported that the K-channel openers activate a channel of relatively low conductance (Beech & Bolton, 1989; Kajioka *et al.*, 1990; Okabe *et al.*, 1990) but the ATP-dependence of these channels was never definitively

demonstrated.

Beech and Bolton (1989) reported that cromakalim induced a voltage-independent current carried by a low conductance channel and simultaneously inhibited the voltage-dependent, delayed rectifier current in cells isolated from the rabbit portal vein. In addition, these authors also reported that an identical rank order of potency existed for the inhibition of both the current induced by cromakalim ($I_{KCO}$) and that carried by delayed rectifier channels ($I_{K(V)}$) when tested against a number of K-channel blockers. Both $I_{K(V)}$ and $I_{KCO}$ were carried by channels of relatively low conductance and thus these authors wondered whether both these effects of cromakalim could be mediated by a single action. They considered the possibility that cromakalim could modify the voltage-sensor of the delayed rectifier channel, $K_V$ allowing this channel to be opened independently of voltage and thus to carry $I_{KCO}$, a non-inactivating, voltage-independent current. However, the lack of inhibitory effect of glibenclamide on $I_{K(V)}$ was a major problem and the possibility of K-channel opener-induced conversion of $K_V$ into $K_{ATP}$ was concluded to be unlikely (Beech & Bolton, 1989).

Similar findings to those of Beech and Bolton (1989) were reported by Noack *et al.* (1992a) who also found that levcromakalim induced a non-inactivating K-current whilst simultaneously inhibiting a voltage-dependent current with characteristics similar to those of the delayed rectifier. Noack *et al.* (1992a), however, suggested that these were mutually exclusive effects of the same agent. The current induced by levcromakalim was a voltage-independent current carried by a channel of approximately 17pS (quasi-physiological K-gradient) and was inhibited by glibenclamide. Later experiments suggested that levcromakalim-induced inhibition of $I_{K(V)}$ might be caused by dephosphorylation of this channel (Noack *et al.* 1992d).

Noack *et al.* (1992d) reported that removal of tricarboxylic acid intermediates from the intracellular solution and glucose from the PSS, a procedure designed to induce cellular ATP depletion, caused the induction of a K-current of relatively low conductance (10-20pS) that was inhibited by

glibenclamide. In addition, following run-down of this metabolic current, levcromakalim had no effect. Induction of this metabolic current also resulted in a decrease in the magnitude of $I_{K(V)}$. This study indicates that levcromakalim and metabolic inhibition both induced the opening of a voltage-independent, ATP-sensitive K-channel.

It therefore seems that K-channel openers such as cromakalim exert their relaxant effects on smooth muscle via the opening of plasmalemmal K-channels that are closed as concentrations of intracellular ATP rise. These are low conductance, voltage-independent channels that are blocked by the sulphonylureas. The vast majority of work to date has examined the mechanism of action of the prototype K-channel opener, cromakalim, or its active enantiomer, levcromakalim. Whether other structurally-diverse K-channel openers exert their relaxant mechanisms via activation of the same channel is as yet unclear.

### 3.1.2. Chemically diverse K-channel openers

Edwards and Weston (1990a) recently highlighted the structural diversity within the K-channel openers. At present it is unclear whether these agents all possess a common nucleus around which different structures are based but the apparent differences in chemical structures may be indicative of different mechanisms of action. Discrepancies do exist in the pharmacological profiles of agents such as nicorandil, diazoxide and minoxidil sulphate when compared to cromakalim (see Introduction). However, cromakalim, pinacidil and RP 49356 are all K-channel opening agents with fundamental differences in their structures despite which they all possess similar pharmacological profiles. All are potent relaxant agents of smooth muscle preparations and all are inhibited by glibenclamide. Thus it is possible that these agents do act in a similar manner and therefore do activate the same channel. However, electrophysiological evidence for a common mechanism of action for these agents has never been demonstrated.

### 3.1.3. Mode of action of the K-channel openers

The mechanism of action by which the K-channel openers act has never been established. it is unclear whether they interact directly with the $K_{ATP}$ channel complex or whether they in some way modulate the mechanism by which the channel is controlled. Thuringer and Escande (1989) suggested that cromakalim may act, in cardiac myocytes, by displacing ATP from an inhibitory site on the channel complex. It may be therefore that a common mechanism is involved in the opening of $K_{ATP}$ and in the inhibition of $I_{K(V)}$. In both cases, the observed effect may arise from interference by the K-channel opener with an ATP binding site. Such an interference with ATP binding could cause $K_{ATP}$ to open. Alternatively, such an action could be exerted on a protein kinase thus preventing phosphorylation of the delayed rectifier channel and preventing its opening. The *inhibition* of $I_{K(V)}$ by these agents is therefore (paradoxically) potentially diagnostic in determining the mechanism by which K-channel *opening* occurs.

The action of the K-channel openers is not confined to K-channels. It has recently been reported that agents such as cromakalim and diazoxide are able to *close* an ATP-regulated chloride channel ($Cl_{ATP}$; Sheppard & Welsh, 1992). It is a defect in this channel that is thought to result in cystic fibrosis and thus the channel is often referred to as the cystic fibrosis transmembrane regulator (CFTR). This channel is activated as intracellular levels of ATP increase and is also subject to cAMP-dependent phosphorylation. Thus, the ability of K-channel openers to close this channel is consistent with an interaction between these agents and an ATP-binding site on the channel. Interestingly, the most potent inhibitor of the $Cl_{ATP}$ channel in this study was found to be glibenclamide.

The mechanism by which the sulphonylureas block the effects of the K-channel openers is unknown. Angel and Bidet (1991) reported that the K-channel openers were unable to displace [$^3$H]-glibenclamide binding from the sulphonylurea receptor in rat cortical membranes. It therefore seems that inhibition of the K-channel openers by agents such as glibenclamide does not

occur via a direct interaction with the same site but via a more distinct allosteric mechanism (Edwards & Weston, 1993).

### 3.1.4. K-channel openers; requirement for phosphorylation of $K_{ATP}$

Noack *et al.* (1992d) recently suggested that levcromakalim could cause dephosphorylation of the delayed rectifier channel, $K_V$, in vascular smooth muscle cells. In addition, they argued that the inability of this agent to increase the open probability of $K_{ATP}$ to values greater than 0.5 was due to its ability also to dephosphorylate this channel i.e. levcromakalim is initially able to open and then effectively to *close* $K_{ATP}$. Such a view is consistent with the reports that the K-channel openers can accelerate the run-down of $K_{ATP}$ in pancreatic β-cells in $Mg^{++}$- and ATP-free conditions (Kozlowski *et al.*, 1989). Wollheim *et al.* (1988) reported that activators of protein kinase C (PKC) caused a decrease in the open state probability of $K_{ATP}$ channels in pancreatic β-cells and also caused a depolarisation in these cells. PKC may thus exert an inhibitory phosphorylating action on $K_{ATP}$ in this tissue. Finally, Tung and Kurachi (1991) reported that the $K_{ATP}$ channel in cardiac myocytes required phosphorylating in order to be maintained in an operative state. Futhermore, these workers showed that a number of nucleotide diphosphates could substitute for phosphorylation and maintain channel activity in ATP-free solutions. The requirement for phosphorylation, both to maintain this channel in an operative state and to cause closure is therefore evident. The ability of K-channel openers to affect this phosphorylation is indicated by the reports of Kozlowski *et al.* (1989) and also by Fan *et al.* (1990) who reported that pinacidil possessed multiple modulatory actions on the $K_{ATP}$ channel in ventricular myocytes. These included the ability both to activate and to inhibit the channel.

### 3.1.5. Specific aims

The aims of the experiments described in this chapter were to determine the mechanism of action of the K-channel openers and of their inhibition by glibenclamide. Primarily, this study was designed to examine whether the structurally-dissimilar K-channel openers, P1060, aprikalim, pinacidil, minoxidil sulphate and levcromakalim act via a common target channel. The nature of the channel activated was studied and the ability of glibenclamide to inhibit the effects of these agents was also investigated.

## 3.2. Methods

### 3.2.1. Cell isolation

All experiments were performed on single smooth muscle cells freshly isolated from the hepatic portal vein of male Sprague-Dawley rats (150-200g), previously killed by stunning and cervical dislocation.

Portal veins were dissected into a nominally $Ca^{++}$-free physiological salt solution (Klöckner & Isenberg, 1985) and cleaned of extraneous connective tissue under a dissecting microscope. The vein was opened along its longitudinal axis and placed in a collagenase / pronase solution originally described for the separation of guinea-pig bladder smooth muscle cells (Klöckner and Isenberg, 1985). The tissue was agitated for 35min in the enzyme solution at 37°C, washed using the same solution free of enzyme and cut into 4 segments. These segments were then triturated using a wide bore, smooth tipped pipette in Kraftbrühe (KB-medium; Klöckner and Isenberg, 1985). Cells were stored at 4°C in KB-medium and used within 9h of separation. All experiments were performed at room temperature (22-26°C).

### 3.2.2. Single-cell electrophysiology

The whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981) was employed using an Axopatch-1C amplifier (Axon Instruments). The settling time of the system was less than 500µs.

Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an Axon TL-1 interface. For cell stimulation and for recording and analysing data, the pClamp 5.5 programme was used (Axon Instruments). Data were stored on a digital audio tape recorder (Sony) and the evoked membrane currents were monitored continuously on a Gould Windograf recorder. The data described were not corrected by linear leak subtraction. Patch pipettes were made from Pyrex glass (H15/10, Jencons, UK) and had a resistance of 2-4MΩ when filled with internal solution.

The bath containing the isolated cells was perfused with a

$Ca^{++}$-free physiological salt solution at approximately 0.7ml $min^{-1}$ using a peristaltic pump (LKB Microperpex); a second identical pump was used to remove the solution from the recording chamber. The effects of drugs were examined by adding appropriate quantities into the reservoir perfusing the bath so that responses could be examined under equilibrium conditions. A minimum of 10min was allowed for drug / tissue contact time or until maximum drug effect was observed. Antagonist studies were conducted by either pre-applying the antagonist to the recording chamber and subsequently applying the agonist (K-channel opener) or reversal experiments were conducted by adding the antagonist once a full response to the agonist had been obtained.

### 3.2.3. Establishing the whole-cell configuration

Patch pipettes filled with intracellular solution were attached to a micromanipulator (Leitz) and positive pressure applied through the pipette as it was lowered into the bathing solution to prevent contamination of the internal solution. This pressure was continually applied until the tip of the patch pipette lay adjacent to the cell. A 1mV pulse (stepping from -1mV to 0mV for 50ms) was then repeatedly applied via the pipette tip as this was placed on the cell surface. Gentle suction was then applied through the pipette tip until the current flow associated with this voltage step was negligible (ie. a gigaseal had been formed). The current level under these conditions was then adjusted to zero using the manual adjustment of the junction null on the Axopatch and also on the oscilloscope and chart recorder. This manual adjustment compensates for any change in junction potential (the potential between the pipette and the bath / cell) that occurs during seal formation. The injected pulse was then increased to 50mV (stepping from -50mV to 0mV for 50ms) and again suction applied until the current evoked by this voltage pulse was negligible. In certain cases, the measured current gradually decreased without suction being applied. Under these conditions, the seal resistance was allowed to develop without aid. A current of 12.5pA when the voltage step of 50mV was applied is indicative of a

seal resistance of 4 GΩ.

Once the gigaseal had been established, short pulses of suction were applied through the electrode until a sudden increase in the size of the capacitive transients was observed (Figure 3.1). This indicates destruction of the patch of membrane below the pipette tip and reflects the contribution of the cell membrane to the pipette input capacitance following the destruction of the membrane patch. In some cases, a gradual increase in the input resistance was observed as a progressive decrease in the magnitude of the measured currents. This was associated with an increase in the time constant, $\tau$, of the transient capacitive current (Figure 3.1). In these cases the pipette tip was raised slightly to alleviate any pressure on the cell surface and suction reapplied to the pipette tip until the value for $\tau$ decreased and the measured currents returned to their previous magnitude.

In the experiments of this type, whole-cell recordings were made for a maximum of 1h and over this period there were no obvious signs of deterioration in the currents measured

### 3.2.4. Voltage protocols

Two voltage protocols were employed in these studies. Firstly, cells were held at -60mV, prepulsed to -90mV and then stepped to a range of test potentials between -80mV and +30mV (10mV increments). Under these conditions the total whole-cell currents were measured comprising both inactivating and non-inactivating currents. In addition, cells were also held at -10mV and stepped to identical test potentials. When this protocol was employed only non-inactivating currents were available.

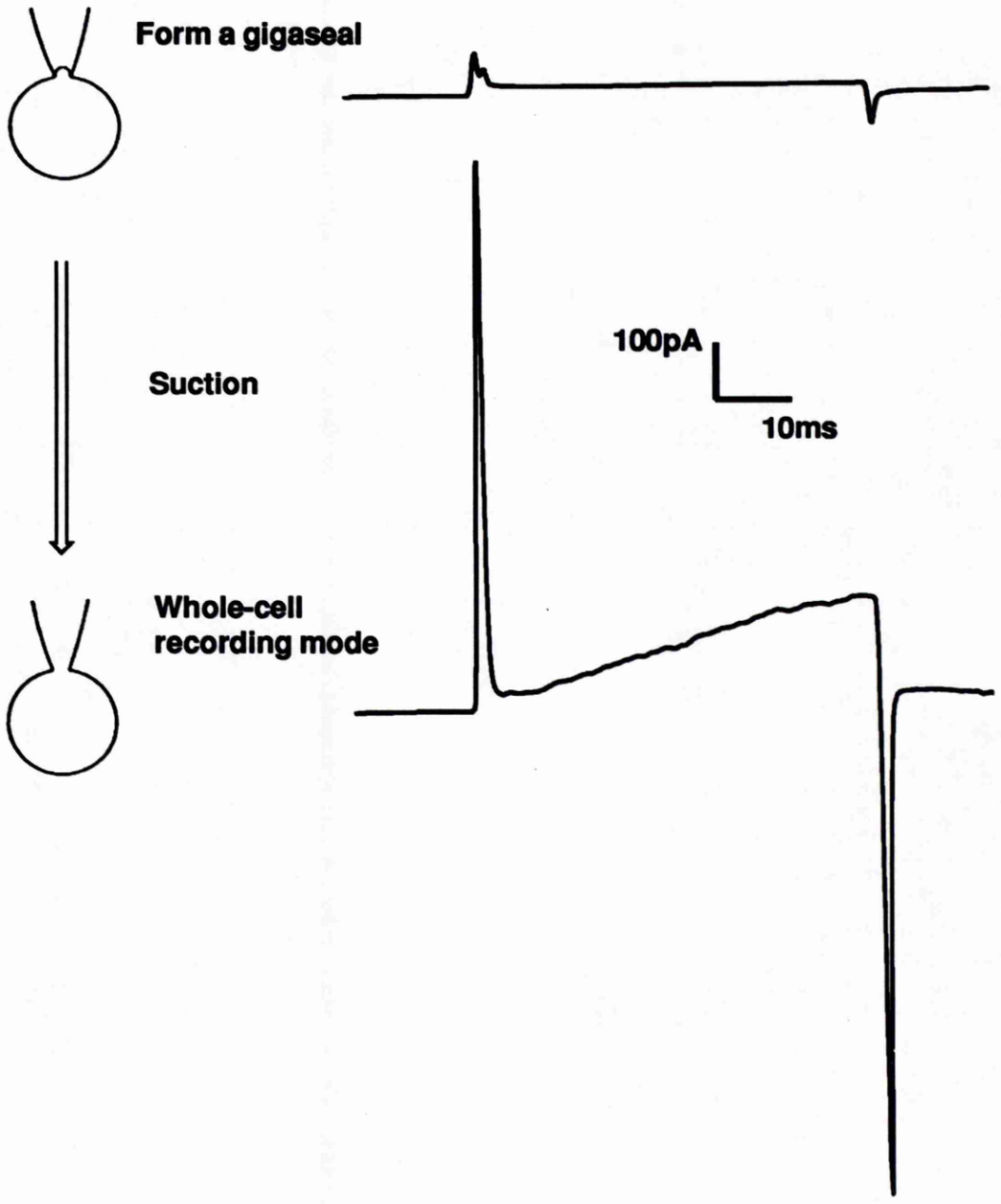



**Figure 3.1.** This figure illustrates the observed changes in the capacitance transient upon patch disruption following formation of a gigaseal between the patch pipette and the cell membrane. In both cases the system was stepped from -50mV to 0mV for 50ms at a frequency of 1Hz. The upper panel illustrates the observed currents prior to patch disruption and the lower panel the currents following patch disruption. Note the large increase in the capacitance transient reflecting the contribution of the cell membrane to the input capacitance and the rising phase of the current due to the time-dependent induction of the delayed rectifier K-current, $I_{K(V)}$ which was induced on stepping from -50mV to 0mV.

### 3.2.5. Single channel noise fluctuation analysis

Analysis of changes in current noise level associated with changes in the holding current was carried out using single channel noise fluctuation analysis (Neher & Stevens, 1977). The theory for noise analysis is based upon three assumptions. All channels are assumed to exist either in a conducting or a non-conducting state with a probability *Po*, of being in the conducting state. Secondly, the gating of each channel is assumed to be statistically independent of other channels and finally the population of channels activated by the agent in question is assumed to be homogeneous. Under these conditions, the variance of the current, $\sigma$ , is expressed as a function of the single channel current, *i*, the mean current, $\mu$, and the number of channels, *N*, by the following equation (Neher & Stevens, 1977; Noack *et al.*, 1992b)

$$\sigma^2 = i.\mu - \mu^2/N \qquad - \qquad \text{eqn 1}$$

Segments of current trace each of 2s duration were sampled for noise analysis from a constant holding potential of -10mV. Current traces were displayed as amplitude histograms using the Fetchan program (pClamp 5.5) and these histograms were then computer fitted with a normal Gaussian distribution (pStat; pClamp 5.5). Values of $\mu$ and $\sigma$ were calculated as the mean and standard deviation of these Gaussian distributions, respectively. Values obtained prior to addition of the K-channel opener were employed as controls and subtracted from subsequent values. Mean current ($\mu$) values were then plotted against $\sigma^2$ and the value for single channel current, *i* was derived from the tangent to the curve where both values equalled zero. Single channel conductance, *g*, was then obtained by substitution of *i* into the Goldman-Hodgkin-Katz equation (eqn 2; for derivation and further explanation see Hille, 1992).

$$E_{rev} = \frac{RT}{F} \ln \frac{PK[K]o + PNa[Na]o + PCl[Cl]i}{PK[K]i + PNa[Na]i + PCl[Cl]o} \quad - \quad \text{eqn 2}$$

Where $E_{rev}$ is the reversal potential or zero current potential, R is the gas constant (8.315 $J.K^{-1}$ $mol^{-1}$), T the absolute temperature (K) and F is Faraday's constant, (9.648 x $10^4$ $C.mol^{-1}$). The prefix 'P' represents the permeability of the membrane to that particular ion (ie. PK means the membrane permeability to potassium ions). The suffix 'i' or 'o' following square brackets denotes the intracellular or extracellular concentration, respectively, of the species inside the brackets.

### 3.2.6. Statistical analysis

To test for significant changes in the magnitude of current / voltage relationships following one or more individual treatments, the multiple analysis of variance (MANOVA) test was employed (Statistica mac). Delayed rectifier current / voltage relationships were tested for significant differences between -20mV and +30mV inclusive, whilst changes in the non-inactivating component were tested for significant differences between -80mV and +30mV.

The rate of onset of the increases in holding curent induced by the K-channel openers was tested for significance using Students unpaired t-test for the comparison of means (Statworks). In both cases, p values less than 0.05 were considered significant.

### 3.2.7. Drugs and solutions

The composition of the solutions used was as follows:

Pipette (intracellular) solution comprised (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, $K_2HPO_4$ 1.2, glucose 11, HEPES 10, EGTA 1.2, oxaloacetic acid 5, sodium pyruvate 2, sodium succinate 5, pH 7.3. In certain experiments MgATP (5mM) was also included in the pipette solution from which $MgCl_2$, oxaloacetic acid, sodium pyruvate and sodium succinate were then omitted;

Bath (extracellular) solution comprised (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, $KH_2PO_4$ 1.2, glucose 11, HEPES 10, EGTA 1, pH 7.3 aerated with $O_2$.

The enzyme solution for cell separation comprised (mM): KOH 130, $CaCl_2$ 0.05 taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (1mg $ml^{-1}$; Sigma Type 8), pronase (0.2 mg $ml^{-1}$; Calbiochem), fatty acid-free albumin (1mg $ml^{-1}$; Sigma). The pH was adjusted to 7.4 with methanesulphonic acid. An identical solution was also prepared without either the collagenase, pronase or fatty acid free albumin ('enzyme free' solution) and used for washing the tissue free of enzyme.

'KI solution' (Klöckner and Isenberg, 1985) comprised (mM): NaCl 100, KCl 10, $KH_2PO_4$ ($\cdot 3H_2O$) 1.2, $MgCl_2$ ($\cdot 6H_2O$) 5, glucose 20, taurine 50, MOPS 5. The pH was adjusted to 6.9 with NaOH.

'KB solution' (Kraftbrühe solution, Klöckner & Isenberg, 1985) comprised (mM): KCl 84.9, $KH_2PO_4$ 38, $MgSO_4$ 4.8, $Na_2ATP$ 4.6, Na-pyruvate 6.2, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid-free human albumin, 1mg/ml. The pH was adjusted to to 7.3 with KOH.

Pipette solution, enzyme and enzyme free solutions and 'KB' and 'KI' solutions were all made up in 100ml batches and then dispensed into appropriately sized aliquots for a single day's work and then kept at -20°C until required. External bath solution was made up from stock solutions (kept at 4°C) and used within 72h. Glucose was not added to the bathing solution until the day of use.

The following drugs were used: aprikalim (formerly RP 52891, Rhône-Poulenc Rorer), P1060 (N'-cyano-*N*-(3-pyridyl)-N"-(*t*-butyl)guanidine; Leo), glibenclamide (Sigma). Okadaic acid and PKI(6-22)amide were all obtained from Sigma. Minoxidil sulphate was obtained from Zeneca Pharmaceuticals and levcromakalim ((-)6-cyano-3,4-dihydro-2,2dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol) was obtained from SmithKline Beecham or from Pfizer Central Research. Taurine, oxaloacetic acid, creatine, Na-pyruvate, Na-succinate, MgATP and $Na_2ATP$ were all obtained from Sigma. All other chemicals were purchased from BDH chemicals Ltd.

Aprikalim, P1060, levcromakalim and pinacidil were all made up in DMSO as 20mM stock solutions. Glibenclamide (10mM) was also made up in DMSO. Minoxidil sulphate (1mM) was made up in 70% ethanol. All stock solutions were subsequently diluted in $Ca^{++}$-free PSS. PKI(6-22)amide (16µM) was dissolved directly into intracellular solution. Okadaic acid (1mM) was dissolved in DMSO and then diluted in intracellular solution.

## 3.3. Results

### 3.3.1. Whole-cell K-currents

Whole-cell currents were recorded from freshly isolated rat portal vein cells under $Ca^{++}$-free conditions. From a holding potential of -60mV, cells were prepulsed to -90mV and then stepped to a range of test potentials between -80mV and +30mV in 10mV increments. Under these conditions, cells displayed a basal current level that was available over the entire test potential range and which did not inactivate. This basal current level possessed a linear current / voltage relationship and was designated the non-inactivating current, $I_{NI}$ (Figure 3.2). The relationship crossed the voltage axis at between -10mV and -30mV and was probably carried by cation channels. In addition, when stepping to test potentials positive to -40mV a slowly activating, slowly inactivating current was also observed. This current increased exponentially with voltage and was designated the delayed rectifier current, $I_{K(V)}$ (Figure 3.2). Finally, a fast activating, fast inactivating current was also observed under these conditions. This current possessed activation and inactivation time-course similar to that described for the A-current in neurones (Connor & Stevens, 1971; Figures 3.5 & 3.6). This current was present in approximately 50% of cells and was of variable magnitude. It ranged from a fairly small current (approximately 50pA at 0mV) to one even larger than the delayed rectifier current, $I_{K(V)}$ (approximately 400pA at 0mV).

When cells were clamped at -10mV, the voltage-dependent currents described above became activated and then inactivated. Following complete inactivation of these currents, only non-inactivating currents were available upon stepping to the same test potentials as outlined above (see Figures 3.5 & 3.6). Holding cells at -10mV and stepping to test potentials therefore allowed measurement of only the non-inactivating currents, $I_{NI}$.

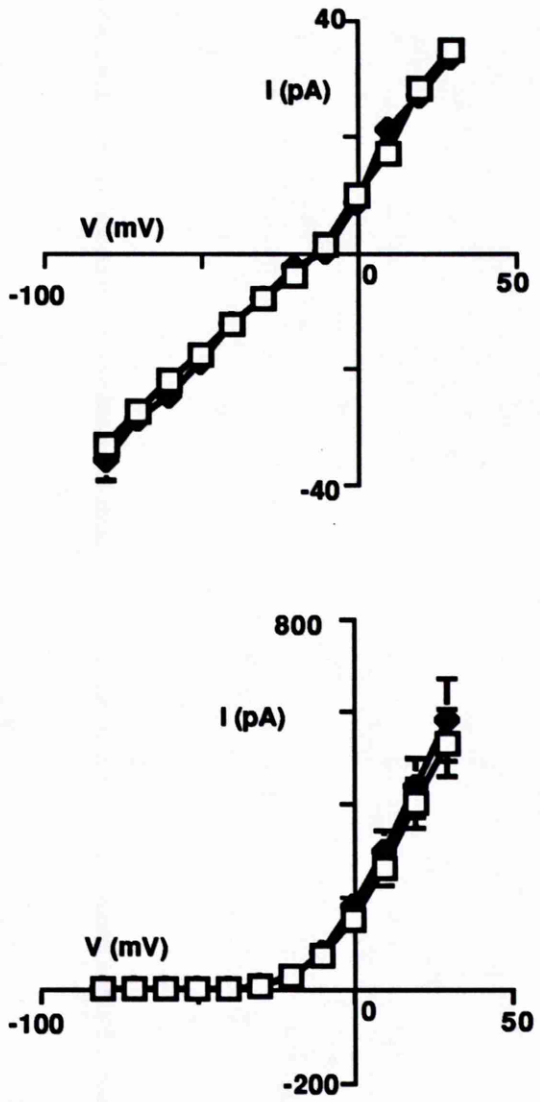



**Figure 3.2.** The effect of drug vehicle (dimethylsulfoxide: DMSO; 0.05%) on the whole-cell K-currents of the rat portal vein. DMSO (◆) had no effect on either $I_{NI}$ (HP -10mV, upper panel) or $I_{K(V)}$ (HP -90mV, lower panel) when perfused into the recording chamber when compared with the control currents (❑). Points are the mean of 3 recordings and vertical bars represent the s.e. mean. Note the difference between the magnitude of $I_{NI}$ and $I_{K(V)}$.

Digital subtraction of the currents evoked when stepping to test potentials from a holding potential of -10mV, from those evoked when stepping to test potentials from -90mV, yielded values for the voltage-dependent component of these whole-cell currents only, ie. $I_{K(V)}$ and the A-current. $I_{K(V)}$ and the A-current could easily be separated by their kinetic differences. The fast inactivation of the A-current meant that current level measurements 250ms into the test potential pulse only comprised $I_{K(V)}$ as by this point the A-current had completely inactivated. The aims of experiments using this two holding potential design were thus to investigate the effects of a variety of treatments on the magnitude of $I_{NI}$ and $I_{K(V)}$. Changes in the A-current were not measured since preliminary experiments showed that none of the treatments / procedures had a significant effect on this current component.

### 3.3.2. The K-channel openers

When cells were held at -10mV, approximate $EC_{90}$ concentrations (for their mechano-inhibitory effects) of the K-channel openers, levcromakalim (1μM), P1060 (1μM), aprikalim (3μM) and pinacidil (3μM) each caused an increase in the holding current which was accompanied by an increase in the current noise level (Figures 3.3). The time to maximum effect of these agents varied with P1060 (1μM) reaching a maximum (6.6 ± 0.6min; n=6) significantly faster than any of the other K-channel openers (Student's t-test, $p<0.05$ in each case; levcromakalim (1μM) 10.89 ± 0.6min, n=7; aprikalim (3μM) 10.94 ± 1.04min, n=5; pinacidil (3μM) 12.07 ± 0.73min, n=3). Application of minoxidil sulphate (1μM) to the recording chamber had no effect on the holding current.

#### 3.3.2.1. Single channel noise fluctuation analysis

The increase in holding current magnitude was accompanied by an increase in the level of noise of the holding current. Single channel noise fluctuation analysis of these increases in noise level was performed to determine the conductance of the channel underlying the effects of P1060 and aprikalim.

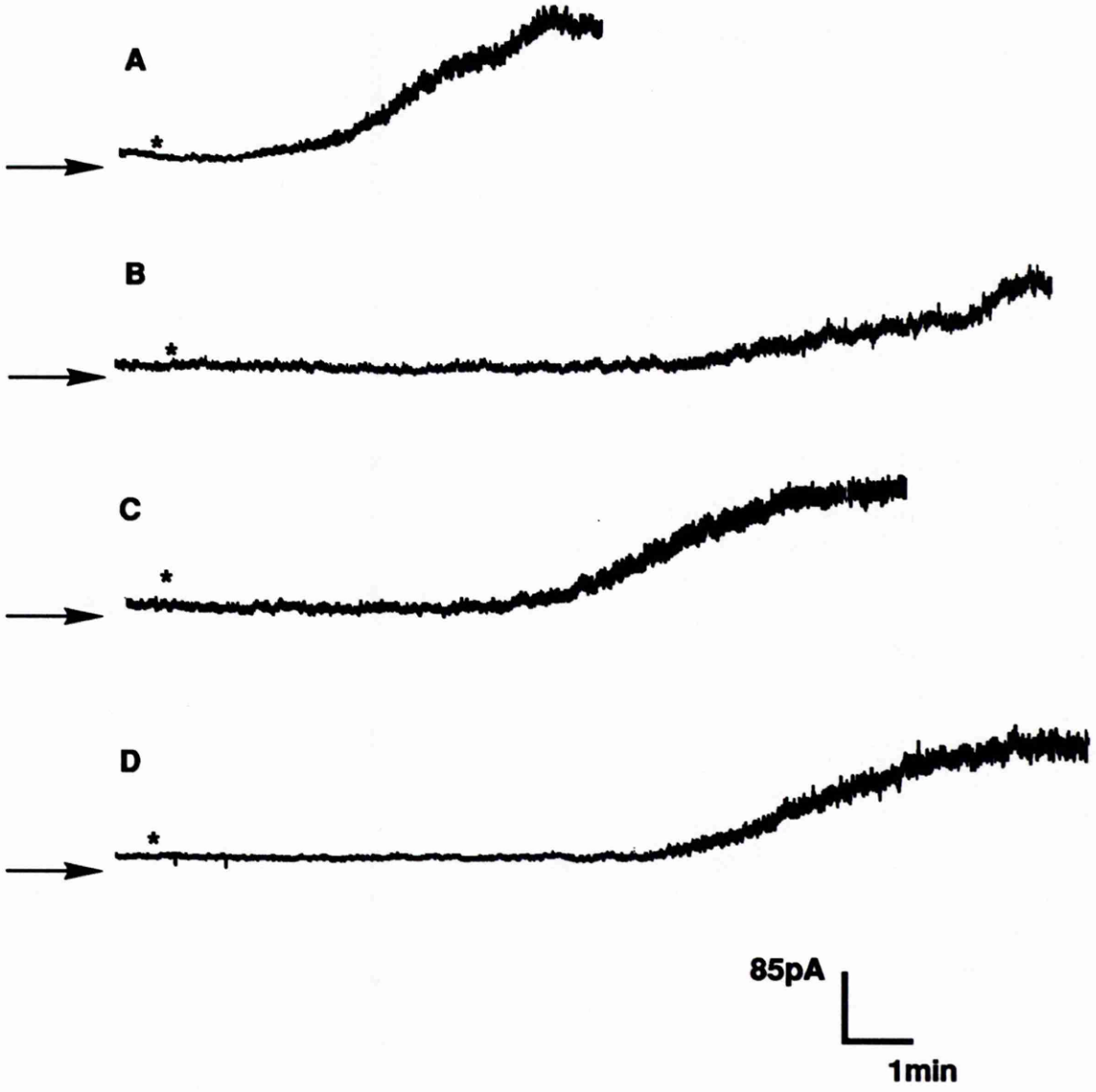



**Figure 3.3.** Typical traces derived from four different cells each clamped at a holding potential of -10mV illustrating the increase in holding current caused by application of P1060 (1μM; A), aprikalim (3μM; B), levcromakalim (1μM; C) or pinacidil (3μM; D). In all cases the increase in holding current was associated with an increase in the noise level of the current. The point at which drugs were applied to the perfusing solution is indicated by the appropriate asterisk (*) and arrows indicate the zero current level. The rate of onset of the increase in holding current was significantly greater for P1060 than for the other K-channel openers.

From the current noise signals, segments of 2s duration were sampled for further analysis. From these, amplitude histograms were constructed and Gaussian distributions fitted to them by computer. For each cell, the maximum number of possible observations was obtained and ranged from 5 - 54 (mean = 14, n = 11). This number was dependent on the rate of onset of the increase in holding current for each individual cell following exposure to the K-channel opener. The mean current ($\mu$) and the current variance ($\sigma$) were obtained for each 2s sample and analysed as described in the methods.

Equation 1 predicts a parabolic shape of the current variance - mean plot with the apex of the curve increasing, and the right hand intercept with the abcissa shifting to the right, as the value for the number of channels, *N* increases. For both agents, best-fitted curves gave values for *N* ranging from 200 - 2000 per cell. The theory for noise analysis predicts a decrease in current noise when the mean value for the open channel probability (*Po*) is larger than 0.5. In 6 out of 11 cells *Po* values greater than 0.5 were obtained (4 out of 7 for P1060 and 2 out of 4 for aprikalim) but only 1 cell (P1060) achieved a *Po* value greater than 0.75. The increase in *Po* to values greater than 0.5 is thus indicative of a slight decrease in the current noise level.

In some cases it was not possible to fit a curve to the larger $\mu$ and corresponding $\sigma^2$ values with great accuracy (see Figure 3.4). Nevertheless, this fact did not markedly influence the calculated values for the single channel current, *i* and thus the single channel conductance, *g*. The data in Figure 3.4 were best-fitted with single channel currents of 0.38pA (curve A) and 0.4pA (curve B). The uncertainty of the fit was related to *N* which was calculated to be 830 and 510 respectively. In spite of the variation found in the number of channels, the data is well-fitted over the initial part of the curve and yields single channel current values that only vary by 5%.

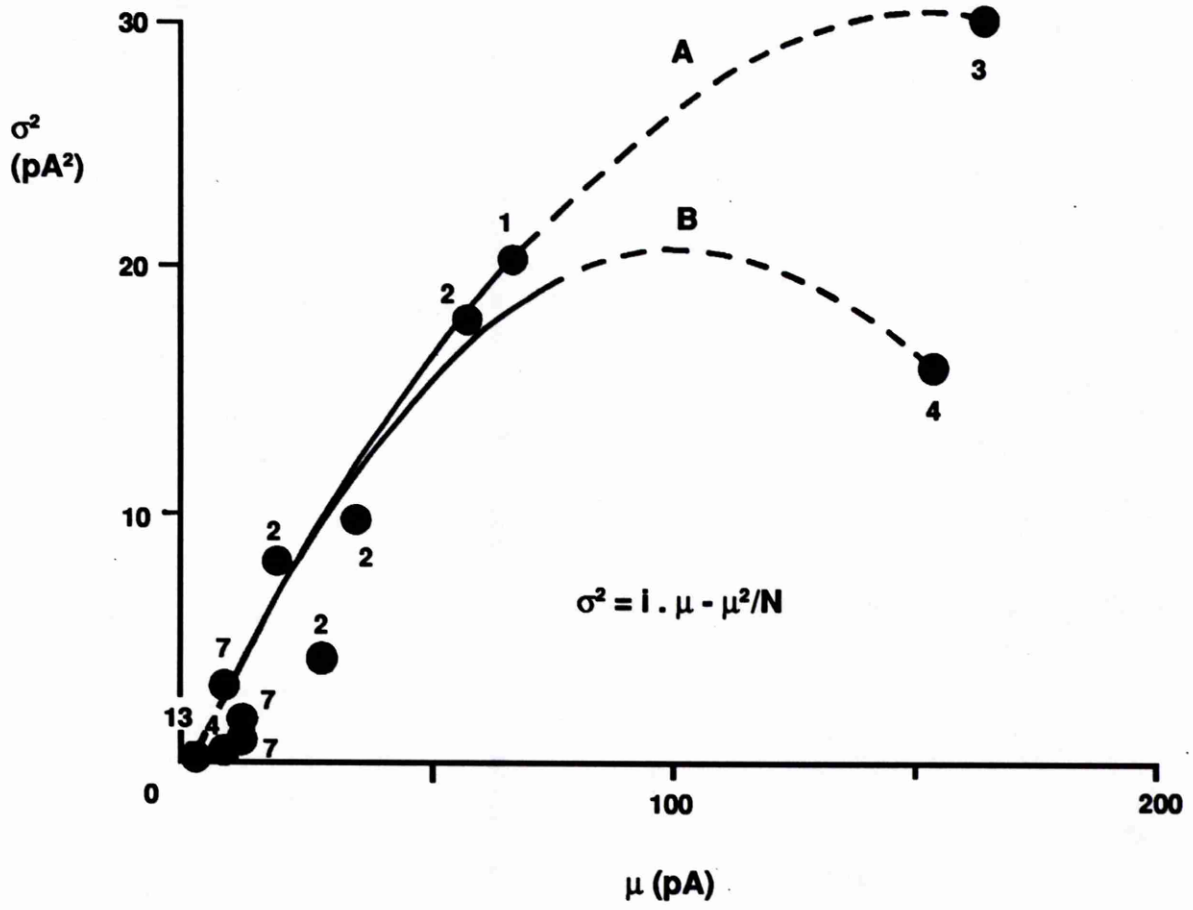



**Figure 3.4.** Current variance ($\sigma^2$) / mean current ($\mu$) plot for the generation of $I_{KCO}$ by P1060 (1$\mu$M). The left-hand data points are well-fitted by the two curves, A and B which were calculated according to the equation $\sigma^2 = i.\mu - \mu^2/N$ (where $i$ is the single channel current, 0.38pA, and $N$ is the number of channels). The single channel current is calculated from the slope of the tangent to the curve at 0pA. These two curves represent the extremes of the obtained data and the value for the single channel current from these two curves differs by only 5%. The error is incurred by the inability to fit the data accurately to the right hand points. Thus reducing $N$ from 830 to 510 results in the two curves A and B, respectively. The numbers near the data points indicate the numbers of observations (2s samples).

Using data derived from 11 cells of comparable size, the single channel current was calculated as 0.41 ± 0.08pA for P1060 (n=7) and 0.41 ± 0.05pA for aprikalim (n=4). When the single channel conductance was calculated according to the Goldman-Hodgkin-Katz equation a value of 10.5pS at 0mV was obtained for both P1060 and aprikalim.

### 3.3.2.2. Effects of K-channel openers on current / voltage relationships

Current / voltage experiments were conducted in the presence of P1060 (1μM), aprikalim (3μM), levcromakalim (1μM), pinacidil (3μM) or minoxidil sulphate (1μM) to determine the effects of these agents in more detail. The effects of P1060 (1μM) and aprikalim (3μM) on whole-cell currents are shown in Figures 3.5 and 3.6 respectively. P1060, aprikalim, levcromakalim and pinacidil each caused an increase in the magnitude of the total non-inactivating currents over the entire test potential range (Figures 3.7, 3.8, 3.9 & 3.10). Control I/V curves reversed at approximately -30mV but in the presence of the K-channel openers this reversal potential was shifted in a hyperpolarising direction by approximately 30mV to -60mV. In addition, test and control curves crossed at approximately -78mV, a value close to the theoretical potassium equilibrium potential ($E_K$) in these cells (-83mV, Noack *et al.*, 1992c). Thus the non-inactivating current induced by the K-channel openers was different from the control non-inactivating current ($I_{NI}$) and was likely to be carried by K-channels. It was thus designated $I_{KCO}$. For P1060 (0.3-10μM) and aprikalim (3-30μM) the observed effects of these agents were concentration-dependent with a decrease in the maximum response to both agents when concentrations were increased to the upper limit of the doses tested (Figures 3.11 and 3.12).

The same K-channel openers also caused a significant decrease in the magnitude of the voltage-dependent current, $I_{K(V)}$ ($p<0.05$ in each case, MANOVA; Figures 3.7, 3.8, 3.9 & 3.10). For aprikalim, levcromakalim and pinacidil this inhibition of $I_{K(V)}$ was evident at all potentials at which $I_{K(V)}$ was activated (Figures 3.8, 3.9 & 3.10 respectively) but in the case of P1060, inhibition of $I_{K(V)}$ was only seen at test potentials positive to 0mV (Figure 3.7).

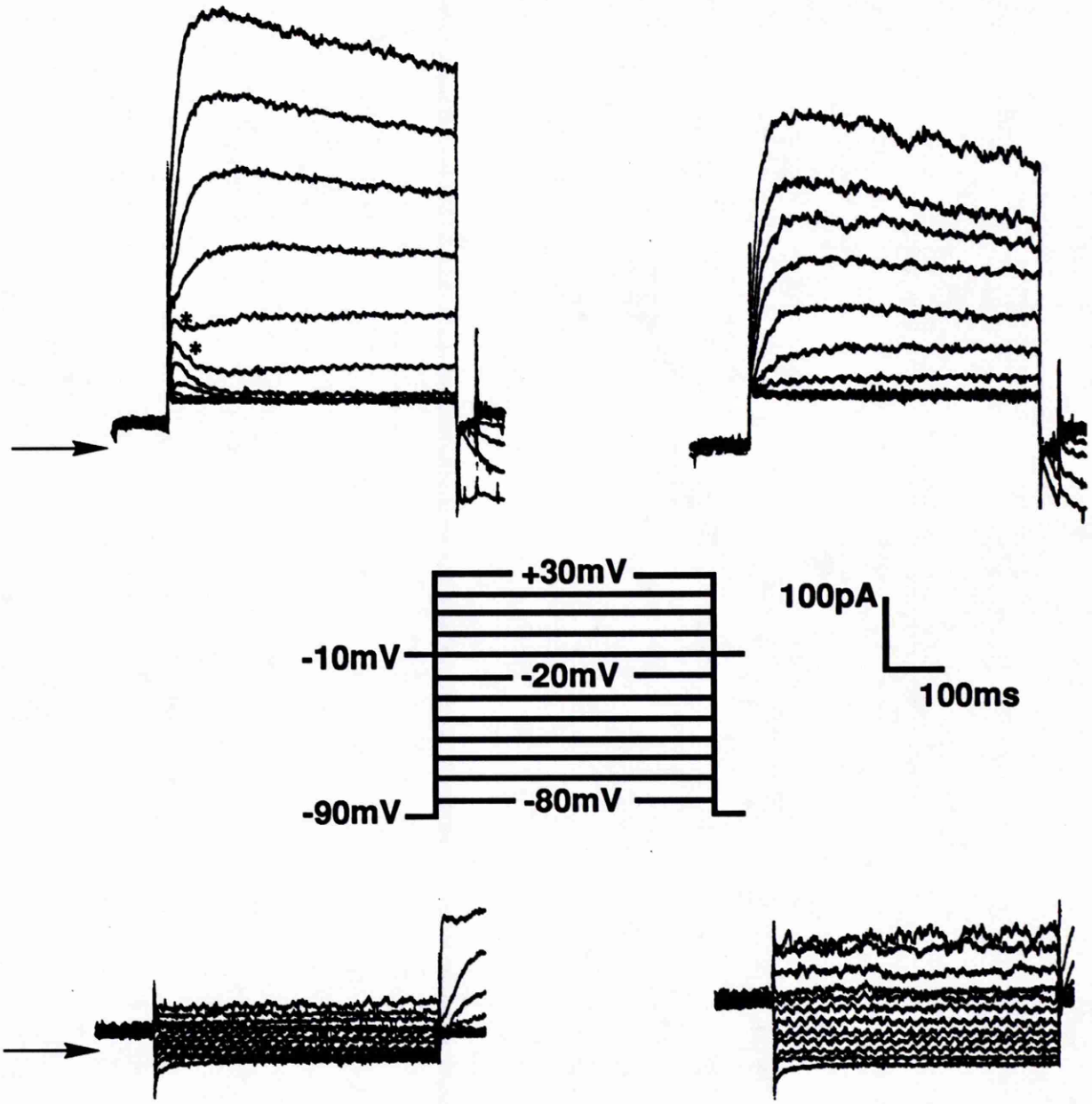



**Figure 3.5.** The effect of P1060 (1μM) on whole-cell K-currents recorded from rat portal vein cells. The upper panels illustrate the delayed rectifier current, $I_{K(V)}$ generated from a holding potential of -90mV. The fast inactivating A-current is also visible in the control panel at -10mV and -20mV (*). P1060 caused a decrease in the magnitude of $I_{K(V)}$ (right-hand panel) when compared to control currents (left-hand panel). The lower panels illustrate the non-inactivating component of the whole-cell currents obtained when stepping to test potentials from a holding potential of -10mV. P1060 caused an increase in the magnitude of non-inactivating currents (right-hand panel) when compared to the control currents (left-hand panel). The centre panel illustrates the two protocols used to evoke these currents and arrows represent the zero current level.

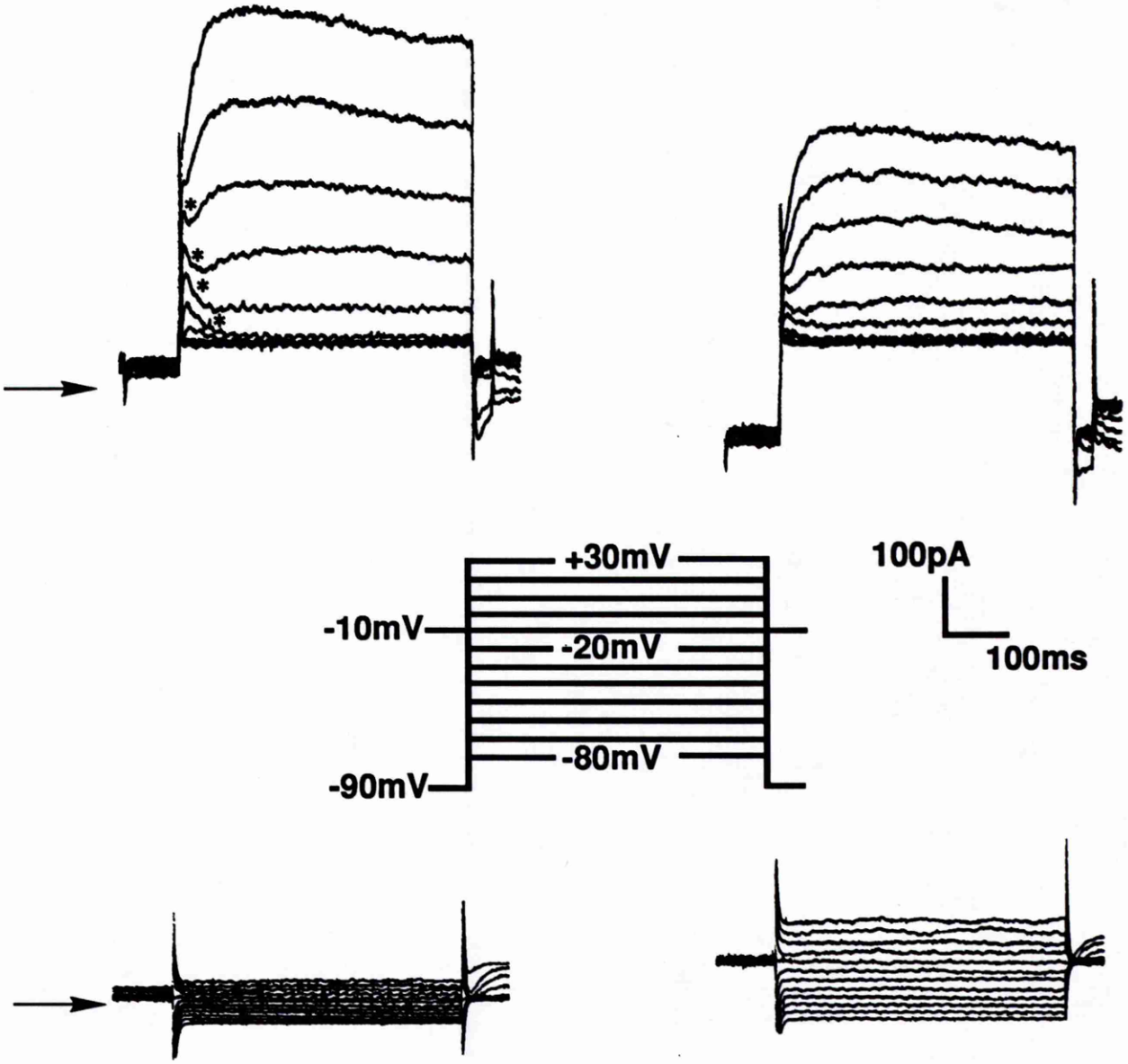



**Figure 3.6.** The effect of aprikalim (3μM) on whole-cell K-currents recorded from rat portal vein cells. The upper panels illustrate the delayed rectifier current, $I_{K(V)}$ generated from a holding potential of -90mV. The fast inactivating A-current is also visible at potentials positive to -20mV (*). Aprikalim caused a decrease in the magnitude of $I_{K(V)}$ (right-hand panel) when compared to control currents (left-hand panel). The lower panels illustrate the non-inactivating component of the whole-cell currents obtained when stepping to test potentials from a holding potential of -10mV. Aprikalim caused an increase in the magnitude of non-inactivating currents (right-hand panel) when compared to the control currents (left-hand panel). The centre panel illustrates the two protocols used to evoke these currents and arrows represent the zero current level.

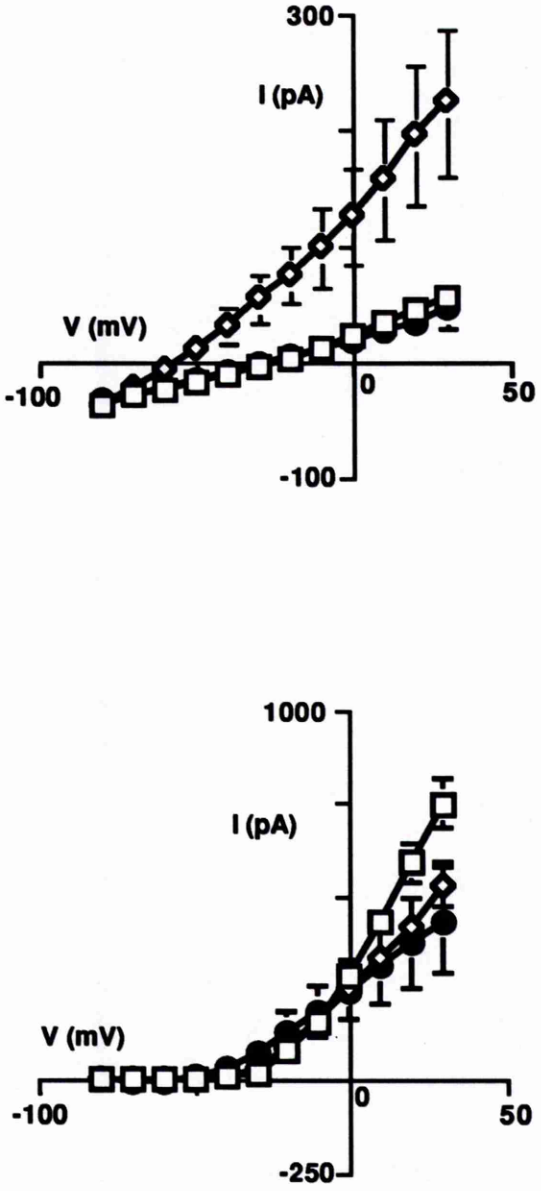



**Figure 3.7.** The effects of P1060 (1μM) on whole-cell K-currents and their reversal by glibenclamide (1μM). Upper panel, HP -10mV: P1060 (1μM) caused an increase in the magnitude of the total non-inactivating component (◊) when compared to control values (❑). These curves crossed at -78mV, a value close to the theoretical K-equilibrium potential in these cells (-83mV). The induced current is designated $I_{KCO}$. Subsequent administration of glibenclamide (1μM; ●) to these cells caused a full inhibition of $I_{KCO}$. Lower panel, HP -90mV: P1060 (1μM) also caused a significant decrease in the magnitude of $I_{K(V)}$ (◊; $p<0.05$, MANOVA) when compared to control currents (❑) and this inhibition was unaffected by subsequent administration of glibenclamide (1μM; ●). Points are the mean of 3 observations and the vertical bars represent the s.e. mean.

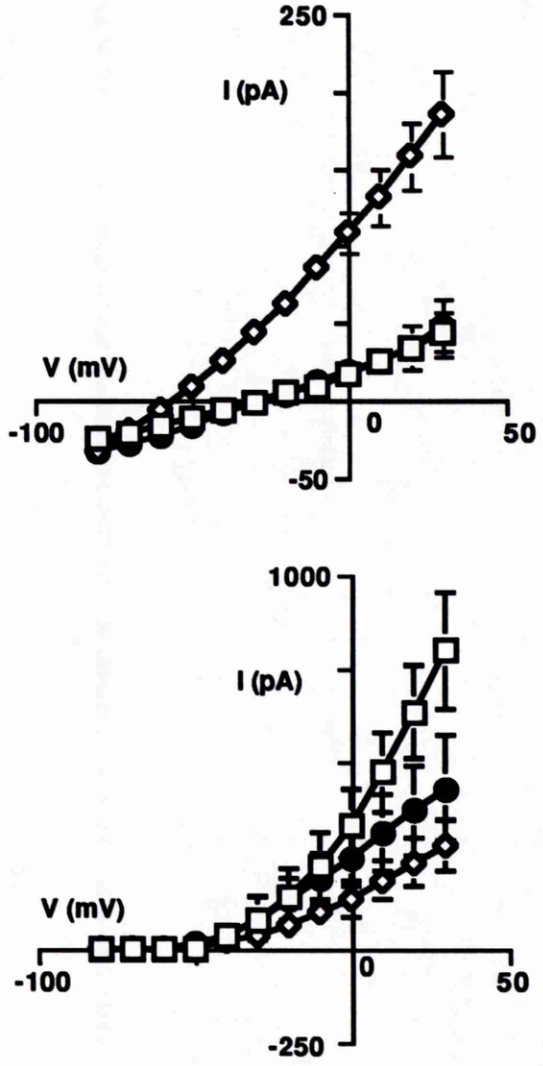



**Figure 3.8.** The effects of aprikalim (3μM) on whole-cell K-currents and their reversal by glibenclamide (1μM). Upper panel, HP -10mV: aprikalim (3μM) caused an increase in the magnitude of the total non-inactivating component (◊) when compared to control values (□). These curves crossed at -75mV, a value close to the theoretical K-equilibrium potential in these cells (-83mV). The induced current is designated $I_{KCO}$. Subsequent administration of glibenclamide (1μM; ●) to these cells caused a full inhibition of $I_{KCO}$. Lower panel, HP -90mV: aprikalim (3μM) also caused a significant decrease in the magnitude of $I_{K(V)}$ (◊; $p<0.05$, MANOVA) when compared to control currents (□) and this inhibition was partially reversed by subsequent administration of glibenclamide (1μM; ●). Points are the mean of 3 observations and the vertical bars represent the s.e. mean.

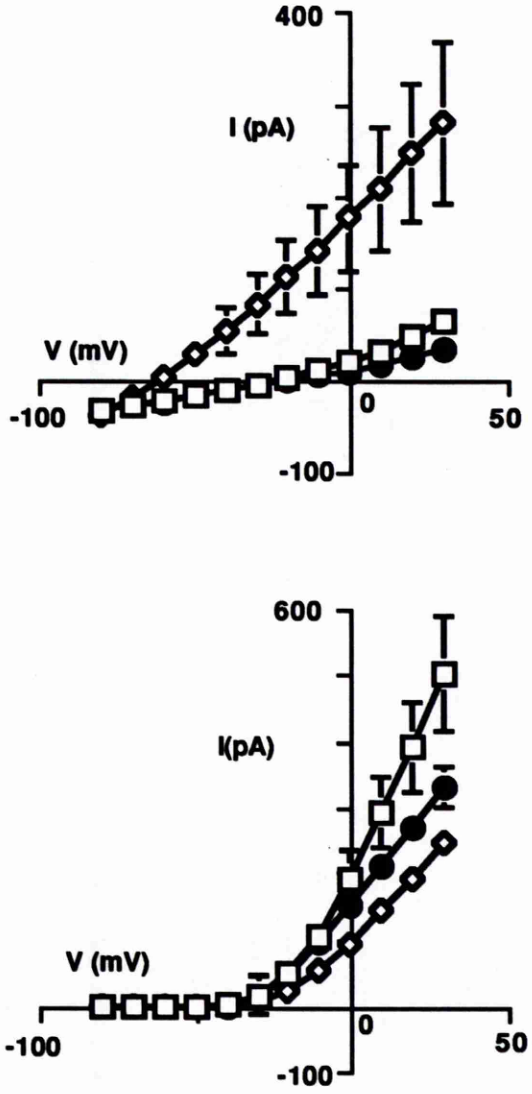



**Figure 3.9.** The effects of levcromakalim (1μM) on whole-cell K-currents and their reversal by glibenclamide (1μM). Upper panel, HP -10mV: levcromakalim (1μM) caused an increase in the magnitude of the total non-inactivating component (◊) when compared to control values (❑). These curves crossed at -78mV a value close to the theoretical K-equilibrium potential in these cells (-83mV). The induced current is designated $I_{KCO}$. Subsequent administration of glibenclamide (1μM; ●) to these cells caused a full inhibition of $I_{KCO}$. Lower panel, HP -90mV: levcromakalim (1μM) also caused a significant decrease in the magnitude of $I_{K(V)}$ (◊; $p<0.05$, MANOVA) compared to control currents (❑) and this inhibition was partially reversed by subsequent administration of glibenclamide (1μM; ●). Points are the mean of 4 observations and the vertical bars represent the s.e. mean.

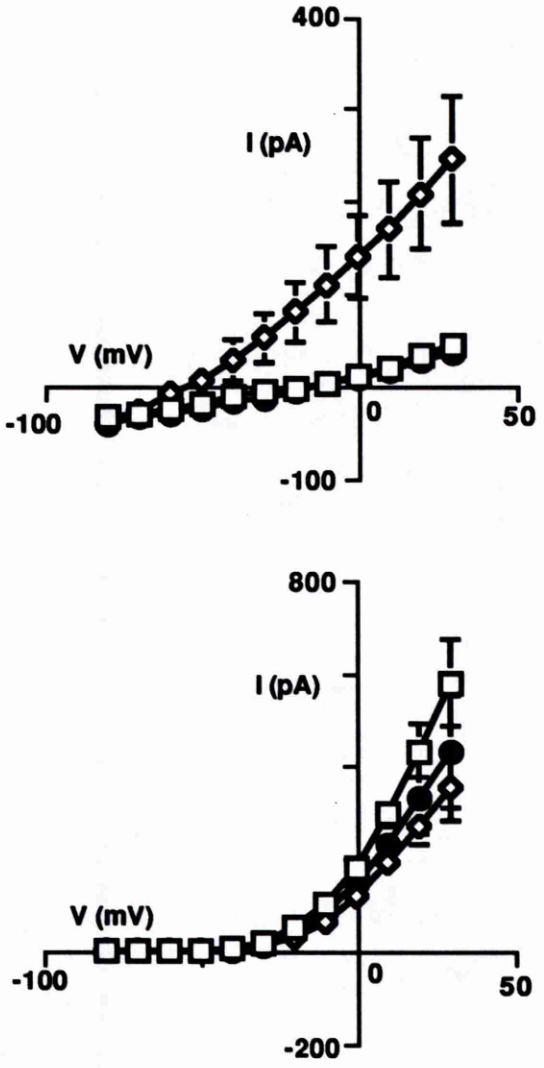



**Figure 3.10.** The effects of pinacidil (3μM) on whole-cell K-currents and their reversal by glibenclamide (1μM). Upper panel, HP -10mV: pinacidil (3μM) caused an increase in the magnitude of the total non-inactivating component (◊) when compared to control values (❑). These curves crossed at -75mV a value close to the theoretical K-equilibrium potential in these cells (-83mV). The induced current is designated $I_{KCO}$. Subsequent administation of glibenclamide (1μM; ●) to these cells caused a full inhibition of $I_{KCO}$. Lower panel, HP -90mV: pinacidil (3μM) also caused a significant decrease in the magnitude of $I_{K(V)}$ (◊; $p<0.05$, MANOVA) when compared to control currents (❑) and this inhibition was partially reversed by subsequent administration of glibenclamide (1μM; ●). Points are the mean of 3 observations and the vertical bars represent the s.e. mean.

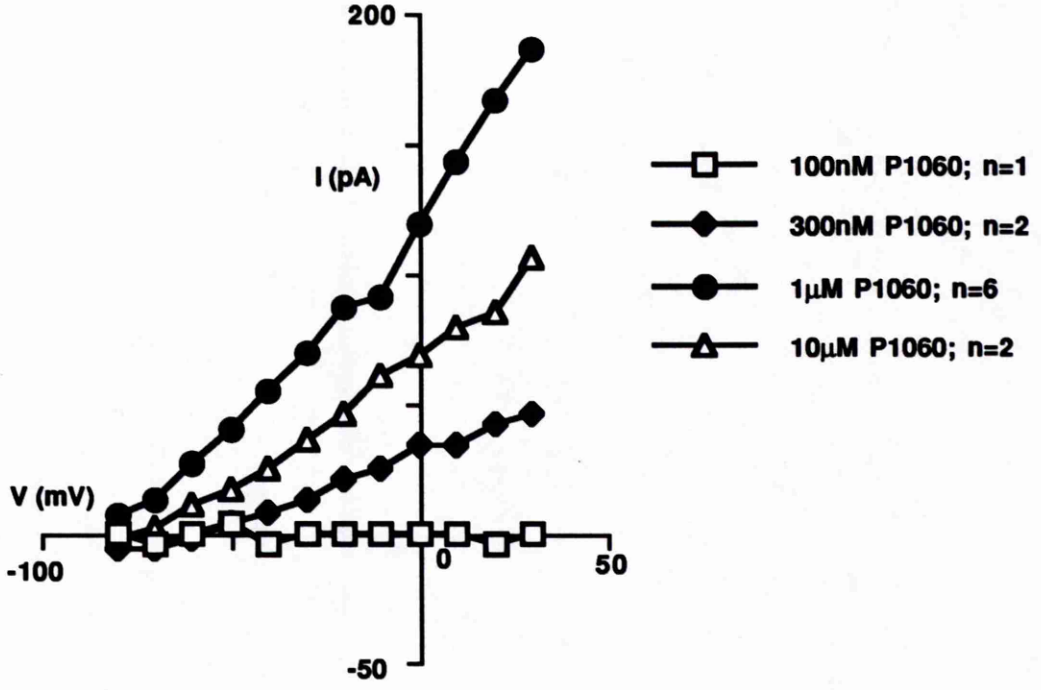



**Figure 3.11.** The concentration-dependent induction of $I_{KCO}$ by P1060 (100nM - 10μM) from a holding potential of -10mV. Values for $I_{KCO}$ were calculated by subtracting control non-inactivating current values from those in the presence of the appropriate concentration of P1060. In all cases the induced currents had a zero current potential of between -70mV and -85mV, values close to $E_K$. Because of the low number of observations, s.e.mean bars have been omitted.

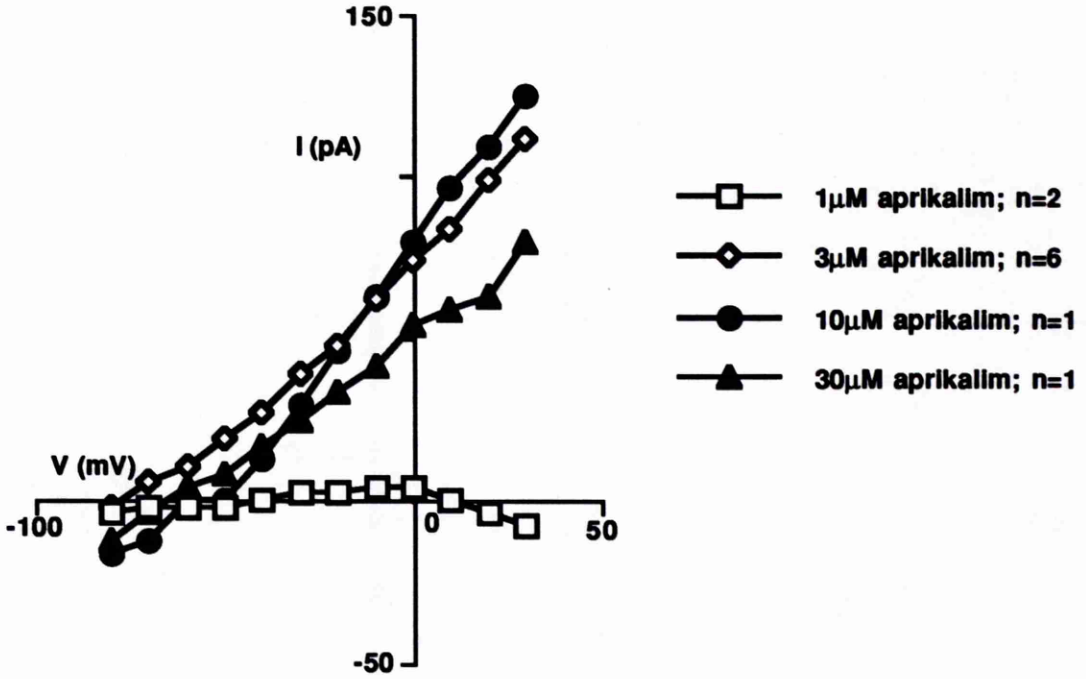



**Figure 3.12.** The concentration-dependent induction of $I_{KCO}$ by aprikalim (300nM - 30μM) from a holding potential of -10mV. Values for $I_{KCO}$ were calculated by subtracting control $I_{NI}$ values from those in the presence of the appropriate concentration of aprikalim. In all cases, the induced currents had a zero current potential of between -65mV and -80mV, values close to $E_K$. Because of the low number of observations, s.e.mean bars have been omitted.

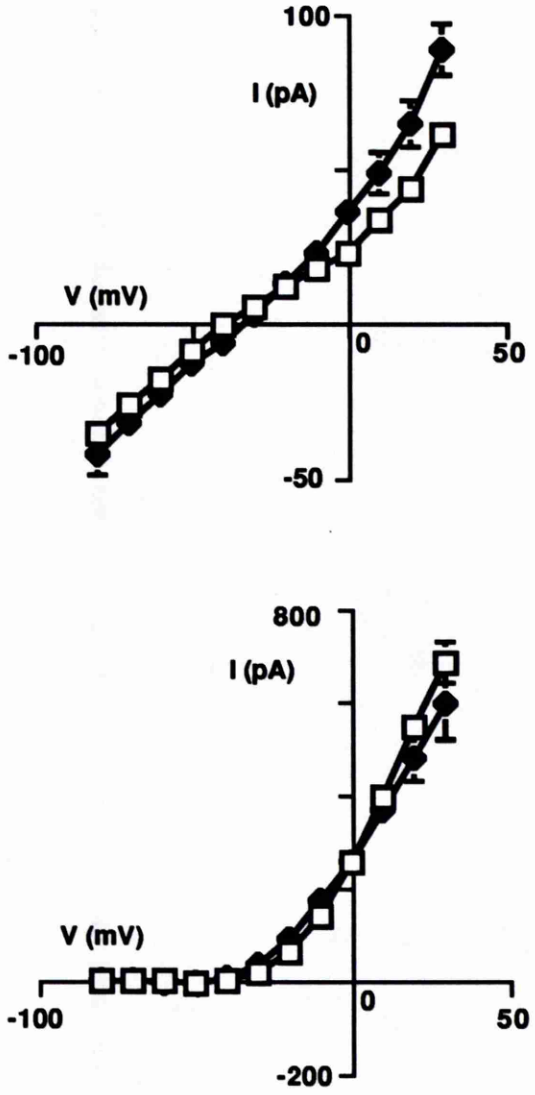



**Figure 3.13.** The effect of minoxidil sulphate (1μM) on whole-cell K-currents from rat portal vein cells. Minoxidil sulphate (1μM; ◆) had little effect, either on $I_{NI}$ (HP -10mV, upper panel,) or on $I_{K(V)}$ (HP -90mV, lower panel; $p>0.05$, MANOVA) when compared to control values (❑). Points are the mean of 5 observations and vertical bars represent the s.e. mean.

Minoxidil sulphate (1μM) also caused a slight increase in the mean magnitude of non-inactivating currents at positive potentials and a simultaneous slight decrease in the magnitude of $I_{K(V)}$ (Figure 3.13). However, these effects were only trends and values did not differ significantly from the control values ($p>0.05$, MANOVA). There was also no effect of minoxidil sulphate at higher concentrations (10μM; n=1, data not shown).

Application of DMSO alone (0.05%) to the recording chamber had no effect on the currents elicited from these cells (Figure 3.2).

### 3.3.2.3. Inhibition of the K-channel openers by glibenclamide.

Application of glibenclamide (1μM) alone to the recording chamber had no effect either on the holding current level or on the magnitude of $I_{NI}$ or $I_{K(V)}$ (Figure 3.14). Glibenclamide (1μM) inhibited $I_{KCO}$ previously generated by exposure to P1060 (1μM; Figure 3.7), aprikalim (3μM; Figure 3.8), levcromakalim (1μM; Figure 3.9) or pinacidil (3μM; Figure 3.10). In all cases this represented a complete return to the control current values. In addition, pre-exposure of cells to glibenclamide (1μM) completely abolished the ability of either P1060 (1μM, Figure 3.15; 300nM, Figure 3.17) or aprikalim (3μM, Figure 3.16) to generate $I_{KCO}$.

Glibenclamide (1μM) partially reversed the inhibition of $I_{K(V)}$ which had been induced by prior exposure to aprikalim (3μM; Figure 3.8), levcromakalim (1μM; Figure 3.9) or pinacidil (3μM; Figure 3.10). However, P1060 (1μM)-induced inhibition of $I_{K(V)}$ was unaffected by addition of glibenclamide (1μM; Figure 3.7). Pre-exposure of cells to glibenclamide (1μM) prevented the inhibition of $I_{K(V)}$ by aprikalim (3μM, $p>0.05$, MANOVA; Figure 3.16) but P1060 (1μM) was still able to significantly inhibit $I_{K(V)}$ in cells previously exposed to, and in the continued presence of glibenclamide ($p<0.05$, MANOVA; Figure 3.15). P1060 (300nM) was also able to inhibit $I_{K(V)}$ following pretreatment of the cell with glibenclamide (1μM; Figure 3.17).

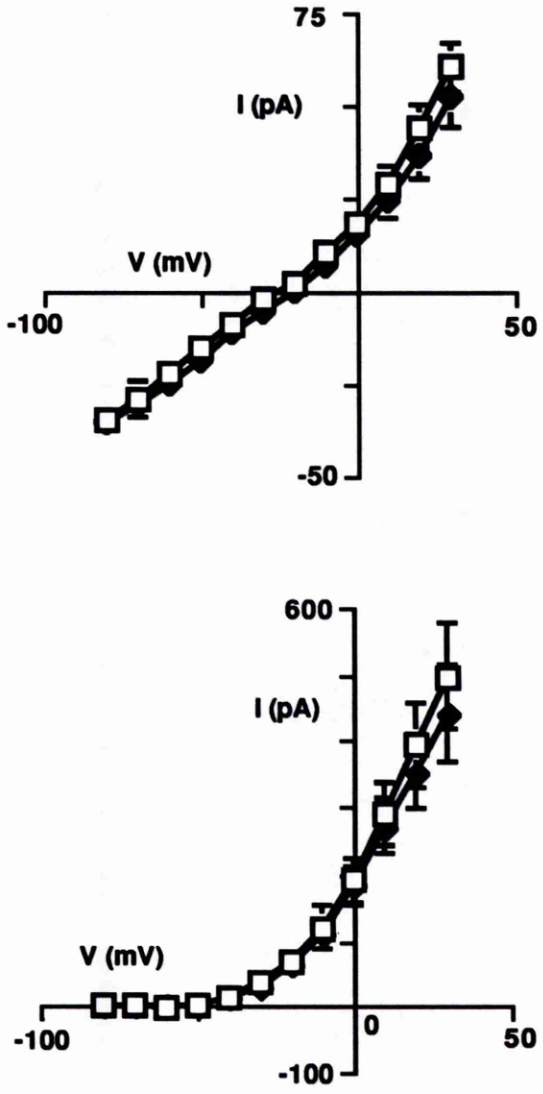



**Figure 3.14.** The effect of glibenclamide (1μM) alone on whole-cell K-currents from rat portal vein cells. Glibenclamide (1μM; ◆) had no effect either on the non-inactivating component (HP -10mV, upper panel) or on $I_{K(V)}$ (HP -90mV, lower panel; $p>0.05$, MANOVA) when compared to control values (❑). Points are the mean of 6 observations and vertical bars represent the s.e. mean.

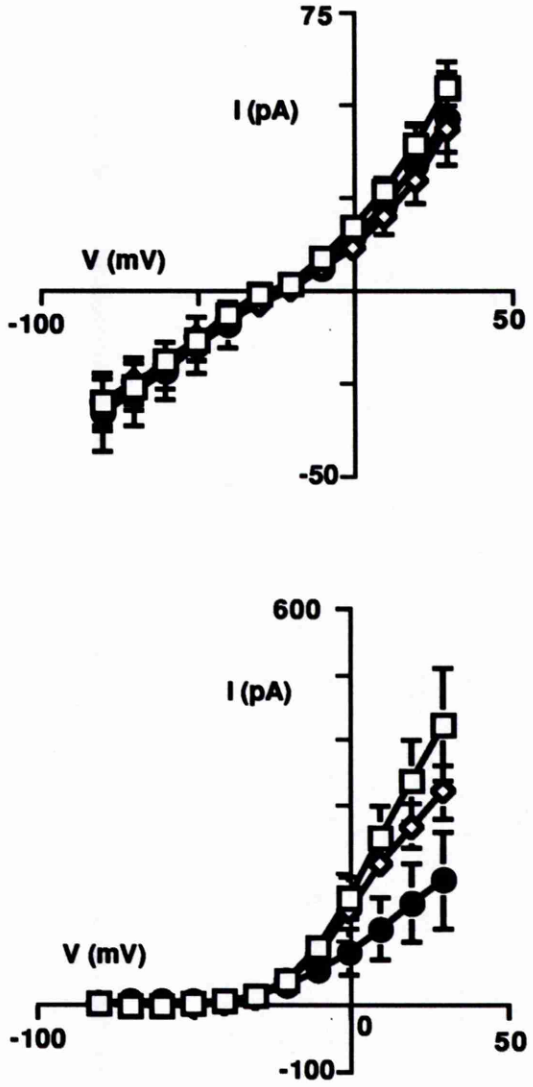



**Figure 3.15.** The effect of pre-exposure to glibenclamide (1μM) on the actions of P1060 (1μM). Glibenclamide (1μM; ◊) had little effect alone on the whole-cell currents when compared to control values (❑). Subsequent addition of P1060 (1μM; ●) to the recording chamber had no effect on $I_{NI}$ (HP -10mV, upper panel) but still caused a significant decrease in the magnitude of $I_{K(V)}$ (HP -90mV, lower panel; $p<0.05$, MANOVA). Points are the mean of 3 observations and vertical bars represent the s.e. mean.

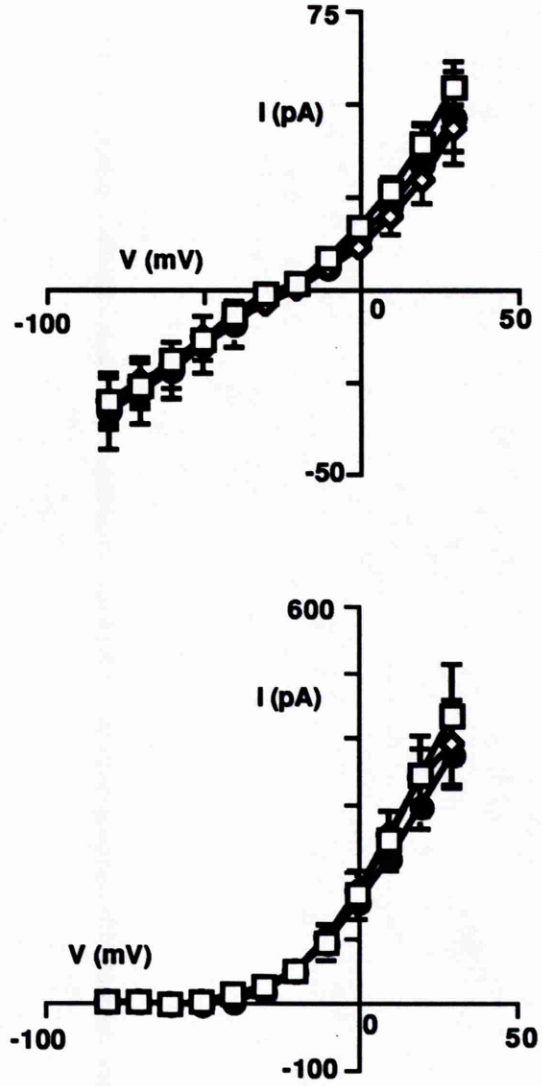



**Figure 3.16.** The effect of pre-exposure to glibenclamide (1μM) on the actions of aprikalim (3μM). Glibenclamide (1μM; ◊) had no effect alone on the whole-cell currents when compared to control values (❑). Subsequent addition of aprikalim (3μM; ●) to the recording chamber had no effect, either on $I_{NI}$ (HP -10mV, upper panel) or on $I_{K(V)}$ (HP -90mV, lower panel, $p>0.05$, MANOVA). Points are the mean of 3 observations and vertical bars represent the s.e. mean.

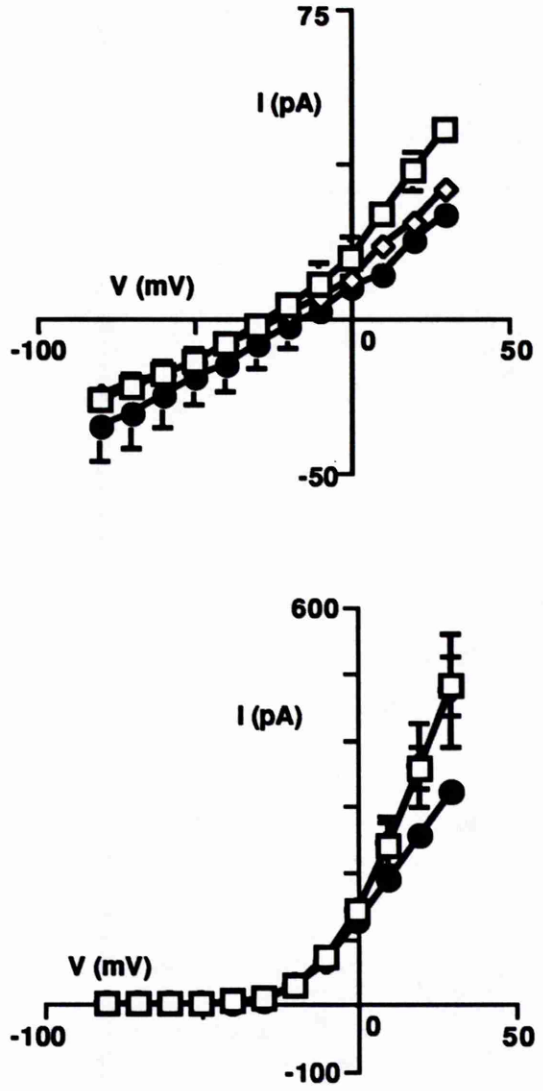



**Figure 3.17.** The effect of pre-exposure to glibenclamide (1μM) on the actions of P1060 (300nM). Glibenclamide (1μM; ◊) had little effect alone on the whole-cell currents when compared to control values (□). Subsequent addition of P1060 (300nM; ●) to the recording chamber had no effect on $I_{NI}$ (HP -10mV, upper panel) but still caused a decrease in the magnitude of $I_{K(V)}$ (HP -90mV, lower panel). Points are the mean of 3 observations and vertical bars represent the s.e. mean.

### 3.3.2.4. Run-down of $I_{KCO}$

Following exposure to levcromakalim (1μM), $I_{KCO}$ did not display any decrease in size (ie. run-down) with time and this current was still at maximum level 20min after achieving its maximum (Figures 3.18 & 3.19). The increased noise associated with induction of $I_{KCO}$ by levcromakalim (1μM) was apparent throughout the duration of cell contact with this agent. $I_{K(V)}$ continued to decrease throughout the duration of levcromakalim contact with the tissue (Figure 3.19).

However, after exposure to levcromakalim (10μM), run-down of $I_{KCO}$ was observed (3 out of 4 cells) and 20min after achieving its maximum effect, $I_{KCO}$ was only approximately 30% (at -10mV) of its previous maximum value (Figures 3.18 & 3.20). In the remaining 1 (out of 4) cell, no run-down of $I_{KCO}$ was observed and the current noise level was well-maintained throughout this period. During the run-down of $I_{KCO}$ in these experiments the noise level returned completely to control levels at holding current values above those in control conditions (Figure 3.18). The concurrent inhibition of $I_{K(V)}$ by levcromakalim (10μM) again continued to develop throughout the duration of contact with the tissue (Figure 3.20).

### 3.3.2.5. Effect of increasing intracellular ATP levels

The effect of P1060 was investigated in cells in which MgATP (5mM) was included in the intracellular solution and from which the tricarboxylic acid cycle intermediates, succinate, pyruvate and oxaloacetate were absent. Under these conditions, P1060 (1μM) had no effect on the holding current at -10mV. In addition, evaluation of the current / voltage relationships in the presence of P1060 indicated that there was only a small increase in $I_{KCO}$ and this was accompanied by only a small and non-significant decrease in the magnitude of $I_{K(V)}$ ($p>0.05$, MANOVA; Figure 3.21).

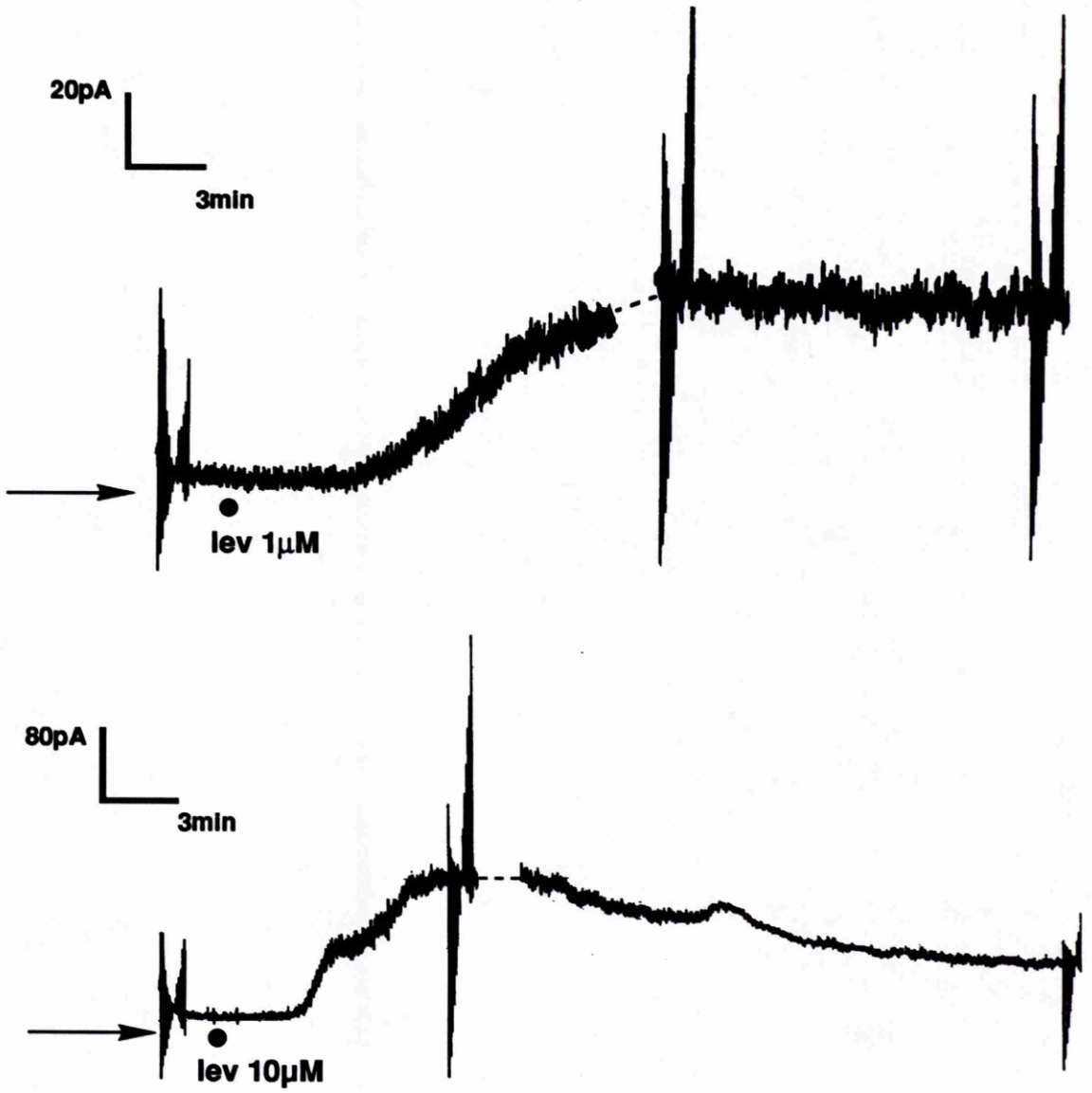



**Figure 3.18.** Typical traces demonstrating the response to levcromakalim (lev, 1μM, upper trace and 10μM, lower trace) on the holding current of two cells clamped at -10mV. The levcromakalim (1μM)-induced increase in holding current was well maintained and the level of current noise was noticeably increased throughout exposure to the drug. In contrast, the levcromakalim (10μM)-induced increase in holding current was not well-maintained and displayed 'rundown'. In addition, the current noise level decreased to control values at current levels which were still substantially elevated from control values. Dotted lines indicate points at which the holding potential was changed to -90mV to analyse $I_{K(V)}$ and arrows represent the zero current level. Note the different current calibration bars in the upper and lower traces

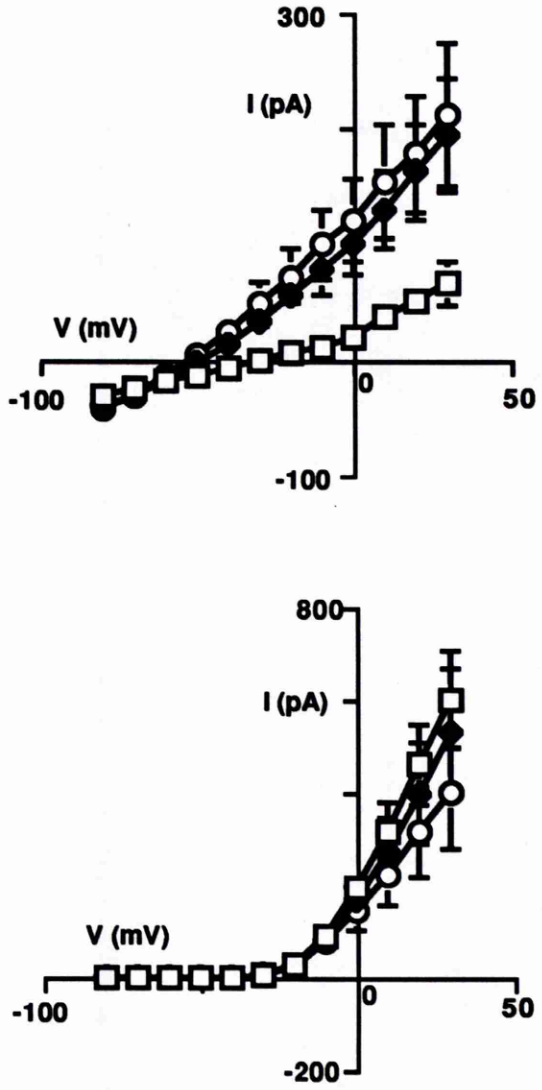



**Figure 3.19.** The effect of prolonged exposure to levcromakalim (1μM) on whole-cell currents recorded from rat portal vein cells. Upper panel, HP -10mV: Levcromakalim (1μM; ◆) increased the non-inactivating currents compared to control values (❑), an effect which was well-maintained 20min following initial exposure to the drug (○). Lower panel, HP -90mV: levcromakalim (◆) inhibited $I_{K(V)}$ when compared to control values (❑) and this inhibition increased following 20min of drug exposure (○). Points are the mean of 3 observations and vertical bars represent the s.e. mean.

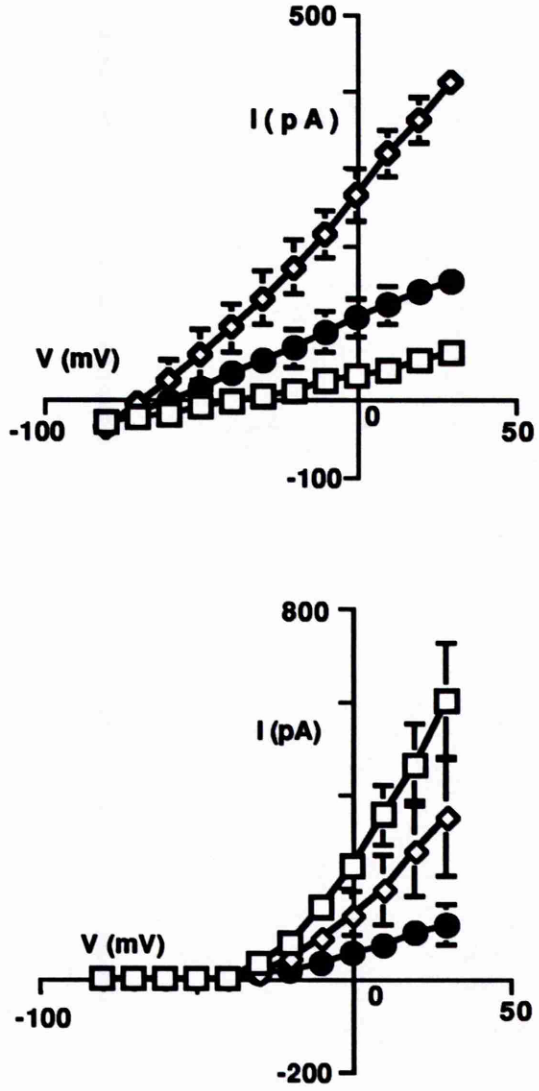



**Figure 3.20.** The effect of prolonged exposure to levcromakalim (10μM) on whole-cell currents recorded from rat portal vein cells. Upper panel, HP -10mV: levcromakalim (10μM; ◊) caused an increase in the total non-inactivating component when compared to control values (❑). This increase was not maintained and 20min following the initial exposure to levcromakalim, the non-inactivating currents had run-down to approximately 30% (●) of control levels. Lower panel, HP -90mV: levcromakalim (10μM; ◊) also inhibited $I_{K(V)}$ compared to control values (❑) and this was further evident after 20min exposure (●). Points are the mean of 3 observations and vertical bars represent the s.e. mean.

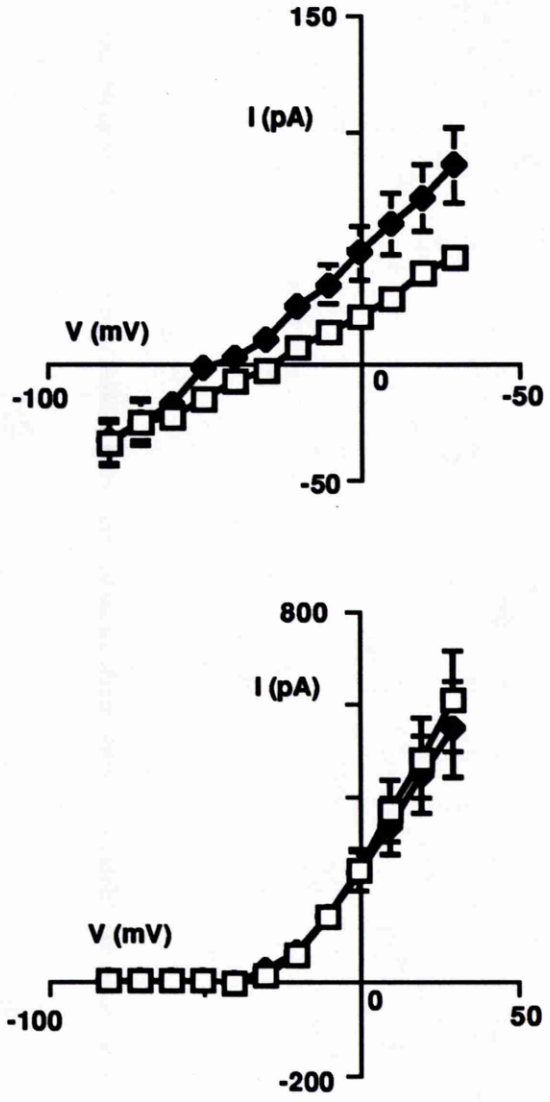



**Figure 3.21.** The effect of P1060 (1μM) on whole-cell currents recorded from rat portal vein cells with the inclusion of MgATP (5mM) in the recording pipette. Upper panel, HP -10mV: P1060 (1μM; ◆) caused an increase in the non-inactivating currents when compared to control values (❑). This induction of $I_{KCO}$ was substantially smaller than the induction of $I_{KCO}$ caused by P1060 (1μM) under normal recording conditions (see Figure 3.7). Lower panel, HP -90mV: P1060 (1μM; ◆) had no effect on $I_{K(V)}$ when compared to control values (❑). The P1060-induced inhibition of $I_{K(V)}$ (see Figure 3.7) was virtually abolished by inclusion of MgATP (5mM) in the recording pipette ($p>0.05$, MANOVA). Points are the mean of 3 observations with the vertical bars representing the s.e.mean.

### 3.3.2.6. Effect of modifying agents on the induction of $I_{KCO}$ and the inhibition of $I_{K(V)}$ by levcromakalim

The response to levcromakalim (1μM) was examined under a variety of differing conditions. Inclusion of the phosphatase inhibitor, okadaic acid (1μM) in the intracellular solution resulted in significantly larger values for $I_{K(V)}$ than those measured under control conditions ($p<0.05$, MANOVA; Figure 3.22). In contrast $I_{NI}$ was unaffected (not shown). The ability of levcromakalim to induce $I_{KCO}$ and to inhibit $I_{K(V)}$ was reduced in those cells in which okadaic acid (1μM) had been included in the intracellular solution ($p>0.05$ for inhibition of $I_{K(V)}$, MANOVA; Figure 3.25). The ability of glibenclamide to inhibit $I_{KCO}$ and to reverse the inhibition of $I_{K(V)}$ induced by levcromakalim was unaffected by inclusion of okadaic acid in the intracellular solution (Figure 3.23).

The selective inhibitor of protein kinase A, PKI(6-22)amide (40nM), had no effect on the ability of levcromakalim (1μM) to generate $I_{KCO}$ or to inhibit $I_{K(V)}$ (Figures 3.24 & 3.25) when included in the intracellular solution. Currents recorded with PKI(6-22)amide in the patch pipette did not differ from currents under control conditions (not shown). In addition, PKI(6-22)amide did not affect the ability of glibenclamide to inhibit the generation of $I_{KCO}$ by levcromakalim. However, inclusion of PKI(6-22)amide in the pipette did inhibit the ability of glibenclamide to reverse the levcromakalim-mediated inhibition of $I_{K(V)}$ (Figure 3.24).

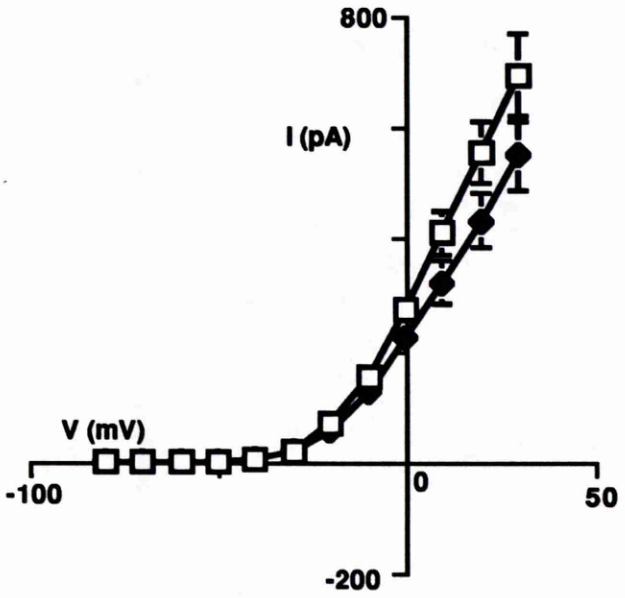



**Figure 3.22.** The effect of inclusion of okadaic acid (1μM) in the recording pipette on the delayed rectifier current. Okadaic acid (1μM; ◆) caused a significant increase in the magnitude of $I_{K(V)}$ (holding potential -90mV; $p<0.05$, MANOVA) when compared to control values (□) recorded from different cells. Points are the mean of between 6 and 8 observations and vertical bars represent the s.e.mean.

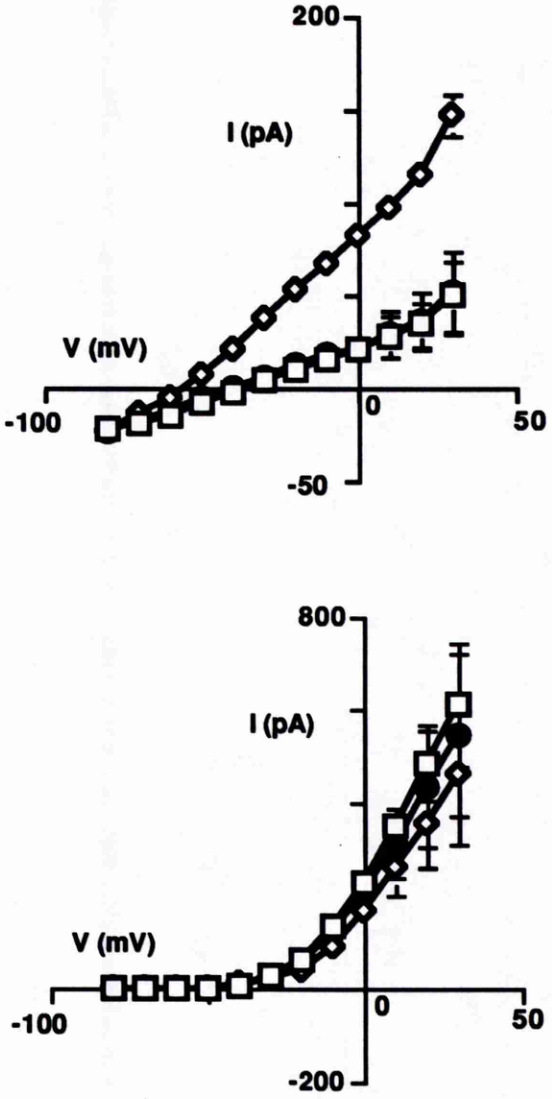



**Figure 3.23.** The effect of inclusion of okadaic acid (1μM) in the intracellular solution on the response to levcromakalim (1μM) and its subsequent reversal by glibenclamide (1μM). Upper panel, HP -10mV: levcromakalim (1μM; ◊) caused an increase in the magnitude of total non-inactivating currents when compared to control values (❑) and this was fully reversed by subsequent application of glibenclamide (1μM; ●, hidden). Lower panel, HP -90mV: levcromakalim (1μM; ◊) caused a slight reduction in $I_{K(V)}$ compared to control values (❑, $p>0.05$, MANOVA) which was partially reversed by subsequent application of glibenclamide (1μM; ●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

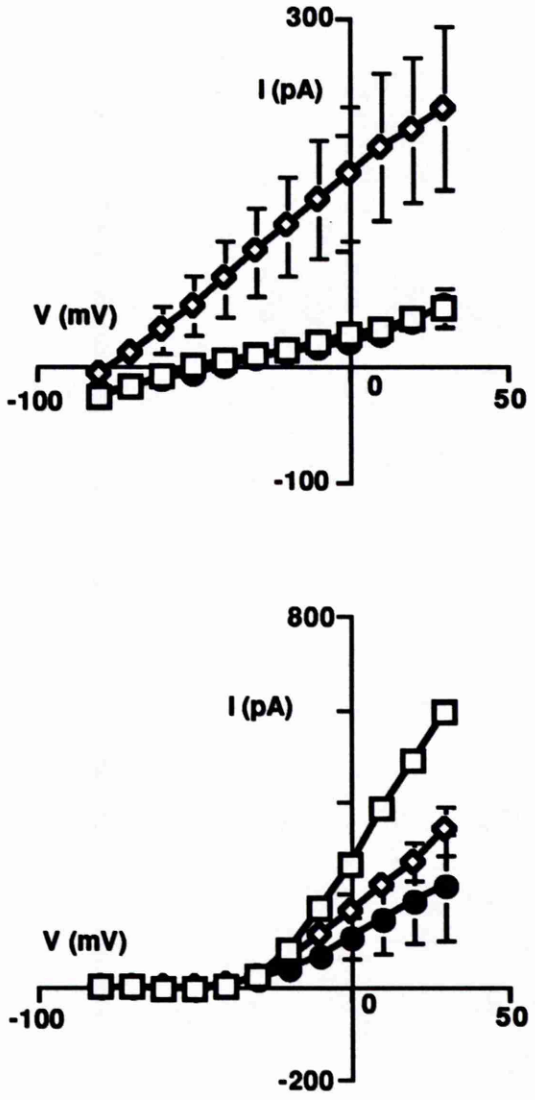



**Figure 3.24**. The effect of inclusion of protein kinase A inhibitor, PKI(6-22)amide (PKI; 40nM) in the recording pipette on the response to levcromakalim (1μM) and its reversal by glibenclamide (1μM). Upper panel, HP -10mV: levcromakalim (1μM; ◊) increased the total non-inactivating currents when compared to control values (□) and this was fully reversed by subsequent application of glibenclamide (1μM; ●). Lower panel, HP -90mV: levcromakalim (1μM; ◊) inhibited $I_{K(V)}$ compared to control values (□), an effect which was not reversed by subsequent application of glibenclamide (1μM; ●; compare with Figure 3.9). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

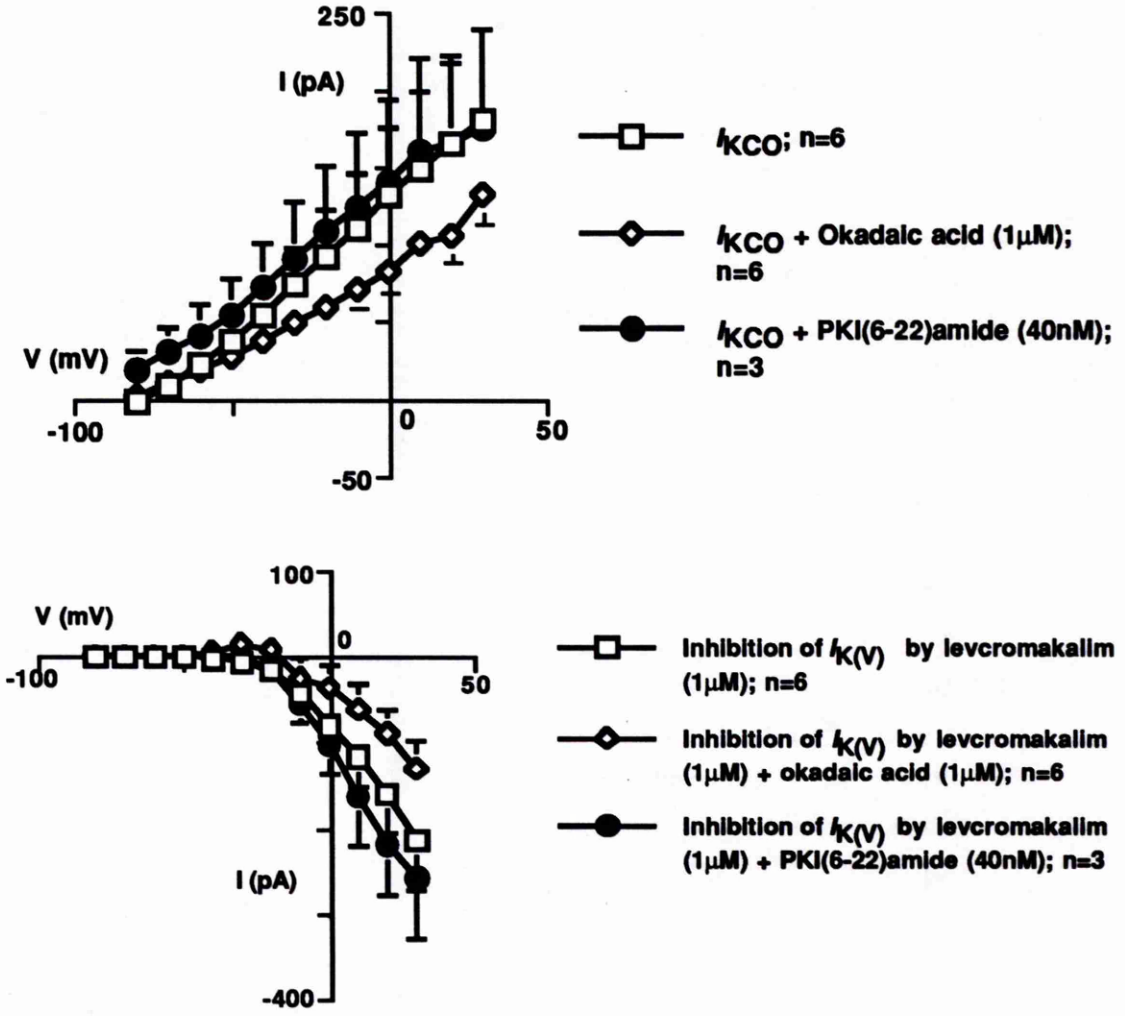



**Figure 3.25** The effect of inclusion of okadaic acid (1μM) or PKI(6-22)amide (40nM) in the intracellular solution on the action of levcromakalim. Upper panel, HP -10mV. The generation of $I_{KCO}$ by levcromakalim (1μM; ❑) was not affected by inclusion of PKI(6-22)amide (40nM; ●) in the intracellular solution. However, inclusion of okadaic acid (1μM; ◊) in the intracellular solution significantly decreased the magnitude of $I_{KCO}$ induced by levcromakalim ($p<0.05$, MANOVA). Values for $I_{KCO}$ were calculated by subtracting control values for the non-inactivating component from the non-inactivating current values in the presence of the levcromakalim. Lower panel, HP -90mV. The levcromakalim-induced inhibition of $I_{K(V)}$ was slightly increased by PKI(6-22)amide ($p>0.05$, MANOVA) but in the presence of okadaic acid the inhibition of $I_{K(V)}$ was significantly reduced ($p<0.05$, MANOVA) compared to that inhibition in the absence of any modifying agent. Inhibition of $I_{K(V)}$ is calculated by subtracting control values for $I_{K(V)}$ from those current values in the presence of levcromakalim.

## 3.4. Discussion

### 3.4.1. Cell quality and viability

Rat portal vein cells were isolated using a modification of a method originally described for the isolation of guinea-pig bladder smooth muscle cells (Klöckner & Isenberg, 1985). This method yielded a large number of good quality portal vein cells which varied in shape and size but which were generally between 10-25µm in length. These cells generated contractions to both noradrenaline and caffeine when placed in $Ca^{++}$-containing medium and under these conditions also displayed spontaneous transient outward currents (STOCs) probably due to the opening of the large, calcium-activated K-channel ($BK_{Ca}$; A.J. Kirkup, personal communication).

Pronase is a non-specific proteolytic enzyme known to affect the gating properties of certain channels. Thus it is possible that the currents recorded from the cells obtained using this isolation technique may not represent those currents of the 'normal' cell. Pronase has been reported to destroy the inactivation gate of the voltage-dependent Na-channel in squid axon (Armstrong & Bezanilla, 1977) and the fast inactivation of the A-current (Matteson & Carmeliet, 1988). However, comparison of the evoked currents in cells of the present study with the currents recorded from rat portal vein cells isolated using a non-pronase based technique (Noack *et al.*, 1992a,c,d) does not indicate that the use of pronase affected any of the currents. Indeed, the effects of pronase on the gating properties of the Na-channel and the A-current (in squid axon and GH3 cells respectively) are only evident when the pronase is applied to the intracellular solution of the cell. The portal vein cell membrane is relatively impermeable to this proteolytic enzyme and therefore its access to the intracellular environment will be limited. Based upon these arguments it is unlikely that the use of pronase in the cell isolation medium is having any direct effect upon the activation or inactivation kinetics of the currents measured in this study.

If the degree of phosphorylation of the channels is important in observing the effects of the K-channel openers, the metabolic integrity of these cells is

clearly of vital importance. In the present experiments, attempts were made to maintain intracellular ATP concentrations by the addition of a number of tricarboxylic acid (TCA) cycle intermediates (oxaloacetic acid, Na-pyruvate and Na-succinate) to the intracellular solution. However, the cells were maintained at room temperature during the experiments and therefore it is possible that the enzymes of the TCA cycle did not function optimally. Thus the cells could have become metabolically compromised ie. intracellular ATP levels could have been lower than under 'normal' conditions.

A number of reasons exist, however, to suggest that this was not the case. Firstly, Noack *et al.* (1992d) reported that the depletion of intracellular ATP following removal of the TCA intermediates from the intracellular solution caused the induction of a K-current, designated $I_{met}$. Noack *et al.* (1992d) obtained evidence that $I_{met}$ was carried by $K_{ATP}$ which opened as a consequence of the fall in intracellular ATP and removal of the inhibition that ATP imposes on this channel. In the cells used in the present experiments, the development of $I_{met}$ was not observed suggesting that intracellular ATP levels were at a sufficiently high level to prevent $K_{ATP}$ opening. In addition, substitution of the TCA intermediates in the intracellular solution with ATP (1mM) also resulted in the gradual development of $I_{met}$ (G. Edwards, personal communication). This would therefore indicate that cellular ATP concentrations in the present experiments was maintained in excess of 1mM by the TCA intermediates. Finally, inclusion of ATP (5mM) in the intracellular solution inhibited the induction of $I_{KCO}$ by the K-channel openers (present study, see later).

Thus the ability of the K-channel openers to exert their characteristic effects indicates that under the conditions of the present study, cellular metabolic viability is sufficiently intact to maintain intracellular ATP levels between 1 and 5mM. Finally, as mentioned earlier, the ability of the cells used in the present study to contract to noradrenaline and caffeine in $Ca^{++}$-containing conditions implies that cellular ATP levels (and other factors) must be sufficiently high to allow normal cellular functioning.

### 3.4.2. Generation of $I_{KCO}$

Noack *et al.* (1992a) reported that application of levcromakalim to cells of the rat portal vein induced a non-inactivating K-current, $I_{KCO}$, with an underlying unitary conductance of 17pS at 0mV under quasi-physiological K-conditions. In the present study, the ability of several structurally-dissimilar K-channel openers to generate $I_{KCO}$ was investigated. Levcromakalim, aprikalim, P1060 and pinacidil each caused an increase in the holding current when cells were clamped at -10mV. This increase in holding current is caused by a movement of the cell membrane potential away from -10mV towards $E_K$ and thus an increase in the injected current was required to maintain the holding potential at -10mV. The current increase generated by P1060 reached a maximum in a significantly shorter time than that produced by levcromakalim, aprikalim or pinacidil all of which had similar time-courses of action. The possible reasons for this are discussed later (section 3.4.7).

Noack and coworkers (1992a) reported that the channel underlying the effects of levcromakalim possessed a unitary conductance of 17pS at 0mV. To determine whether a similar channel was opened by aprikalim and by P1060, single channel noise fluctuation analysis was performed on the increase in holding current noise induced by these agents. Under the quasi-physiological conditions of the experiment, both P1060 and aprikalim were estimated to open a K-channel with an underlying unitary conductance of 10.5pS at 0mV. This value correlates well with the findings of Kajioka *et al.* (1990), Okabe *et al.* (1990) and Noack *et al.* (1992a) who all demonstrated the ability of the K-channel openers to activate a relatively low conductance channel. This value is also similar to reported conductances of Type 1 $K_{ATP}$ channels (Ashcroft & Ashcroft, 1990) in cardiac myocytes and in pancreatic β-cells of 50-80pS (symmetrical K-gradient).

These calculated single channel conductances derived from the present study do not, however, support the findings of Standen *et al.* (1989), Hu *et al.* (1990) and Carl *et al.* (1992) all of whom suggested that the K-channel openers

activated a large-conductance channel. Standen *et al.* (1989) reported that the open probability of the channel activated by cromakalim (135pS, $[K]_o$ = 140mM, $[K]_i$ = 60mM) decreased as concentrations of intracellular ATP were increased. Such a large conductance however, is inconsistent with any previously described $K_{ATP}$ channel. Hu *et al.* (1990) and Carl *et al.* (1992) both suggested that the K-channel openers activate the 'maxi-K' channel or $BK_{Ca}$. Wickenden *et al.* (1991) however, demonstrated that the relaxant effects of the K- channel openers were not blocked by the selective inhibitor of $BK_{Ca}$, charybdotoxin, and thus it seems that even if the K-channel openers are able to open $BK_{Ca}$ this action does not play a pivotal role in the relaxant response to these agents.

Analysis of the current / voltage relationships of $I_{KCO}$ was also conducted. $I_{KCO}$ generated by levcromakalim, P1060, aprikalim or pinacidil was characterised by an increase in the magnitude of the non-inactivating current component and a shift in the reversal potential of this current in a hyperpolarising (negative) direction. Control non-inactivating currents reversed at approximately -30mV but in the presence of the K-channel openers this value was shifted to approximately -60mV. In addition, control curves and curves in the presence of the K-channel opener crossed at approximately -78mV a value close to the theoretical K-equilibrium potential in these cells (-83mV; Noack *et al.*, 1992c). This indicates that the current induced by the K-channel openers ($I_{KCO}$) is carried exclusively by $K^+$ ions whereas the control non-inactivating current ($I_{NI}$) is probably associated with a cation channel.

Further studies were conducted using P1060 and aprikalim to determine the dose-response relationship between the concentration of K-channel opener and the induction of $I_{KCO}$. P1060-induced $I_{KCO}$ was found to be dose-dependent between 300nM and 10μM. P1060 (100nM) gave no response whilst at 10μM the magnitude of $I_{KCO}$ was smaller than at 1μM. Aprikalim-induced $I_{KCO}$ was dose-related between 3μM and 30μM. No response was observed with aprikalim (1μM) whilst the response to aprikalim (30μM) was smaller than that to 10μM. With both K-channel openers therefore, some decrease in the

maximum attainable response was observed when concentrations were increased to levels considered supramaximal for the mechano-inhibitory actions of these agents. The possible reasons for this are discussed in section 3.4.5.

The lack of effect of minoxidil sulphate on the K-currents in rat portal vein cells may indicate that the relaxant mechanism of action of this agent differs from that of the other K-channel openers. Newgreen *et al.* (1990) reported an $EC_{50}$ of 7.1μM for minoxidil sulphate-induced inhibition of the spontaneous activity of the rat portal vein but that minoxidil sulphate (100μM) was ineffective at causing an increase in the efflux of $^{86}Rb^{+}$ from this tissue. It is therefore possible that the concentration of minoxidil sulphate used in the present study was not sufficient to induce $I_{KCO}$. The inhibition of minoxidil sulphate-mediated relaxation of rat portal vein by glibenclamide is of a non-parallel type compared to other K-channel openers in which the inhibition is of a parallel nature (Newgreen *et al.*, 1990). The available information defines a different pharmacological profile for minoxidil sulphate when compared to other K-channel openers and may therefore be indicative of this agent causing vasorelaxation in a manner different to that of other K-channel opening agents.

### 3.4.3. Inhibition of $I_{KCO}$ by glibenclamide

$I_{KCO}$ induced by levcromakalim, P1060, pinacidil and aprikalim was inhibited by the sulphonylurea, glibenclamide. This represented a shift of the reversal potential in a depolarising direction, back to control values and a concomitant decrease in the size of the evoked currents.

Application of glibenclamide alone to the recording chamber had no effect on either $I_{NI}$ or $I_{K(V)}$. This indicates that glibenclamide is a selective inhibitor of the channel activated by the K-channel opener with no effects on any other $Ca^{++}$-independent channels. This is consistent with the reports of Noack *et al.* (1992c) and Schwietert *et al.* (1992) who demonstrated that application of glibenclamide to intact portal veins mounted for isometric tension recording had no effect on the spontaneous activity of these tissues. These experiments were

conducted in $Ca^{++}$-containing PSS which further suggests that any effect of glibenclamide on $Ca^{++}$-dependent currents is negligible.

The lack of effect of glibenclamide alone on the evoked currents implies that the channel activated by the K-channel openers is actually closed under resting conditions in these cells. This is thought to be true also for $K_{ATP}$ channels which are closed in the presence of physiological concentrations of ATP and this therefore reinforces the idea that $K_{ATP}$ is the target channel of the K-channel openers.

Pre-exposure of cells to glibenclamide completely blocked the ability of either P1060 or aprikalim to generate $I_{KCO}$. The ability of glibenclamide to inhibit the current generated by the K-channel openers and also to block the mechano-inhibitory effects of these agents implies that it is the induction of $I_{KCO}$ in the intact tissue that is responsible for the vasorelaxant properties of this class of drugs.

### 3.4.4. The effect of increasing intracellular ATP levels on the generation of $I_{KCO}$

The influence of intracellular ATP on the effects of P1060 was examined by increasing the ATP concentration in the intracellular to 5mM. In control experiments, ATP was not included in the pipette solution. However, a number of TCA cycle intermediates were added hopefully to allow the cell to synthesise ATP, and in addition, glucose was included in the bathing solution. Under these conditions, P1060 induced a large $I_{KCO}$ current and also inhibited $I_{K(V)}$. Inclusion of ATP (5mM) in the intracellular solution (MgATP substituted for $MgCl_2$, and removal of TCA intermediates and glucose) caused a marked reduction in the generation of $I_{KCO}$ and the inhibition of $I_{K(V)}$ by P1060. The reduced effect of P1060 under conditions of high intracellular ATP is consistent with the mechanism suggested by Thuringer and Escande (1989) to account for the actions of the K-channel openers. These authors suggested that these agents act by displacing ATP from the inhibitory regulatory site on the $K_{ATP}$ channel

complex. Further evidence in support of this view has recently been obtained from two other groups. Noack *et al.* (1992d) demonstrated that removal of all metabolic substrates from the pipette solution and the assumed subsequent fall in intracellular ATP levels caused the induction of a current very similar to that induced by levcromakalim. This current was glibenclamide-sensitive and carried by a low conductance K-channel. Secondly, Sheppard and Welsh (1992) reported that the K-channel openers are able to *inhibit* a chloride current that is *activated* as intracellular levels of ATP *rise*. When this evidence is considered together it seems possible that the K-channel openers effectively reduce the effects of ATP. Thus the $K_{ATP}$ channel in smooth muscle opens and the ATP-sensitive Cl-current (Sheppard & Welsh, 1992) is reduced. Addition of ATP to the recording pipette shifts the balance in favour of ATP binding to the inhibitory site and thus the ability of P1060 to generate $I_{KCO}$ is consequently reduced.

### 3.4.5. K-channel opener-induced *'inhibition'* of $K_{ATP}$

Kozlowski *et al.* (1989) reported that the K-channel openers were able to increase the rate of rundown of $K_{ATP}$ channels. This run-down is thought to be due to dephosphorylation of the channel as it can be reversed be exposure to solutions containing MgATP (Findlay & Dunne, 1986; Kozlowski *et al.*, 1989). In addition, Noack *et al.* (1992d) suggested that the ability of levcromakalim to inhibit the delayed rectifier channel was mediated via dephosphorylation. Evidence has emerged from the present study to suggest that this group of compounds are also able to *close* the channel that they are thought to exert their mechano-inhibitory effects through, ie. K-channel openers are able to initially *open* and subsequently *close* the same channel. This *inhibition* of $K_{ATP}$ may, like the inhibition of $I_{K(V)}$, result from K-channel opener-induced dephosphorylation.

As mentioned earlier, the K-channel openers exhibit a decrease in the maximum size of $I_{KCO}$ when concentrations are increased to values considered excessive for their mechano-inhibitory effects. This may be due to the K-channel openers causing dephosphorylation of the $K_{ATP}$ channel and therefore decreasing the number of channels available for opening. Increasing

concentrations of K-channel opener above that required for a maximal induction of $I_{KCO}$ will only serve to increase the rate of dephosphorylation, decrease the number of available channels and thus reduce the magnitude of the generated $I_{KCO}$.

When $I_{KCO}$ is generated by levcromakalim (10μM), the increase in holding current is not well-maintained and displays run-down. This run-down may be attributed to a progressive dephosphorylation of the $K_{ATP}$ channel by the high concentration of levcromakalim used. Noack *et al.* (1992d) reported that $I_{met}$ also displays run-down in a manner similar to that observed with levcromakalim (10μM). These authors suggested that the run-down of $I_{met}$ was at least partially due to dephosphorylation of the $K_{ATP}$ channel.

Additional information may be obtained from examining the changes in the level of current noise that occur during the run-down of the levcromakalim-induced current. During the run-down of $I_{KCO}$, there is a marked decrease in the current noise at a current level above control current level. This fall in current noise may indicate either a *P*o value approaching 1 (all channels are open) or approaching 0 (all channels are closed). Because the current level is still substantially elevated, it is unlikely that *P*o values will be approaching 0 and thus the observed decrease in current noise may be indicative of an increase in channel open probability (*P*o approaching 1). Thus, when applied at sufficiently high concentrations, levcromakalim may actually open all the *available* channels (ie. all channels that have not been dephosphorylated). Run-down is then observed as dephosphorylation (and hence closure) of these open channels progresses.

Evidence therefore exists suggesting that the K-channel openers are able, not only to open, but also to close the channel carrying $I_{KCO}$. This closure may be caused via dephosphorylation of the channel as suggested by Noack *et al.* (1992d) but clearly further experiments are required to determine the manner in which the K-channel openers are able to close $K_{ATP}$.

### 3.4.6. Inhibition of $I_{K(V)}$ by the K-channel openers

Noack *et al.* (1992a) reported that the K-channel opener, levcromakalim inhibited the time- and voltage-dependent, delayed rectifier current, $I_{K(V)}$. The present study demonstrates that this property of levcromakalim is also common to P1060, aprikalim and pinacidil all of which caused a decrease in the magnitude of $I_{K(V)}$. The P1060-induced inhibition of $I_{K(V)}$ was only evident at potentials positive to 0mV whilst inhibition of $I_{K(V)}$ by levcromakalim, aprikalim and pinacidil was evident over the full range of potentials at which this current was activated. The lesser effect of P1060 may be due to the shorter contact time required for P1060 to induce a full development of $I_{KCO}$ (see section 3.4.2) compared to the other K-channel openers and thus consequently less time for the inhibition of $I_{K(V)}$ to develop.

Noack *et al.* (1992d) proposed that the ability of levcromakalim to inhibit $I_{K(V)}$ was due to dephosphorylation of the channel. This is consistent with the data obtained by Kozlowski and coworkers (1989) who found that the K-channel openers accelerated the run-down of $K_{ATP}$ in excised patches of insulin-secreting cells. This was also thought to be due to channel dephosphorylation. Beech and Bolton (1989) suggested that this decrease in the size of the delayed rectifier current might be due to conversion of the delayed rectifier channel into a channel with the characteristics of $K_{ATP}$ (ie. non-inactivating, voltage-independent). The possible mechanisms involved in the inhibition of the delayed rectifier current will be discussed more thoroughly in the next section (3.4.7).

### 3.4.7. Reversal of K-ohannel opener-induced inhibition of $I_{K(V)}$ by glibenclamide

Noack *et al.* (1992a) reported that glibenclamide was unable to *reverse* the levcromakalim-induced inhibition of $I_{K(V)}$ once this had developed. In the present study, the inhibition of $I_{K(V)}$ by levcromakalim, aprikalim and pinacidil was found to be partially reversed by glibenclamide, however, P1060-induced inhibition of $I_{K(V)}$ was unaffected by glibenclamide. To clarify this position further,

the ability of P1060 and aprikalim to inhibit $I_{K(V)}$ was examined in cells pretreated with glibenclamide. Under these conditions, aprikalim had no effect on the magnitude of $I_{K(V)}$ whereas P1060 was still able to inhibit this current. In addition, P1060 was also able to inhibit $I_{K(V)}$ under the same conditions (pre-exposure to glibenclamide) when used at sub-micromolar concentrations.

Glibenclamide is thus able to reverse the inhibition of $I_{K(V)}$ caused by the K-channel openers (except P1060) and also to prevent this inhibition when pre-applied to tissues. The precise mechanism by which glibenclamide is able to block the inhibition of $I_{K(V)}$ by the K-channel openers is unclear. However, Caro (1990) reported that glibenclamide was able to inhibit the A-kinase involved in the control of the tandem enzyme, fructose 2,6-bisphosphatase in the glycolytic pathway. Inhibition of this enzyme results in an increase in the cellular levels of fructose 2,6-bisphosphate, one of the key regulators of carbohydrate metabolism. The overall effect is a decrease in glycogenolysis and an increase in glycolysis leading to net synthesis of ATP. If, as speculated by Noack *et al.* (1992d), reduction in the size of $I_{K(V)}$ does arise as a consequence of channel dephosphorylation, a glibenclamide-driven increase in intracellular ATP concentrations could serve to prevent or reverse the K-channel opener-induced inhibition of $I_{K(V)}$. This therefore indicates that a critical balance may exist between the ATP concentration and channel phosphorylation. Thus, if this type of functional antagonism does exist, the relative contact times of K-channel opener and glibenclamide will be critical in observing the degree of antagonism and therefore explain why this phenomenon was not observed by Noack *et al.* (1992a) who employed relatively brief glibenclamide contact times.

The reason for the insensitivity of the P1060-induced inhibition of $I_{K(V)}$ to glibenclamide is unclear. P1060-induced $I_{KCO}$ reached a maximum significantly quicker than any of the other K-channel openers and thus the drug / tissue contact times were consequently shorter. Thus, the difference cannot be explained by *extended* exposure to P1060.

Thuringer and Escande (1989) suggested that the K-channel openers exert their mechano-inhibitory effects by competing with ATP for an inhibitory site

on the $K_{ATP}$ complex. The significantly faster induction of $I_{KCO}$ by P1060 may indicate that this agent possesses a higher affinity for the ATP binding site than the other tested K-channel openers. If we then translate this to the inhibition of $I_{K(V)}$, it is possible that the K-channel openers are also able to compete with ATP for its binding site on the kinase responsible for phosphorylating the delayed rectifier channel and in so doing prevent channel phosphorylation. If P1060 also possesses a higher affinity than the other K-channel openers for the kinase ATP binding site then glibenclamide may be unable to increase intracellular ATP to a level sufficient to displace P1060. The other K-channel openers may have lower affinities for the kinase ATP binding site and thus glibenclamide-mediated increases in intracellular ATP are at least partially able to alleviate the dephosphorylation caused by these agents.

Alternatively, it may be that P1060 is able to inhibit $I_{K(V)}$ by more than one mechanism. The ability of many imidazoline- and *guanidine*-based compounds, such as phentolamine, to inhibit $I_{K(V)}$ is now recognised (see Chapter 4) as is the ability of the K-channel openers. P1060 is a cyano*guanidine* derivative and may therefore be able to inhibit $I_{K(V)}$ by two distinct mechanisms. Firstly, in a manner similar to the K-channel openers through dephosphorylation as discussed above, and secondly in a manner similar to phentolamine (because of the structural similarities between these two agents). Reversal by glibenclamide may alleviate the inhibition due to dephosphorylation (K-channel opener-induced inhibition) but not affect the secondary mechanism (phentolamine-like inhibition). To test this hypothesis another cyanoguanidine K-channel opener, pinacidil was employed and the glibenclamide sensitivity of the pinacidil-induced inhibition of $I_{K(V)}$ was tested. Pinacidil displayed a similar profile to levcromakalim and aprikalim in that pinacidil-induced inhibition of $I_{K(V)}$ was partially reversed by glibenclamide. Thus, it does not seem feasible that the guanidine moiety within the P1060 molecule is responsible for the observed difference in profile. However, the small differences in the structures of pinacidil and P1060 do give rise to differences in binding properties, eg. pinacidil is an IGR site ligand but P1060 is unable to bind to this site (see Chapter 2). These

differences in binding properties may indicate that possible functional differences could exist between pinacidil and P1060.

Based on the current evidence it seems likely that the differences observed between P1060 and the other K-channel openers arise from differences in the affinity between these agents for their site of action and not from different actions.

### 3.4.8. The effect of increasing intracellular ATP on the inhibition of $I_{K(V)}$

The P1060-induced inhibition of $I_{K(V)}$ is significantly decreased with the inclusion of high ATP in the intracellular solution (5mM ATP was substituted for the TCA intermediates in the intracelluiar solution and glucose in the bath solution, see section 3.4.4). This is consistent with the view that the K-channei opener-induced inhibition of $I_{K(V)}$ is mediated via dephosphorylation of the $K_V$ channel (Noack *et al.*, 1992d). By increasing intraceiiular ATP levels, the potential for phosphorylation will increase and thus the inhibitory effect of the K-channel openers will be decreased.

### 3.4.9. The importance of channel phosphorylation in the action of levcromakalim

Okadiac acid is a poiyether derivative first isolated from the marine sponge *Halichondria* (Tachibana *et al.*, 1981). It is a potent inhibitor of protein phosphatases 1 and 2a (Hescheier *et al*, 1988), the enzymes responsible for cleaving phosphate groups from target proteins.

Inclusion of okadaic acid in the intraceiiular solution had no effect on $I_{Ni}$ but caused an increase in the magnitude of $I_{K(V)}$ when compared to control cells. This increase in $I_{K(V)}$ may be indicative of the requirement of the $K_V$ channel for phosphorylation as suggested by Perozo and Bezaniila (1991) who reported that the delayed rectifier channel in squid axon required phosphorylation for opening. By removing the route of dephosphorylation (by inclusion of okadaic acid and thus inhibiting phosphatase activity), the channel will remain in a more complete phosphorylated state and therefore the capacity

for opening is potentially increased. This view is consistent with the report of Hescheler (1988) who reported that the delayed rectifier current in guinea-pig cardiac myocytes was aiso increased in the presence of okadaic acid. Karaki *et al.* (1989) reported that okadaic acid could augment the effects of cAMP-dependent phosphorylation. This is thought to arise from inhibition of the phosphatase involved in the dephosphorylation of cAMP-dependent phosphorylation.

Inclusion of okadaic acid in the patch pipette decreased both the ability of levcromakallm to induce $I_{KCO}$ and to inhibit $I_{K(V)}$. This is consistent with the view that levcromakalim exerts its effects both on $K_{ATP}$ and on $K_V$ by causing a decrease in the channel phosphorylation state. By decreasing the potential for dephosphorylation (or Increasing the potential for phosphorylation), via inhibition of phosphatase activity, the effects of ievcromakalim are reduced. As regards the inhibition of $I_{K(V)}$, this is consistent with the hypothesis that ievcromakaiim-induced inhibition of $I_{K(V)}$ occurs via channel dephosphorylation. By reducing the potential for dephosphorylation via inhibition of phosphatase activity, the inhibition of $I_{K(V)}$ by levcromakalim is reduced.

The reason for the reduction in the magnitude of $I_{KCO}$ in the presence of okadaic acid, however is not so clear. This result implies that levcromakalim activates $K_{ATP}$ through a dephosphoryiating action and therefore upon inhibition of phosphatase activity (inhibition of dephosphorylation), the generation of $I_{KCO}$ is reduced. The role of dephosphorylation in the ability of ievcromakaiim to induce $I_{KCO}$ is discussed more thoroughly in Chapter 5.

PKI(6-22)amide (PKI) is the regulatory subunit associated with the enzyme cAMP-dependent protein kinase A (PKA) under normal conditions in the cell. It is dissociation of this (regulatory) subunit away from the catalytic subunit which then permits enzymic activity and phosphorylation. Addition of PKI therefore prevents this kinase from phosphorylating its substrates. Inclusion of PKI in the intraceliuiar solution had no effect on either the control $I_{NI}$ or $I_{K(V)}$ currents. In addition, ievcromakaiim was able to induce $I_{KCO}$ and also to inhibit $I_{K(V)}$ to a similar degree as in control experiments. Glibenclamide reversed the induction

of $I_{KCO}$ in the presence of PKI but in contrast to control experiments, had no effect on the inhibition of $I_{K(V)}$ by levcromakaiim.

The inhibition of $K_{ATP}$ ($I_{KCO}$) by the sulphonylureas is thought to be mediated via an interaction with a closely associated membrane protein, the suiphonylurea receptor (for review see Ashcroft & Ashcroft, 1992; Edwards & Weston, 1993). These data indicate that phosphorylation of the sulphonylurea binding site by PKA is not necessary for the binding of, and hence inhibitory action of the sulphonylureas on the $K_{ATP}$ channel.

In contrast, PKA activity is evidently necessary for giibenclamide to reverse the inhibition of $I_{K(V)}$ by levcromakalim. As previously recounted, the ability of glibenclamide to reverse the levcromakaiim-induced inhibition of $I_{K(V)}$ is suggested to be mediated though an increase in intraceliuiar ATP concentrations (via inhibition of an A-kinase leading to an increase in glycolysis - see section 3.4.7). This ATP may then be utilised as the substrate for PKA, which can then re-phosphoryiate and re-activate the delayed rectifier channel. Inhibition of PKA activity would thus prevent the increased intracellular ATP levels being employed in the rephosphoryiation of the delayed rectifier channel.

### 3.4.10. General discussion

The information in this chapter describes the effects of a number of K-channel opening agents on the whole-cell K-currents in rat portal vein cells. Despite the structural heterogeneity of these agents, all the K-channel openers studied (with the exception of minoxidil sulphate) activate a K-channel of unitary conductance in the range 10-20pS (Noack *et al.*, 1992a; present study). This channel has a linear current / voltage relationship and is inhibited by glibenclamide. Furthermore, the actions of the K-channei openers are inhibited by increasing intracellular concentrations of ATP and can be mimicked by lowering intraceiiuiar ATP levels (Noack *et al.*, 1992d). Thus all the indications are that this class of agents activate what has come to be referred to as the $K_{ATP}$ channel, ie. a small conductance channel that is inhibited by high intraceiiuiar ATP concentrations.

In addition, these compounds are also able to inhibit the voltage- and time dependent, delayed rectifier K-channel. This is thought to occur through channel dephosphorylation (Noack *et al.*, 1992d) and the ability of glibenclamide to reverse this phenomenon may provide an interesting insight into the mechanism by which glibenclamide exerts its actions. A glibenclamide-driven increase in intracellular ATP may well aid the recovery of $I_{K(V)}$ from K-channel opener-induced dephosphorylation and also potentially cause closure of $K_{ATP}$ (increasing intracellular ATP leading to channel closure).

The phosphorylation state of the channels involved are of obvious importance to their functioning. The ability of the K-channel openers to possibly modulate this state led us to conduct further experiments in which we attempted to experimentally manipulate the phosphorylation state of the channel. The data in the present study provides little information on the role of phosphorylation in the control of $K_{ATP}$ and the action of the K-channel openers in generating $I_{KCO}$. However, some information is available regarding the role of phosphorylation in the control of the delayed rectifier channel.

Firstly, the ability of okadaic acid to increase $I_{K(V)}$ suggests that this channel requires phosphorylation for opening. Karaki *et al.* (1989) reported that okadaic acid could augment the effects of cAMP-dependent phosphorylation.

Secondly, the ability of okadaic acid to decrease the levcromakalim-induced inhibition of $I_{K(V)}$ suggests that levcromakalim may inhibit this current through dephosphorylation of the $K_V$ channel.

Finally, the dependency of the glibenclamide-mediated reversal of the levcromakalim-induced inhibition of $I_{K(V)}$ on PKA activity further suggests that the phosphorylation required for opening of $I_{K(V)}$ may be mediated via protein kinase A.

# Chapter 4:

# Modulation of K-channel function by imidazoline- and guanidine-based ligands; putative involvement of the imidazoline / guanidine receptor site

## 4.1. Introduction

### 4.1.1. The imidazoline / guanidine receptor site: evidence for a functional link with K-channels

The imidazoline / guanidine receptor (IGR) site has been identified in a wide number of tissues of both neuronal and non-neuronal origin (for review see Atlas, 1991) including smooth muscle (Yablonsky *et al.*, 1988). The majority of these studies have been conducted using radio-ligand binding and the selectivity of this site for imidazolines and guanidines but not for catecholamines is now unequivocal. Despite the well-described structure-activity relationships between agents which interact with this binding site, little evidence exists as to its functional role. However, many of the putative physiological effects linked with this site could occur via modulation of membrane potential in excitable cells and in particular with the modulation of K-channels.

Zonnenschein *et al.* (1990) reported that [$^3$H]-idazoxan which was bound to the IGR site in the rat liver was displaced by both TEA and 4-AP. These agents are non-selective blockers of K-channels but are also known to displace many adrenoceptor ligands (including clonidine, an IGR site ligand) from their binding site (Drukarch *et al.*, 1989). Whether the displacement of [$^3$H]-idazoxan observed at the IGR site is a direct effect of TEA or 4-AP at this binding site or is due to the interaction of TEA or 4-AP with a K-channel is unclear.

It has been speculated that pre-junctional (negative feedback) mechanisms involved in the modulation of neurotransmitter release involve the opening of plasmalemmal K-channels in the neurone terminal and a consequent hyperpolarisation of the pre-junctional membrane (Zoltay & Cooper, 1990). This would lead to a decreased influx of calcium and a decrease in transmitter release. Such a mechanism has been associated with the presynaptic $\alpha_2$-adrenoceptor in neurones of the sub-mucous plexus (North & Surprenant, 1985). Göthert and Molderings (1991) reported that IGR site ligands, but not noradrenaline could inhibit the release of [$^3$H]-noradrenaline from postganglionic sympathetic nerve

terminals of the rabbit pulmonary artery following blockade of adrenoceptors with phenoxybenzamine or rauwolscine. These authors thus concluded that an IGR site exists on the pre-junctional terminal of sympathetic neurones that is involved in the control of neurotransmitter release. This was corroborated by Smith and Weston (1993) who reported that the $\alpha_2$-adrenoceptor agonist, guanabenz was abie to inhibit [$^3$H]-noradrenaline release from the rat anococcygeus muscle and that this was only partially sensitive to the selective $\alpha_2$-adrenoceptor antagonist, RX 821002. They also concluded that an IGR site is present on the pre-junctional nerve terminal that reduces transmitter release when activated. The possibility therefore exists that activation of the IGR site on sympathetic nerve terminals causes K-channel opening and a reduction in transmitter release.

Regunathan *et al.* (1991a) reported that clonidine-displacing substance (CDS), the putative endogenous ligand at the IGR site, was able to release catecholamines from chromaffin cells and that this was partially blocked by the calcium channel blocker, cobalt. The involvement of calcium channels may indicate that this effect of CDS involves depolarisation-induced release of catecholamines. It is therefore possible that it is closure of K-channels that leads to a depolarisation of the membrane and a consequent influx of calcium permitting the release of the catecholamines.

### 4.1.2. Imidazolines and guanidines as K-channel inhibitors

In the pancreatic β-ceii, Schulz and Hasselbiatt (1989a) reported that under conditions of $\alpha_2$-adrenoceptor blockade, clonidine was abie to stimulate insulin release, the opposite effect to that normally associated with activation of $\alpha_2$-adrenoceptors. In a later study, Schulz and Hasselblatt (1989b) reported that this effect of clonidine was shared by other imidazoline derivatives, including the α-adrenoceptor antagonist, phentolamine. Later, Plant and Henquin (1990) reported that this property of phentolamine was attributable to inhibition of ATP-sensitive K-channels ($K_{ATP}$) resulting in membrane depolarisation and insulin release.

Since these initiai reports, a wide range of imidazoline derivatives including such agents as alinidine, antazoline, tolazoline (Jonas *et al.*, 1992), clonidine (Plant *et al.*, 1991) and efaroxan (Chan *et al.*, 1993) has been demonstrated to block $K_{ATP}$ in insulin-secreting cells. Certain authors (Dunne, 1991; Plant *et al.*, 1991) have speculated that the ability of these compounds to modulate K-channel function may be mediated via an IGR site, but direct evidence in support of this view has not been forthcoming. In fact efaroxan, which inhibits $K_{ATP}$ in the pancreatic β-celi (Chan *et al.*, 1993) is not a ligand at the IGR site identified by Remaury and Paris (1992) in the insulin secreting ceil line, RINm5F. Efaroxan therefore shares the ability of the other structuraiiy-simiiar agents to modulate K-channel function but exhibits no affinity for the IGR site in the same tissue.

The ability of this structural class of compound to inhibit K-channels in insulin-secreting cells has since been extended to smooth muscle by McPherson and Angus (1989) who demonstrated that phentolamine blocked the vasorelaxant properties of cromakalim in dog isolated coronary artery. Since this initial report, a range of compounds containing either an imidazoline or a guanidine moiety has been demonstrated to block the mechano-inhibitory effects of the K-channel openers. These agents include naphazoline (Grana *et al.*, 1991), guanethidine (Berry *et al.*, 1992) and ciclazindol (Noack *et al.*, 1992c) in addition to phentolamine (McPherson & Angus, 1989; Murray *et al.*, 1989).

Agents containing an imidazoline moiety also increase the spontaneous myogenic activity of rat portal vein. Schwietert *et al.* (1992) reported that both phentolamine and antazoiine each increased the activity of this tissue and Okumura *et al.* (1992) reported similar effects for clonidine, toiazoiine and idazoxan. Finally, Noack *et al.* (1992c) reported that the imidazo-isoindole anorectic agent, ciclazindol, blocked the vasorelaxant effects of levcromakaiim and increased the myogenic activity of rat portal vein in tissue bath experiments. In whole-cell voltage clamp experiments, ciclazindol inhibited $I_{KCO}$ generated by levcromakalim and aiso inhibited the delayed rectifier channel, $I_{K(V)}$ (Noack *et*

*al.*, 1992c).

Thus it is clear that many pharmacoiogicaliy-diverse agents which possess either an imidazoline or a guanidine moiety are able to modulate K-channel function both in the pancreatic β-ceii and in smooth muscle. It remains to be determined whether the modulation of K-channeis by these agents is mediated via interaction with an IGR site. Compounds such as phentolamine are relatively poor IGR site ligands and yet are able to modulate the the activity of these K-channeis. Also, both efaroxan (Remaury & Paris, 1992) and ciclazindol (see Chapter 1) possess no affinity for the IGR site and yet are stili able to modulate K-channel function in a similar manner.

### 4.1.3. Specific aims

The aims of the experiments described in this chapter were to evaluate the effects of agents possessing either an imidazoline or a guanidine moiety on the whole-cell K-currents of freshly-isolated rat portal vein cells. The role of the IGR site in the modulation of K-channei function was assessed using the imidazolines phentolamine, cirazoiine, antazoline, clonidine and the guanidines, guanethidine and guanabenz. The structures of these agents are illustrated in Figure 4.1.

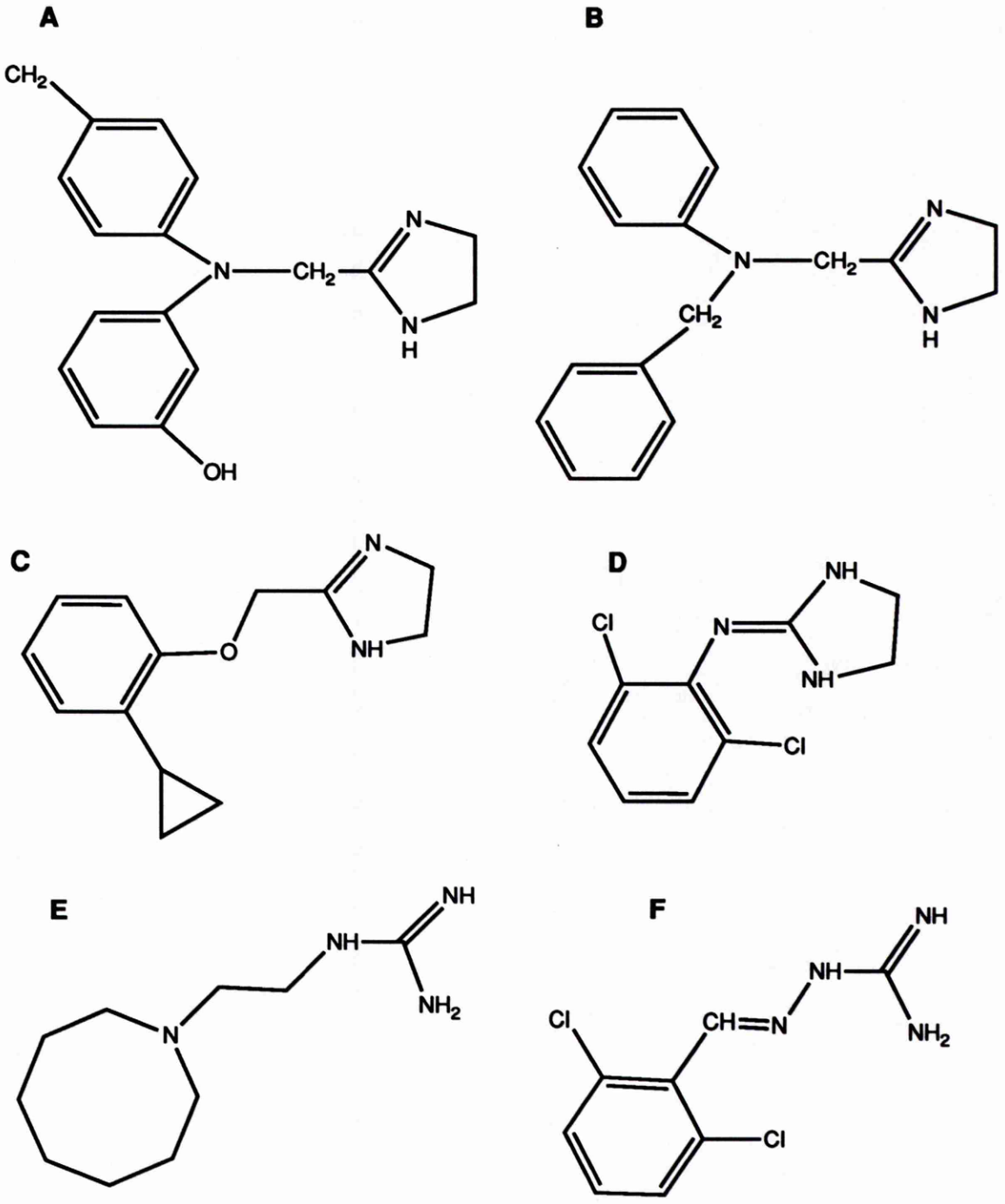



**Figure 4.1.** The structures of the α-adrenoceptor antagonist, phentolamine (A); the histamine $H_1$-antagonist, antazoline (B); the $\alpha_1$-adrenoceptor agonist, cirazoline (C); the $\alpha_2$-adrenoceptor agonist, clonidine (D); the adrenergic neurone blocking agent, guanethidine (E) and the $\alpha_2$-adrenoceptor agonist, guanabenz (F).

## 4.2. Methods

### 4.2.1. Whole-tissue mechanical studies

Male, Sprague Dawley rats (200-350g) were killed by stunning and cervical dislocation and intact portal veins were removed into normal physiological salt solution (PSS). Whole portal veins were then mounted under 10mN tension for isometric recording. The tissues were allowed to equilibrate in Krebs solution (see Drugs and solutions) for 1h and washed at 10min intervals. During the 1h equilibration period, the spontaneous myogenic activity gradually developed. Mechanical responses of the portal vein were recorded and activity integrated with respect to time by use of an Apple Macintosh computer in conjunction with Maclab hardware (Maclab 8) and software (Chart, version 2.5) (Analog Digital instruments).

*Effect on spontaneous activity*: The effect of guanabenz and antazoline on the spontaneous activity of these tissues was investigated by exposing tissues to cumulative additions of either agent (100nM - 100μM), each concentration with a drug / tissue contact time of 6min. In the case of guanabenz, certain tissues were pretreated with RX 821002 (1μM) for 30min to eliminate the effects of $\alpha_2$-adrenoceptor activation on the tissue activity. Antazoiine is devoid of any α-adrenoceptor activity (Schulz & Hasselblatt, 1989b) and therefore no antagonists were employed.

*Inhibition of the K-channel openers*: Rat portal veins were mounted as documented above and following the equilibration period all tissues were exposed to cumulative addition of levcromakallm (1nm-10μM). The veins were then allowed to recover for 1h and were washed every 10min. The tissues were then treated with either antazoline (30μM), guanabenz (30μM), KCl-enriched Krebs solution (20mM; see Drugs and Solutions) or noradrenaline (1μM) for 20min following which the levcromakaiim dose-response experiment was repeated. Each concentration of levcromakalim was in contact with the tissue for

6min and the final 4min of this period was used to calculate the integral of the activity.

### 4.2.2. Single-cell electrophysiology

The electrophysiological recordings were made using the whole-cell configuration of the patch clamp technique as described in Chapter 3. The rat portal vein cells used in these experiments were isolated using the protocol also described in Chapter 3.

### 4.2.3. Statistical analysis

The mean log $EC_{50}$ values for the shifts in the levcromakaiim dose / response curves were compared using Student's unpaired t-test for the comparison of means. To determine the significance of drug treatment on the current / voltage relationships, a 2-way, within subjects (repeated measures) ANOVA test was used. This test was selected because it permitted the paired nature in which the experiments were conducted to be accounted for in the statistical analysis. Inhibition of $I_{K(V)}$ was tested for statistical significance between -20mV and +30mV as described in Chapter 3 (these were the test potentials at which this voltage-dependent current was activated). Both tests were applied using software (Statistica v.3.0a : Statsoft) and p values less than 0.05 were assumed to indicate significance.

### 4.2.4. Drugs and solutions

The Krebs solution used for the whole tissue mechanical studies comprised (mM) NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1. This solution was aerated with a mixture of 95% $O_2$ and 5% $CO_2$ and maintained at 37°C.

The KCl-enriched Krebs solution was of an identical composition to that described for normal Krebs with the following modifications (mM) KCl 18.9, NaCl 100.35.

The solutions used in the cell isolation procedure and in the whole-cell voltage-clamp experiments are described in Chapter 3.

The following substances were used: levcromakalim (Pfizer Central Research), phentolamine hydrochloride, antazoline, clonidine, guanethidine, guanabenz (1-(2,6-dichlorobenzylideneamino)guanidine), noradrenaline bitartrate and okadaic acid (all Sigma), cirazoline (Zeneca). P1060 was obtained from Leo Pharmaceuticals and aprikalim from Rhône-Poulenc Rorer. Phentolamine hydrochloride, antazoline and cirazoline stock solutions (all 10mM) were made in distilled water and then subsequent dilutions were performed in PSS. Clonidine, guanethidine and guanabenz were all dissolved in DMSO (10mM stock solutions) and subsequent dilutions made in PSS. Okadaic acid, P1060, aprikalim and levcromakalim were dissolved in DMSO as detailed in Chapter 3. Noradrenaline bitartrate was dissolved in 70% ethanol (10mM) with ascorbate (1% v/v) added for stability and then diluted in PSS.

## 4.3. Results

### 4.3.1. Whole-tissue mechanical studies

Rat portal veins were mounted for measurement of changes in isometric tension. The $\alpha_2$-adrenoceptor agonist, guanabenz (100nM-100μM) or the histamine $H_1$-antagonist, antazoiine (100nM - 100μM) both caused an increase in the spontaneous myogenic activity of the portal veins (Figure 4.2). This was manifest as an increase in the amplitude and duration of tension waves and a general increase in the complexity of the phasic activity of the tissue. Interaction of guanabenz with $\alpha_2$-adrenoceptors may itself lead to an increase in tissue activity and therefore the effects of this agent were also evaluated in the presence of the selective $\alpha_2$-adrenoceptor antagonist, RX 821002 (1μM). RX 821002 caused a decrease in the response to guanabenz at lower concentrations but the increased spontaneous activity observed at higher concentrations of guanabenz (30μM & 100μM) was unaffected by the antagonist (Figure 4.2). Antazoline is reported to be free of adrenoceptor activity (Schulz & Hasselblatt, 1989b) and therefore an adrenoceptor antagonist was not used.

Both guanabenz (30μM) and antazoline (30μM) caused a rightward shift in the levcromakalim dose-response curve in rat portal veins mounted for isometric tension recording studies (Figure 4.3).

Because of the increase in myogenic activity caused by guanabenz and antazoiine, it is possible that the shift in the levcromakalim relaxation / response curve arises as a result of the increased activity of the tissue and not from a direct inhibition of the K-channel opener. To eliminate this possibility, rat portal veins were pretreated with concentrations of either noradrenaline (1μM) or KCl (20mM substituted Krebs solution) that gave equivalent increases in myogenic activity (5-fold) to that evoked by antazoline (30μM) or guanabenz (30μM) and the mechano-inhibitory effects of levcromakaiim were then investigated. With both noradrenaline and KCl the levcromakalim relaxation / response curve was shifted to the right in a parallel manner (Figure 4.4). However, the shift in the levcromakalim relaxation curve caused by either guanabenz or antazoline (mean

log $EC_{50}$ values of -6.81 $\pm$ 0.04 and -6.28 $\pm$ 0.03 respectively) was significantly greater that the shift in the levcromakalim response curve caused by either KCl or noradrenaline (mean log $EC_{50}$ values of -7.01 $\pm$ 0.13 and -7.08 $\pm$ 0.10 respectively; Student's t-test, $p<0.05$). It therefore appears that the guanabenz- and antazoline-induced inhibition of the mechano-inhibitory effects of levcromakalim can only partially be attributed to functional antagonism and thus is partially due to a direct antagonistic effect.

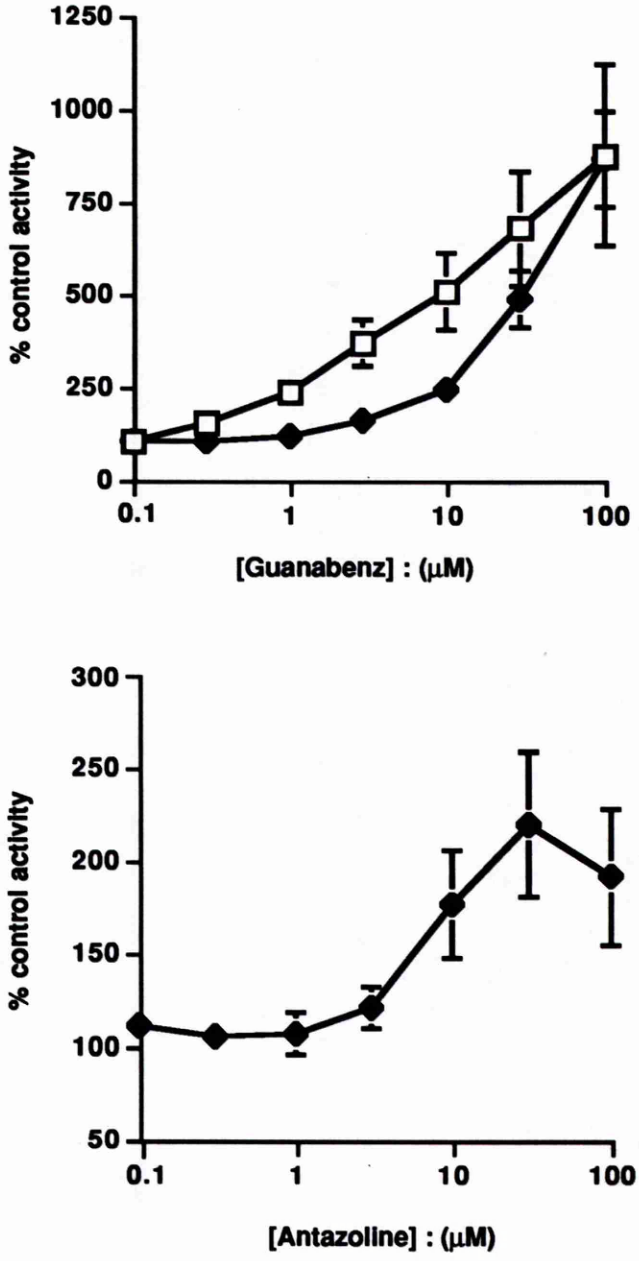



**Figure 4.2.** The effect of guanabenz and antazoline on the integrated spontaneous activity of rat portal vein. The concentration-dependent effect of guanabenz (0.1-100μM, □; upper panel) and antazoline (0.1-100μM, ◆; lower panel) on mechanical activity is expressed as a percentage of the initial control activity. The ability of RX 821002 (1μM, ◆) to antagonise the spasmogenic effect of guanabenz is also demonstrated in the upper panel. Each point is the mean value from 6-8 observations and vertical bars represent the s.e.mean.

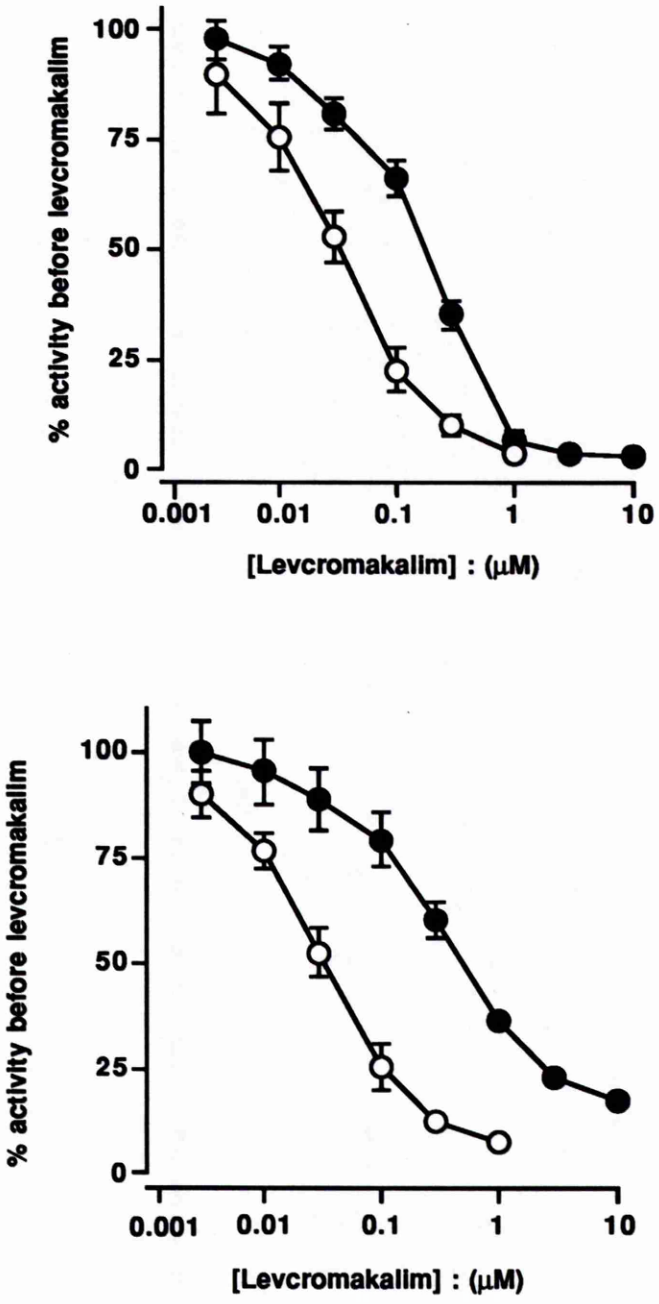



**Figure 4.3.** Antagonism of the mechano-inhibitory effects of levcromakalim by guanabenz and antazoline. Guanabenz (30μM, ●; upper panel) and antazoline (30μM, ●; lower panel) both shifted the levcromakalim dose-response curve to the right when compared to control values (○). Data are expressed as a percentage of control activity immediately before the addition of levcromakalim. Each point is the mean value from 4 observations and vertical bars represent the s.e.mean.

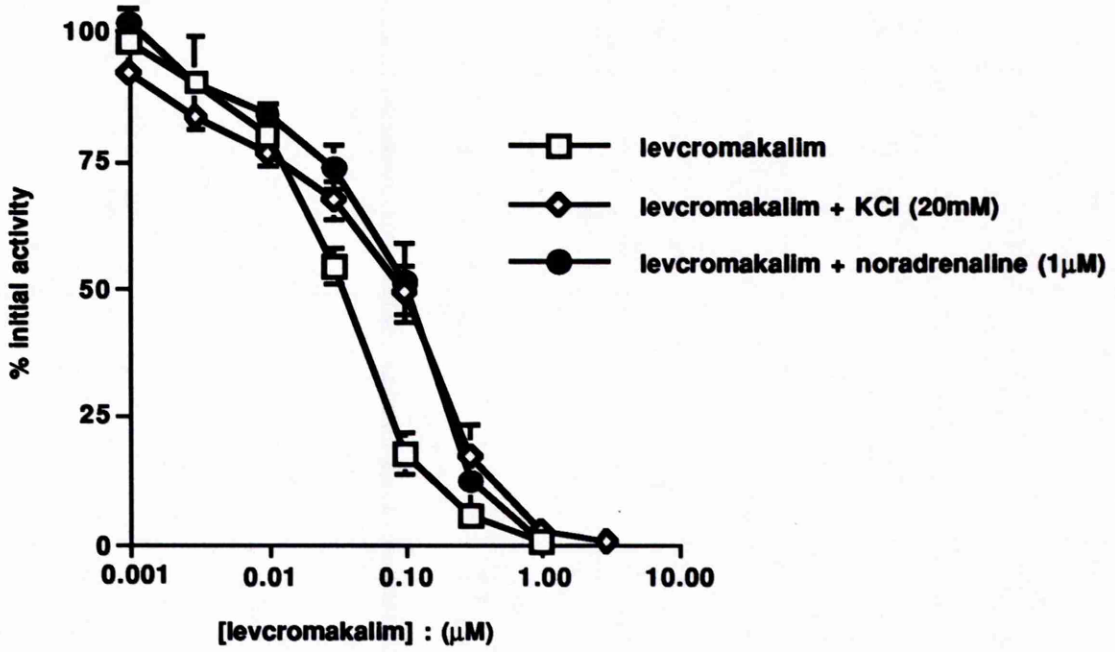



**Figure 4.4.** Antagonism of the mechano-inhibitory effects of levcromakalim by KCl and noradrenaline. Noradrenaline (1μM, ●) and KCl (20mM substituted Krebs, ◊) both shifted the levcromakalim dose-response curve to the right when compared to control values (❑). Data are expressed as a percentage of control activity immediately before the addition of levcromakalim. Each point is the mean value from 4 observations and vertical bars represent the s.e.mean.

### 4.3.2. Single-cell electrophysiology

Levcromakalim (1μM) caused the induction of a non-inactivating K-current ($I_{KCO}$) and a simultaneous decrease in the magnitude of the delayed rectifier current, $I_{K(V)}$ in cells isolated from rat portal vein (Chapter 3). The control non-inactivating current ($I_{NI}$) had a reversal potential of approximately -30mV and was probably carried by cation channel. For further discussion of the currents evoked from these cells and the voltage protocols employed, see Chapter 3.

A series of imidazoline / guanidine ligands were tested both for their ability to inhibit $I_{K(V)}$ and also to block the generation of $I_{KCO}$ by levcromakaiim.

*Phentolamine:* The α-adrenoceptor antagonist, phentolamine (30-100μM) caused a significant, dose-related inhibition of the delayed rectifier current, $I_{K(V)}$ ($p<0.05$, ANOVA) but was without effect on the control non-inactivating component, $I_{NI}$ (Figure 4.5). The generation of $I_{KCO}$ by levcromakalim was reversed by phentolamine (30μM; Figure 4.6), and the levcromakalim-induced inhibition of $I_{K(V)}$ was even greater in the presence of both agents (Figure 4.6).

Similar effects were observed using the structurally-different K-channel openers, P1060 and aprikalim. The generation of $I_{KCO}$ by P1060 (1μM; Figure 4.7) or aprikalim (3μM; Figure 4.8) was reversed by addition of phentolamine (30μM). The inhibition of $I_{K(V)}$ by these K-channel openers (P1060, Figure 4.7; aprikalim, Figure 4.8) was unaffected in the joint presence of both K-channel opener and phentolamine (30μM). Thus, phentolamine demonstrates a similar profile of inhibition regardless of the structural type of K-channel opener.

*Antazoline & cirazoline:* The histamine-$H_1$ receptor antagonist, antazoiine (30μM) and the $\alpha_1$-adrenoceptor agonist, cirazoline (30μM) both contain imidazoline moieties within their structures. Addition of either antazoline (30μM; Figure 4.9) or cirazoline (30μM; Figure 4.10) to the recording chamber had little effect on the non-inactivating component $I_{NI}$, but significantly decreased $I_{K(V)}$ ($p<0.05$, ANOVA). In addition, subsequent exposure of cells pretreated with either

antazoiine (30μM; Figure 4.9) or clrazoline (30μM; Figure 4.10) to levcromakaiim (1μM) failed to generate $I_{KCO}$. The delayed rectifier current was further inhibited in the joint presence of either antazoiine or cirazoline and levcromakalim.

*Guanethidine:* Guanethidine is an adrenergic neurone blocking agent, but the mechanism by which it exerts its sympatholytic effects is unknown. Guanethidine (50μM) had no effect on either the inactivating ($I_{K(V)}$; $p>0.05$, ANOVA) or the non-inactivating ($I_{NI}$) K-currents (Figure 4.11). The generation of $I_{KCO}$ by levcromakaiim, however was reversed by guanethidine (50μM; Figure 4.12) and the levcromakaiim-induced inhibition of $I_{K(V)}$ was slightly larger in the joint presence of both agents.

*Guanabenz:* The $\alpha_2$-adrenoceptor agonist, guanabenz (30μM) significantly decreased the magnitude of the delayed rectifier current, $I_{K(V)}$ ($p<0.05$, ANOVA), but had little effect on $I_{NI}$ (Figure 4.13). In addition, cells pretreated with guanabenz failed to generate $I_{KCO}$ on exposure to levcromakalim (1μM; Figure 4.13). The inhibition of $I_{K(V)}$ in these cells was further evident in the presence of both guanabenz and levcromakalim (Figure 4.13).

*Clonidine:* Application of the $\alpha_2$-adrenoceptor agonist, clonidine (0.1 - 1μM) caused a significant dose-related decrease in the magnitude of the delayed rectifier current, $I_{K(V)}$ ($p<0.05$, ANOVA) but had no effect on $I_{NI}$ in these cells (Figure 4.14).

Addition of clonidine (30μM) caused a slight decrease in the magnitude of the delayed rectifier current although this was only a trend and not significantly different from controls (Figure 4.15; $p>0.05$, ANOVA). Clonidine (30μM) also had little effect on the non-inactivating component, $I_{NI}$. Addition of levcromakalim to those cells pretreated with clonidine (30μM) failed to generate $I_{KCO}$ (Figure 4.15) but the reduction in $I_{K(V)}$ was greater in the joint presence of both clonidine and levcromakaiim (Figure 4.15).

Finally, the generation of $I_{KCO}$ by levcromakalim (1μM) was reversed by clonidine (30μM; Figure 4.16) and in the joint presence of both clonidine and levcromakalim the inhibition of $I_{K(V)}$ was greater (Figure 4.16).

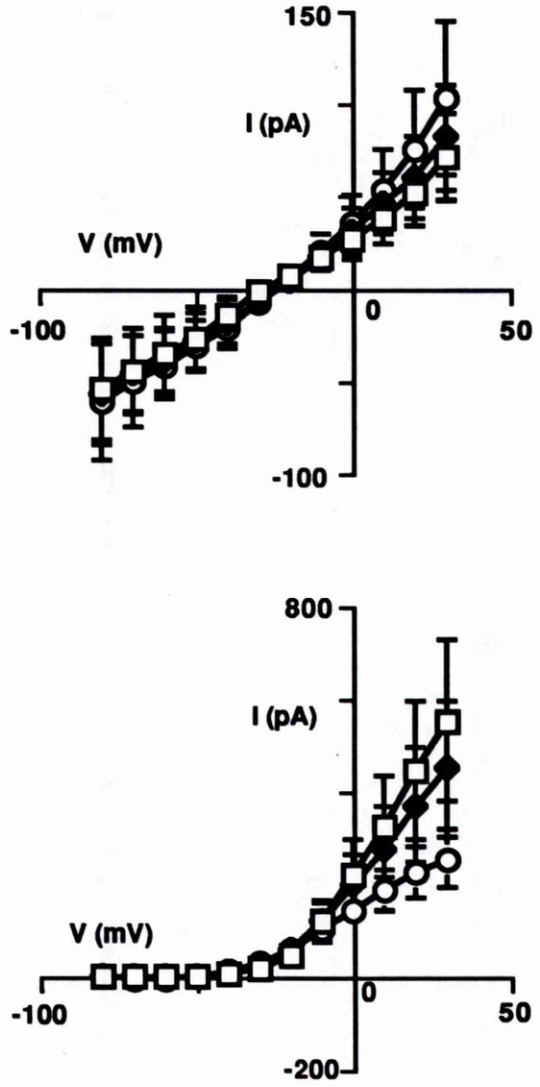



**Figure 4.5.** The effect of phentolamine (30-100μM) on whole-cell currents from rat portal vein. Upper panel, HP -10mV: Phentolamine (30μM, ◆; 100μM, ○) had little effect on the non-inactivating component, $I_{NI}$ when compared to control values (□). Lower panel, HP -90mV: Phentolamine caused a significant, dose-related decrease in the magnitude of the delayed rectifier current, $I_{K(V)}$ compared to control values ($p<0.05$, ANOVA). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

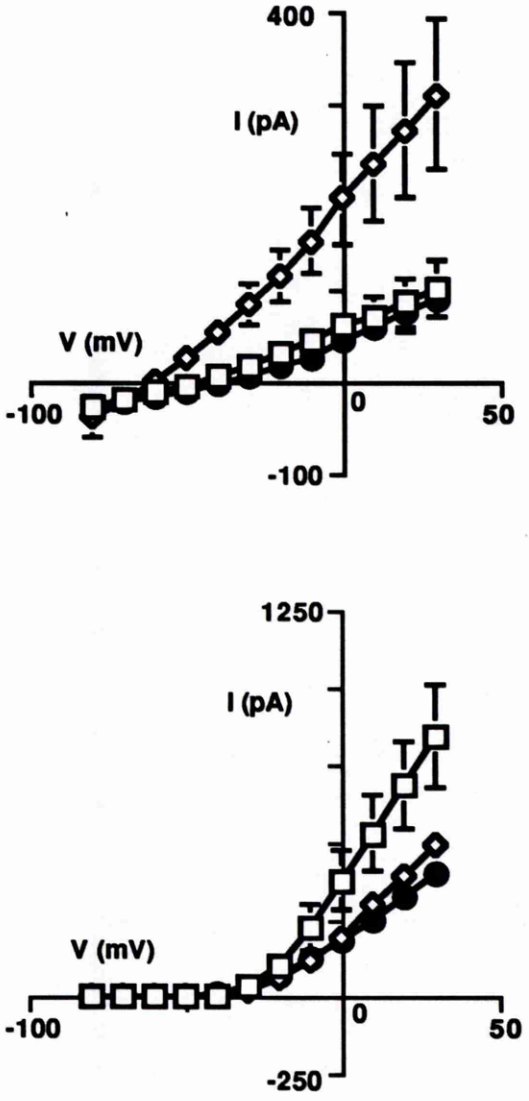



**Figure 4.6.** The effects of levcromakalim on whole-cell currents and their reversal by phentolamine. Upper panel, HP -10mV: Levcromakalim (1μM, ◊) increased the total non-inactivating component when compared to control values (❑). The induced current ($I_{KCO}$) was inhibited by subsequent application of phentolamine (30μM, ●). Lower panel, HP -90mV: Levcromakalim (1μM; ◊) inhibited $I_{K(V)}$ compared to control values (❑) an effect which was greater in the joint presence of both levcromakalim and phentolamine (30μM; ●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

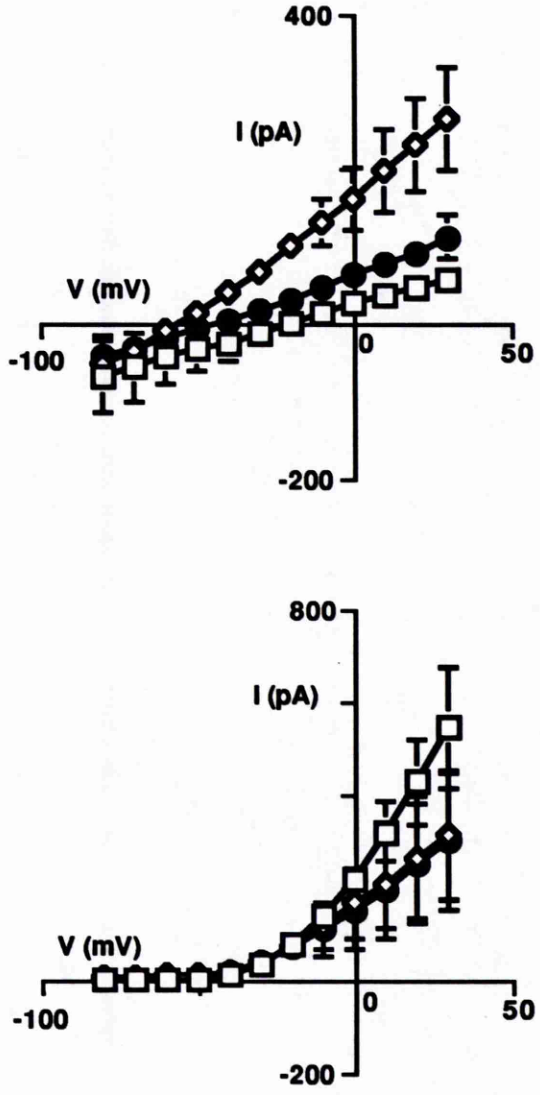



**Figure 4.7.** The effects of P1060 on the whole-cell currents and their reversal by phentolamine. Upper panel, HP -10mV: P1060 (1μM, ◊) increased the total non-inactivating component when compared to control values (□). The induced current ($I_{KCO}$), was inhibited by subsequent addition of phentolamine (30μM, ●). Lower panel, HP -90mV: P1060 (1μM; ◊) inhibited $I_{K(V)}$ compared to control values (□) and this inhibition was unchanged in the joint presence of both P1060 and phentolamine (30μM; ●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

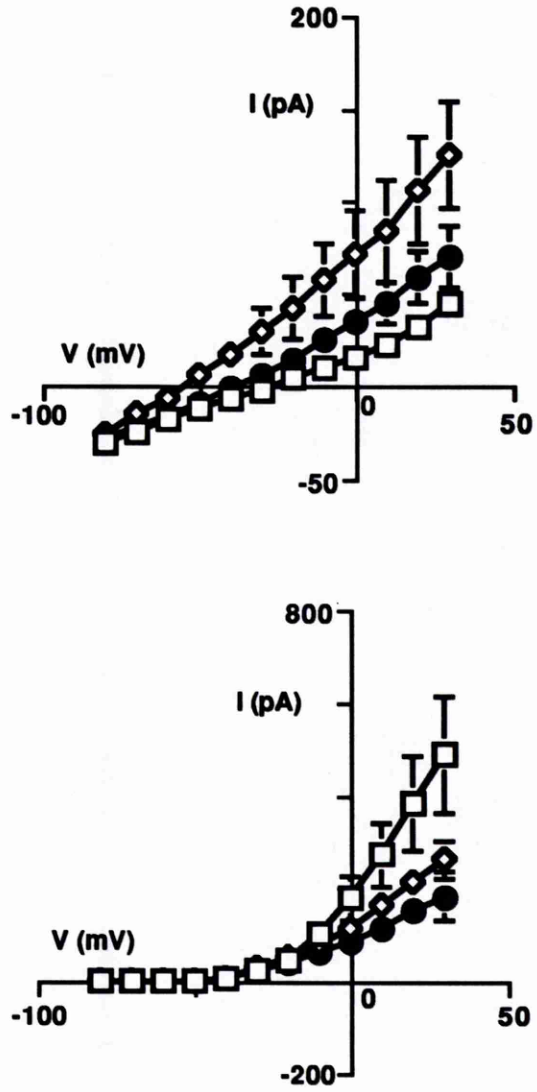



**Figure 4.8.** The effects of aprikalim on the whole-cell currents and their reversal by phentolamine. Upper panel, HP -10mV: Aprikalim (3μM, ◊) increased the total non-inactivating component when compared to control values (❑). The induced current ($I_{KCO}$) was inhibited by subsequent addition of phentolamine (30μM, ●). Lower panel, HP -90mV: Aprikalim (3μM, ◊) inhibited $I_{K(V)}$ compared to control values (❑) an effect which was greater in the joint presence of both aprikalim and phentolamine (30μM, ●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

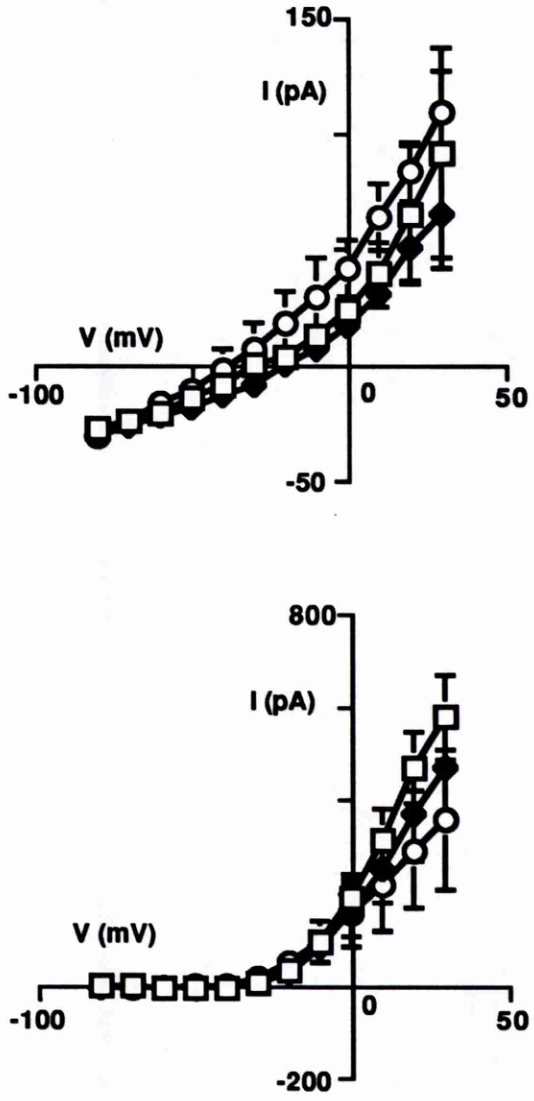



**Figure 4.9.** The effects of pre-exposure to antazoline on the changes in non-inactivating currents or $I_{K(V)}$ produced by levcromakalim. After 15min exposure to antazoline (30μM, ◆), a slight reduction in $I_{NI}$ was observed compared to control currents (❑) and subsequent addition of levcromakalim (1μM, ○) failed to induce $I_{KCO}$ (upper panel, HP -10mV). The same concentration of antazoline significantly inhibited $I_{K(V)}$ (lower panel, HP -90mV; $p<0.05$, ANOVA) and this effect was enhanced by levcromakalim (1μM). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

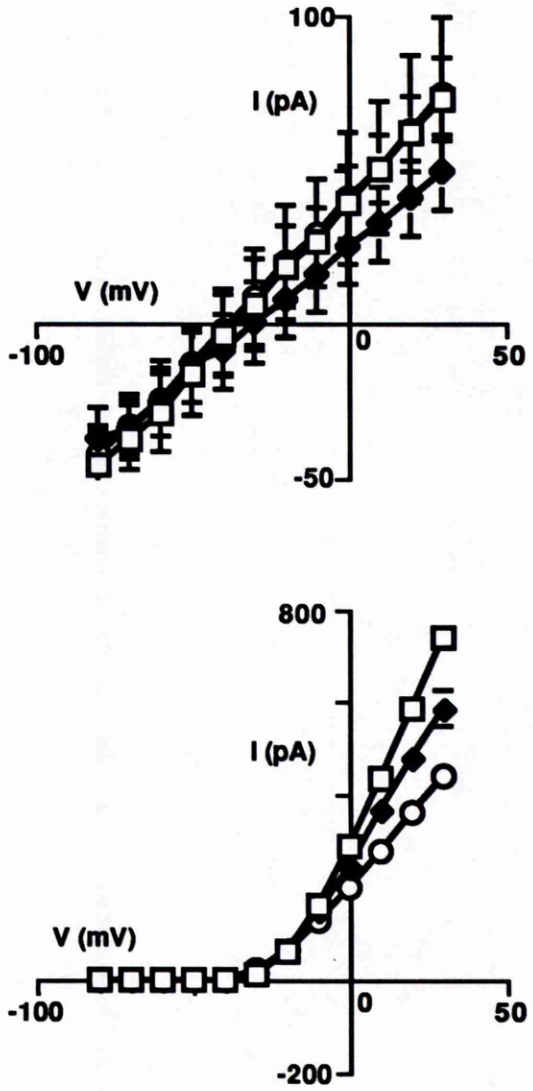



**Figure 4.10.** The effects of pre-exposure to cirazoline on the changes in non-inactivating currents or $I_{K(V)}$ produced by levcromakalim. After 15min exposure to cirazoline (30μM, ◆), a slight reduction in $I_{NI}$ was observed compared to control currents (□) and subsequent addition of levcromakalim (1μM, ○) failed to induce $I_{KCO}$ (upper panel, HP -10mV). The same concentration of cirazoline significantly inhibited $I_{K(V)}$ (lower panel, HP -90mV; $p<0.05$, ANOVA) and this effect was enhanced by levcromakalim (1μM). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

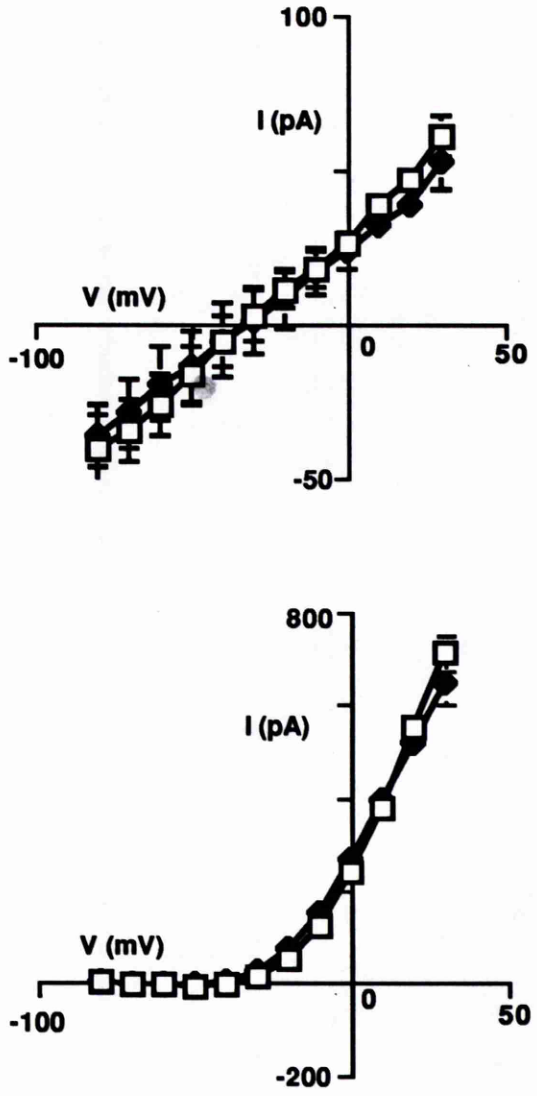



**Figure 4.11.** The effect of guanethidine on the non-inactivating and inactivating currents in rat portal vein. Guanethidine (50μM, ◆) had no effect either on $I_{NI}$ (upper panel, HP -10mV) or $I_{K(V)}$ (lower panel, HP -90mV) when compared with control currents (❑). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

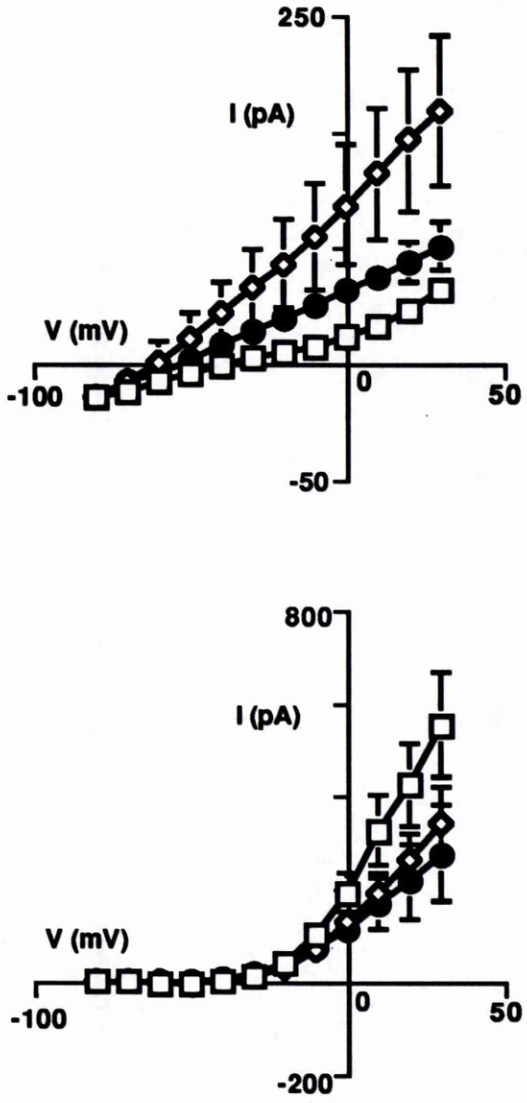



**Figure 4.12.** The effects of guanethidine on the changes in non-inactivating currents and $I_{K(V)}$ produced by levcromakalim. Upper panel, HP -10mV: Levcromakalim (1μM, ◊) increased the non-inactivating currents compared to controls (❑). The induced current ($I_{KCO}$) was inhibited by subsequent addition of guanethidine (50μM, ●). Lower panel, HP -90mV: Levcromakalim (1μM, ◊) inhibited $I_{K(V)}$, an effect that was slightly greater in the joint presence of both levcromakalim and guanethidine (50μM; ●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

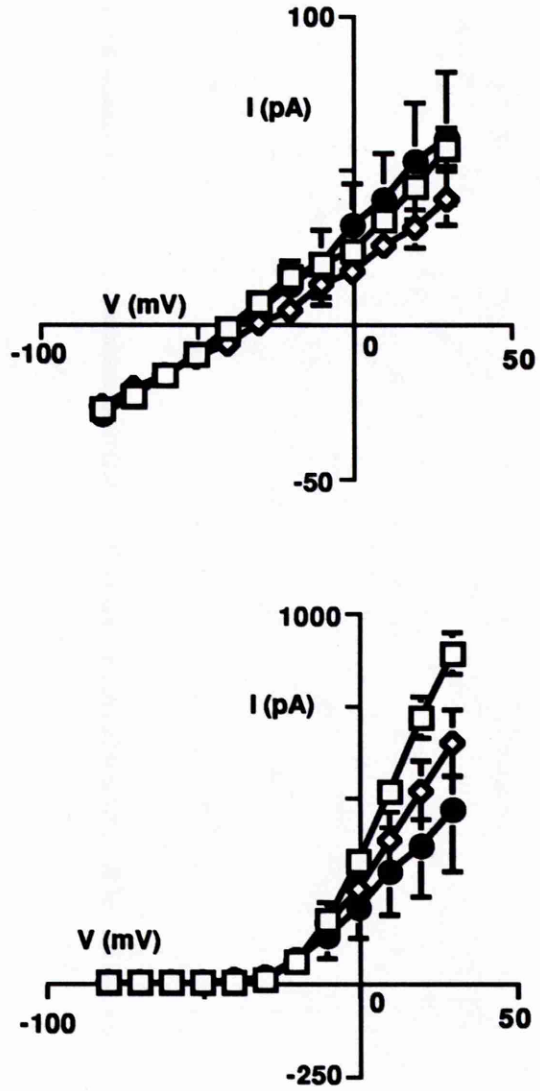



**Figure 4.13.** The effects of pre-exposure to guanabenz on the changes in non-inactivating currents and $I_{K(V)}$ produced by levcromakalim. After 15min exposure to guanabenz (30μM, ◊), a slight reduction in the non-inactivating currents was observed compared to control values (□) and subsequent addition of levcromakalim (1μM, ●) failed to induce $I_{KCO}$ (upper panel, HP -10mV). The same concentration of guanabenz significantly inhibited $I_{K(V)}$ (lower panel, HP -90mV; $p<0.05$, ANOVA) and this effect was enhanced by levcromakalim (1μM, ●). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

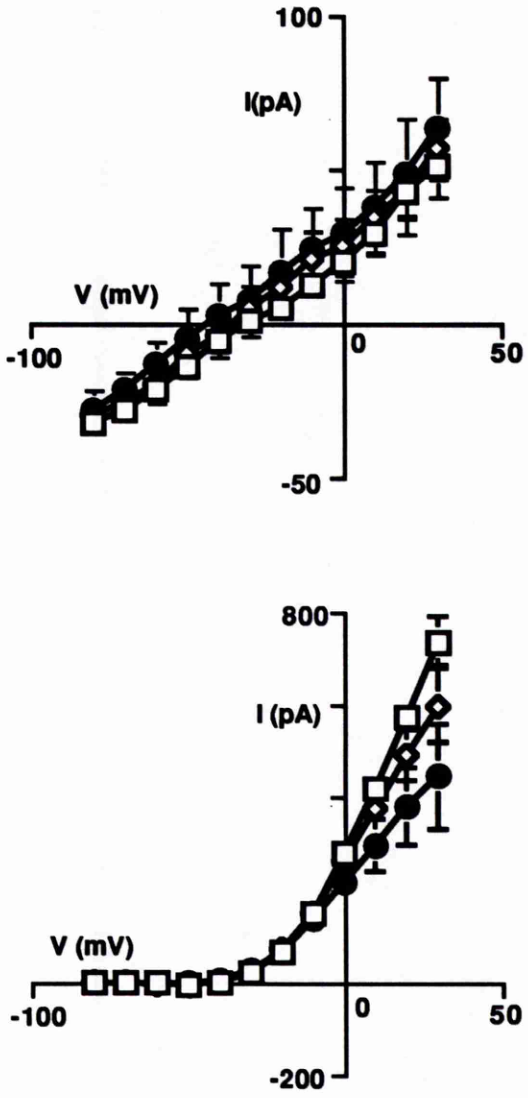



**Figure 4.14.** The effect of clonidine on the non-inactivating and inactivating currents in rat portal vein. Clonidine (100nM, ◊;1μM, ●) had no effect either on $I_{NI}$ (upper panel, HP -10mV) but caused a significant, dose-related decrease in the magnitude of $I_{K(V)}$ (lower panel, HP -90mV; $p<0.05$, ANOVA) when compared with control currents (□). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

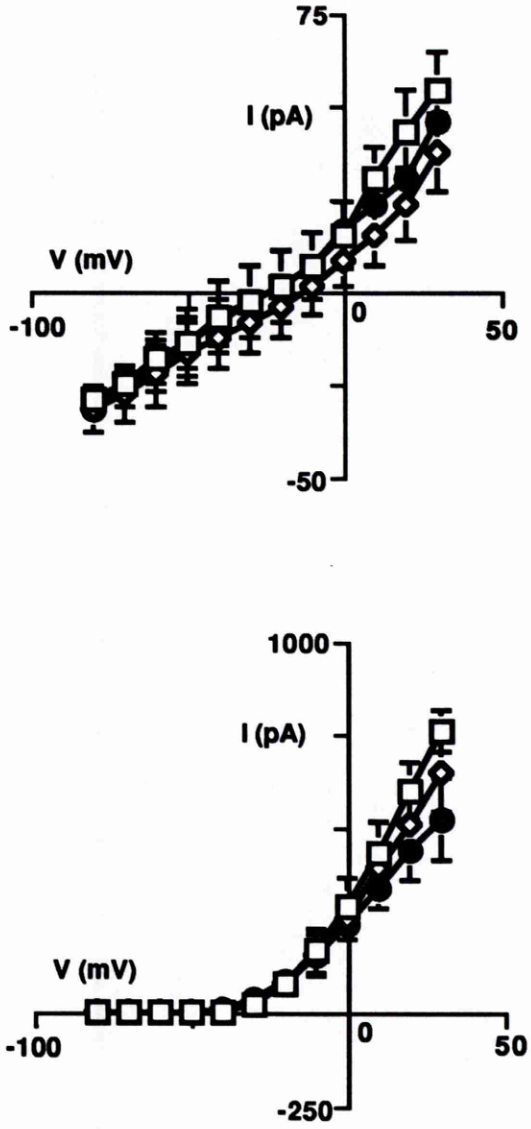



**Figure 4.15.** The effects of pre-exposure to clonidine on the changes in non-inactivating currents and $I_{K(V)}$ produced by levcromakalim. After 15min exposure to clonidine (30μM, ◊), little effect on $I_{NI}$ was observed compared to control currents (❑) but subsequent addition of levcromakalim (1μM, ●) failed to induce $I_{KCO}$ (upper panel, HP -10mV). The same concentration of clonidine slightly reduced $I_{K(V)}$ (lower panel, HP -90mV; $p>0.05$, ANOVA) and this effect was enhanced by levcromakalim (1μM). Each point is the mean value derived from 3 observations and vertical bars represent the s.e.mean.

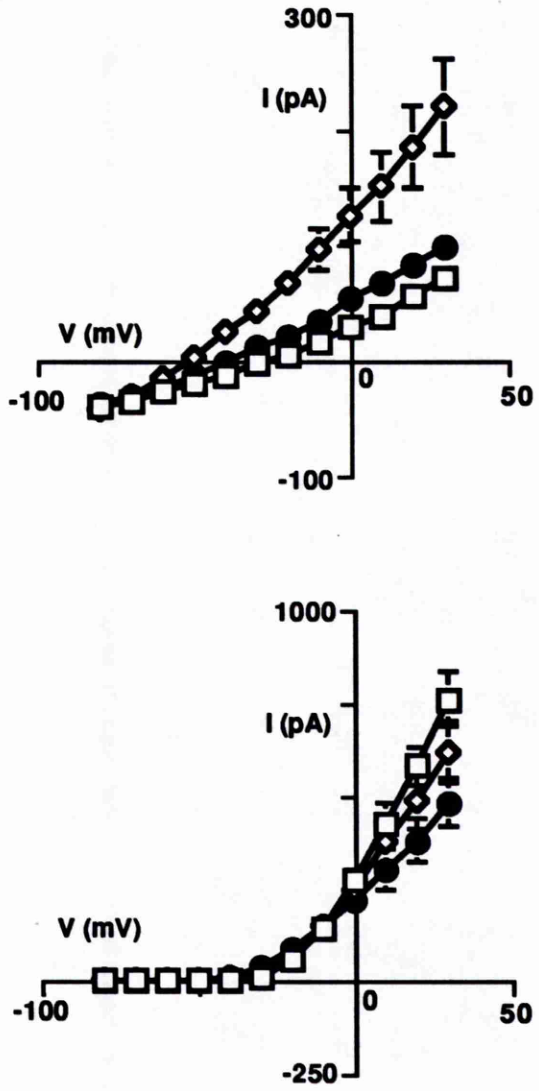



**Figure 4.16.** The effects of clonidine on the changes in non-inactivating currents and $I_{K(V)}$ produced by levcromakalim. Upper panel, HP -10mV: Levcromakalim (1μM, ◊) caused an increase in the the total non-inactivating currents compared to control values (□). The induced current ($I_{KCO}$) was inhibited by subsequent application of clonidine (30μM, ●). Lower panel, HP -90mV: Levcromakalim (1μM, ◊) decreased the magnitude of $I_{K(V)}$, an effect which was greater in the joint presence of both levcromakalim and clonidine (●). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

*Noradrenaline:* To discount the possible involvement of adrenoceptors in the mechanism of action of the imidazoline / guanidine ligands investigated, the effect of noradrenaline on the whole-cell currents was also investigated. Application of noradrenaline (10μM) to the bathing solution had no effect on either $I_{NI}$ or $I_{K(V)}$ when compared to control values (Figure 4.17).

*The effect of okadaic acid on the phentolamine-induced inhibition of $I_{KCO}$ and $I_{K(V)}$*: The inhibitory effects of phentolamine on the response to levcromakalim were also examined with the inclusion of the phosphatase inhibitor, okadaic acid in the recording pipette. This was performed in order to obtain some preliminary information about the mechanism by which phentolamine inhibits these currents. With the inclusion of okadaic acid (1μM; Figure 4.18), $I_{KCO}$ induced by levcromakalim (1μM) and the inhibition of $I_{K(V)}$ by this agent were both reduced (Chapter 3). Induction of $I_{KCO}$ by levcromakalim was reversed by phentolamine (30μM) at potentials between -80mV and +20mV, however at more positive potentials (+30mV - +50mV) the current level was still markedly elevated. The non-inactivating component therefore demonstrated marked outward rectification at positive potentials. In the joint presence of both levcromakalim and phentolamine, the delayed rectifier current, $I_{K(V)}$ was inhibited (Figure 4.18).

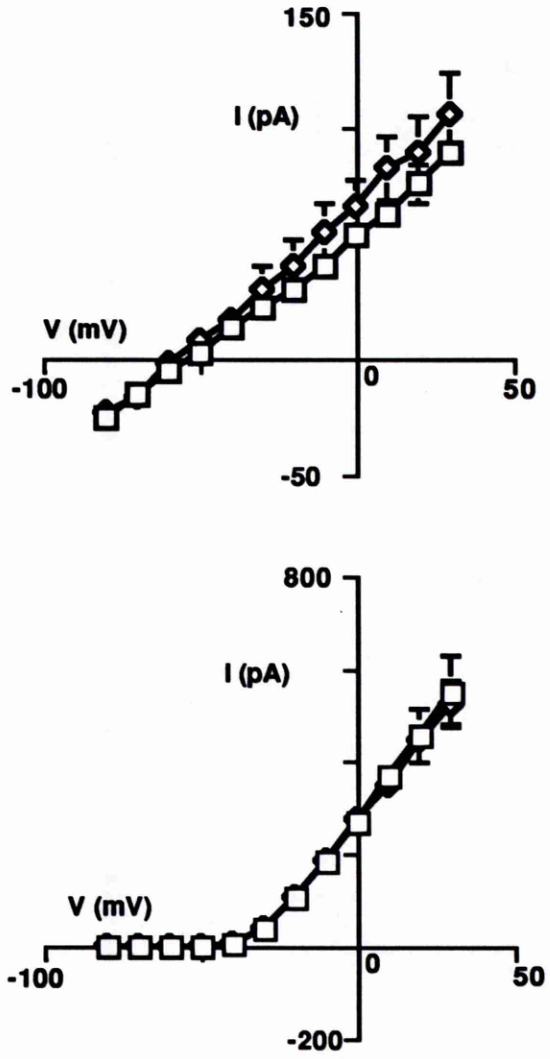



**Figure 4.17.** The effect of noradrenaline (10μM) on whole-cell currents in rat portal vein. Noradrenaline (10μM; ◊) had no effect either on the non-inactivating currents (upper panel, HP -10mV) or on $I_{K(V)}$ (lower panel, HP -90mV) when compared to control values (❑). Points are the mean of 3 observations and vertical bars represent the s.e.mean.

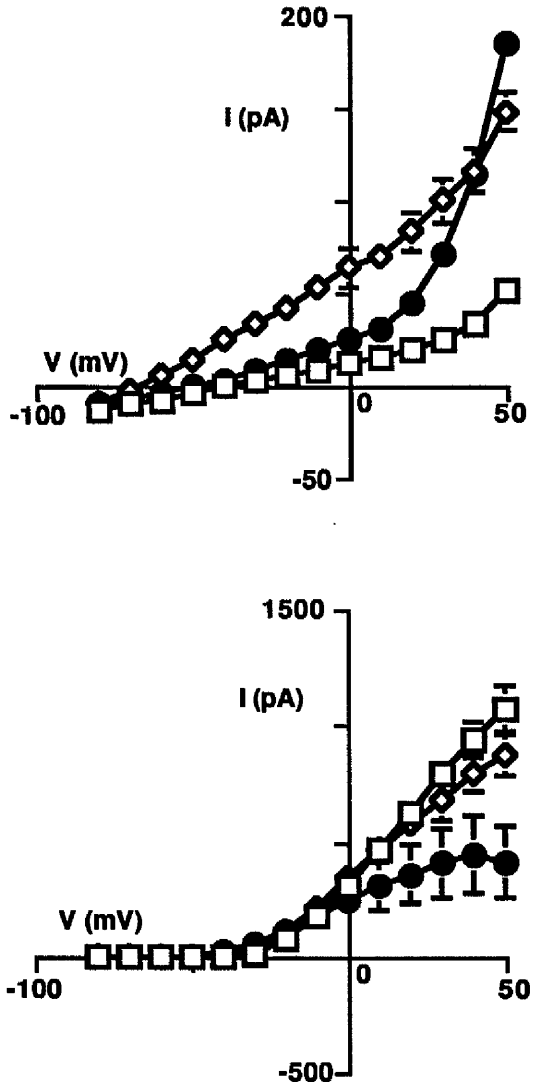



**Figure 4.18.** The effect of phentolamine on the changes in non-inactivating currents and $I_{K(V)}$ produced by levcromakalim with the inclusion of okadaic acid (1μM) in the patch pipette. Upper panel, HP -10mV: Levcromakalim (1μM, ◊) caused an increase in the total non-inactivating currents compared to control values (□). The induced current ($I_{KCO}$) was partially inhibited between -80mV and +20mV by subsequent addition of phentolamine (30μM, ●). At potentials positive to +20mV the current level remained elevated and thus the non-inactivating component displayed outward rectification. Lower panel, HP -90mV: Levcromakalim (1μM, ◊) did not affect the magnitude of $I_{K(V)}$ but in the joint presence of both levcromakalim and phentolamine (●), $I_{K(V)}$ was inhibited. Test potentials between -80mV and +50mV were used in these experiments. Points are the mean of 3 observations and vertical bars represent the s.e.mean.

## 4.4. Discussion

### 4.4.1. Imidazoline- and guanidine-based compounds inhibit K-channels in vascular smooth muscle

These data demonstrate that agents containing either an imidazoline or a guanidine moiety within their structure are capable of modulating K-channel function in vascular smooth muscle. Of the six imidazoline- / guanidine-based agents tested in this study, five (antazoline, cirazoline, clonidine, phentolamine and guanabenz) were able both to inhibit the delayed rectifier current, $I_{K(V)}$ and also to block the induction of $I_{KCO}$ by levcromakalim. In addition, for two selected examples of these agents, antazoline, an imidazoline and guanabenz, a guanidine the effects observed in the single cells were consistent with effects observed in the whole-tissue. Both of these agents increased the spontaneous myogenic activity of the rat portal vein and both also antagonised the mechano-inhibitory effects of levcromakalim in this tissue. These results correspond well with the recent findings of Noack *et al.* (1992c) who demonstrated that ciclazindol, an anorectic agent containing an imidazo- moiety also inhibits both $I_{KCO}$ and $I_{K(V)}$ and that these effects are also consistent with effects observed in the tissue bath. Ciclazindol both increases myogenic activity and blocks the mechano-inhibitory effects of levcromakalim in isolated rat portal vein.

Guanabenz, an $\alpha_2$-adrenoceptor agonist, antazoline, a histamine $H_1$-antagonist and ciclazindol, a monoamine uptake blocker (Becket *et al.*, 1973) are pharmacologically diverse agents. Therefore it is unlikely that the adrenergic system, the histaminergic system or inhibition of amine uptake are responsible for the observed effects of these compounds on $I_{K(V)}$ or on $I_{KCO}$. In addition, noradrenaline (10μM) had no effect on $I_{K(V)}$ or on $I_{NI}$ and this therefore further discounts the possiblility that adrenoceptors are involved in the inhibition of these currents by the imidazoline- / guanidine-based ligands.

Satake *et al.* (1984) reported that phentolamine induced rhythmic contractions in guinea-pig detrusor muscle and that this was blocked by inhibitors of the cyclo-oxygenase enzyme system. These authors concluded that

phentolamine-induced contractile activity in guinea-pig detrusor muscle was, at least partially, attributable to the production of arachidonic acid metabolites via the cyclo-oxygenase enzyme cascade. The structural similarities between phentolamine, guanabenz and antazoline may imply that a similar mechanism of action underlies both guanabenz- and antazoline-induced activity in the portal vein. However, the ability of phentolamine to induce activity in guinea-pig detrusor was not shared by another imidazoline, clonidine. This suggests that the ability to induce the production of arachidonate metabolites is not a feature common to all imidazoline / guanidine-based ligands and it therefore seems unlikely that arachidonate metabolites are responsible for the guanabenz- and antazoline-induced increase in activity in rat portal vein. This however remains to be confirmed by further experimentation.

The data of the present study therefore suggest that it is the inhibition of the delayed rectifier current, $I_{K(V)}$ that underlies the ability of antazoline and guanabenz to increase the spontaneous activity of portal veins.

It is also likely that the induction of $I_{KCO}$ by the K-channel openers is responsible for the mechano-inhibitory effects of these agents in tissue bath experiments. Again, both the mechano-inhibitory effects and the induction of $I_{KCO}$ are blocked by guanabenz and antazoline. In addition, phentolamine also inhibits $I_{KCO}$ induced by P1060 or by aprikalim. This indicates that the effects of the imidazolines and guanidines are not confined to levcromakalim but extend to the other structural groups of K-channel openers.

In contrast to the guanidine, guanabenz (and the imidazolines phentolamine, antazoline, cirazoline and clonidine) guanethidine inhibited the induction of $I_{KCO}$ with little or no effect on $I_{K(V)}$. This is consistent with the report of Berry *et al.* (1992) who found that guanethidine antagonised the relaxant effects of levcromakalim in guinea-pig isolated trachea but that it did not modify the spontaneous tone of this tissue.

Interestingly, the $\alpha_2$-adrenoceptor agonist, clonidine inhibited $I_{K(V)}$ at concentrations lower (0.1μM & 1μM) than those required to inhibit $I_{KCO}$ (30μM).

Clonidine (10μM) was not sufficient to provide a consistent reversal of $I_{KCO}$ induced by levcromakalim (not shown). The ability of low concentrations of clonidine to inhibit $I_{K(V)}$ may reflect the activation of $\alpha_2$-adrenoceptors leading to a decrease in adenylate cyclase activity (Ruffolo *et al.*, 1991), a decrease in cAMP-dependent phosphorylation of the delayed rectifier channel and a consequent decrease in channel opening. However, the lack of effect of noradrenaline on $I_{K(V)}$ would seem to contradict this view as noradrenaline would also be expected to activate the $\alpha_2$-adrenoceptors and thus inhibit $I_{K(V)}$.

Clonidine (30μM) caused a slight decrease in the magnitude of $I_{K(V)}$ however this was only a trend and not significantly different from controls. This is in contrast to the ability of low concentrations of clonidine (0.1μM & 1μM) to significantly inhibit this voltage-dependent current. The lack of significance seen with clonidine (30μM) is due to an *increase* in $I_{K(V)}$ in 1 out of the 3 cells tested. At present it is unclear whether higher concentrations of clonidine exert additional actions that are not observed at the lower concentrations.

Cull-Candy *et al.* (1988) reported that clonidine (2μM-30μM) also inhibits an acetylcholine-induced inward current in chromaffin cells. The ability of clonidine to modulate the functioning of a number of different ion channels directly, in addition to its $\alpha_2$-adrenoceptor agonist actions suggests that care must be taken in drawing conclusions from the use of this agent. Clearly, further experiments are required to determine whether the effects observed at lower concentrations of clonidine are mediated via an $\alpha_2$-adrenoceptor or via a different pathway and whether this agent, at higher concentrations, possesses any additional actions to those observed at lower concentrations.

### 4.4.2. Are the effects of the imidazoline- / guanidine-based ligands on K-channels mediated via an IGR site ?

All the imidazoline- / guanidine-based compounds tested were able to inhibit K-channel function in cells isolated from rat portal vein. In addition, Yablonsky and coworkers (1988) reported that five of these agents, cirazoline,

guanabenz, phentolamine, guanethidine and clonidine all exhibited some affinity for the IGR site identified in rabbit urethral smooth muscle ($K_i$ values of 2.9nM, 4.4nM, 455nM, 701nM and 1265nM respectively). These workers did not examine the affinity of antazoline for this site. The imidazoline- / guanidine-based compounds employed therefore, are all ligands at the IGR site in smooth muscle (with the possible exception of antazoline) and all are able to modulate K-channel function in smooth muscle. These findings are therefore consistent with the view that the IGR site found in smooth muscle may be involved in the control of membrane excitability via the modulation of one or more K-channels. It is unclear, however, whether it is the modulation of $K_{ATP}$ or $K_V$ that may arise as a result of IGR site activation. The inability of guanethidine to inhibit $I_{K(V)}$ may indicate that it is closure of $K_{ATP}$ that occurs in response to stimulation of the IGR site.

A more feasible explanation, however, may be that the IGR site controls the closure of both $K_V$ and $K_{ATP}$. In the present study, five out of the six agents tested (antazoline, cirazoline, phentolamine, clonidine and guanabenz) inhibited both $I_{KCO}$ and $I_{K(V)}$ and this profile is shared by a sixth agent, ciclazindol (Noack *et al.*, 1992c). These data may therefore indicate that a functional link exists between the channels carrying these two currents. IGR site activation may cause the inhibition of only a single channel but this channel may exist in one of two states. Each of these states could thus carry currents with different voltage-dependencies, a possibility which is discussed further in Section 4.4.3.

Although these data suggest that IGR site activation could result in K-channel modulation, a number of anomalies exist. Efaroxan blocks the $K_{ATP}$ channel in the insulin secreting, RINm5F cell-line (Chan *et al.*, 1993) but this agent exhibits no affinity for the IGR site in the same cell line (Remaury & Paris, 1992). It is at present unclear whether efaroxan is a ligand at the IGR site in smooth muscle and whether this agent is able to modulate K-channels in smooth muscle. It is therefore difficult to determine whether the inability of this agent to interact with the IGR site in insulin-secreting cells also applies to the IGR site in smooth muscle.

Similarly, ciclazindol has no affinity for the IGR site in rat liver (see Chapter 1) but is still capable of a similar profile of K-channel modulation as the other recognised IGR site ligands (Noack *et al.*, 1992c). One possible explanation is that the IGR site in smooth muscle differs from that in other tissues and that ciclazindol is a ligand at the site in smooth muscle but not in other tissues such as liver. The existence of subtypes of IGR site has been suggested (Michel & Insel, 1989; Michel & Ernsberger, 1992) and of particular interest are the findings of Wikberg *et al.* (1991) who reported differences between the IGR site in smooth muscle (guinea-pig ileum) and in neuronal tissue (guinea-pig cortex). It thus remains to be determined whether ciclazindol is a ligand at the IGR site in smooth muscle.

Yablonsky *et al.* (1988) reported a rank order of potency for the binding of these agents to the IGR site in smooth muscle (for $K_i$ values see above) whereas, in the present study, all agents appeared to inhibit $I_{K(V)}$ and $I_{KCO}$ with approximate equipotency. The ability of agents such as phentolamine (which possesses a relatively low affinity for the IGR site) to inhibit K-channels equally as well as agents such as guanabenz (which is a high affinity ligand at the IGR site) does not appear consistent with the notion that K-channel inhibition occurs through the IGR site. However, the only common feature linking all the agents of the present study is their possession of either an imidazoline or a guanidine moiety and this remains the single, most convincing piece of evidence to suggest that the actions of these agents are mediated through an IGR site.

### 4.4.3. Are $I_{KCO}$ and $I_{K(V)}$ carried by the same channel ?

Beech and Bolton (1989) suggested that the K-channel openers are able to change the voltage-dependency of $K_V$ and to remove the ability of this channel to inactivate, a change which would markedly alter the gating properties of this channel. This view was based largely upon the finding that both currents possessed an identical rank-order of potency towards a number of relatively non-specific K-channel blockers. Earlier, Fink and Wettwer (1978) had reported that

metabolic exhaustion of skeletal muscle fibres increased the resting K-conductance and that this was due to a change in the properties of $K_V$ channels allowing them to open independently of membrane potential, ie. with a linear current / voltage relationship. The Na-channel activators, N-bromoacetamide and batrachotoxin are also believed to act by inducing a loss of channel inactivation (Hille, 1992). In addition, N-bromoacetamide is also known to remove the inactivation of the voltage-dependent K-current in GH3 cells under whole-cell voltage-clamp conditions (Matteson & Carmeliet, 1988). When considered collectively, the above data shows that modifications in the inactivation of certain voltage-dependent channels, including $K_V$, can lead to profound changes in the characteristics of these channels. These channels lose their ability to inactivate and assume a profile similar to that of voltage-independent channels.

The results of the present study demonstrate five agents (antazoline, cirazoline, clonidine, phentolamine and guanabenz) that are able to inhibit both $K_V$ and $K_{ATP}$. These five agents can be regarded as members of an increasing family of compounds, all of which are able to inhibit both $I_{K(V)}$ and the effects of the K-channel openers. This group of agents includes 4-AP, cibenzoline, ciclazindol, clofilium, dofetilide, phencyclidine, quinidine and tedisamil (Beech & Bolton, 1989; Bray & Quast, 1992; Noack *et al.*, 1992c; Pfründer & Kreye, 1992; Yang *et al.*, 1992; Sakuta *et al.*, 1993) and thus the common pharmacology of these K-channels is becoming increasingly recognised. These data are therefore consistent with the view that both $I_{KCO}$ and $I_{K(V)}$ could be carried by the same channel and that the K-channel openers could cause conversion from the voltage-dependent state ($K_V$) to the voltage-independent state currently known as $K_{ATP}$. Indeed, the ability of nicorandil to shorten the cardiac action potential is inhibited by dofetilide, the delayed rectifier channel blocker in cardiac tissue (Yang *et al.*, 1992). Thus, the imidazolines and guanidines may in reality only block one channel which passes two apparently different currents under different conditions.

Such a possibility could explain the ability of the K-channel openers

simultaneously to induce $I_{KCO}$ and to inhibit the delayed rectifier current, $I_{K(V)}$. The potential of the K-channel openers to cause dephosphorylation is discussed in Chapter 3 and has also been suggested by other authors (Noack *et al.*, 1992d; Kozlowski *et al.*, 1989). The ability of the phosphatase inhibitor, okadaic acid to decrease both the induction of $I_{KCO}$ and the inhibition of $I_{K(V)}$ may indicate that it is through a reduction in phosphorylation of the $K_V$ state that the $K_{ATP}$ state is obtained. It may be, therefore, that a K-channel opener-mediated alteration in channel phosphorylation state causes conversion from the $K_V$ state to the $K_{ATP}$ state of the channel. This 'conversion hypothesis', however, remains controversial and further experiments are clearly required to substantiate this view. These ideas are discussed further in Chapter 5.

Guanethidine did not inhibit $I_{K(V)}$ but this agent was able to reverse the generation of $I_{KCO}$. If the 'conversion hypothesis' is correct, it may be that guanethidine can only bind to $K_V$ when this is in the $K_{ATP}$ conformation. Clonidine (30μM) reduced $I_{K(V)}$ and $I_{KCO}$ but, at relatively lower concentrations, $I_{K(V)}$ was inhibited whereas $I_{KCO}$ was not affected. This could indicate a relatively high affinity of clonidine for $I_{K(V)}$ while still allowing some conversion of $K_V$ into $K_{ATP}$. Further experiments are clearly required to establish how agents such as clonidine inhibit $I_{K(V)}$. Differential inhibition of $K_V$ and $K_{ATP}$ is also possible with glibenclamide (see Chapter 3) and the possible reasons for this are further discussed in Chapter 5.

It is unclear from these data whether the IGR site is part of a K-channel or whether IGR site modulation of K-channel function occurs via an intracellular second messenger. Despite this, the possibility remains that the IGR site in smooth muscle is involved in the modulation of a single K-channel but that this channel is capable of existing in two different forms. The present data is not consistent with the rank-order of potency for these agents binding to the IGR site in smooth muscle (Yablonsky *et al.*, 1988). The differences in the affinity of ligands for the IGR site may thus indicate that particular agents will only bind to the IGR site when it exists in a certain conformation as could be the case with

guanethidine, whilst other agents such as phentolamine are able to bind to this site despite its conformation.

### 4.4.4. Mechanism of inhibition of K-currents by phentolamine

To investigate the mechanism by which IGR site ligands are able to inhibit $I_{K(V)}$ and $I_{KCO}$, the action of phentolamine was examined in the presence of okadaic acid, an inhibitor of protein phosphatase activity. Okadaic acid was chosen because a potential method of inhibition of these channels by IGR site ligands is through dephosphorylation. By inhibiting the natural dephosphorylation of $K_{ATP}$ and $K_V$ (via inhibition of phosphatase activity) it might be possible to decrease the ability of the imidazoline- / guanidine-based ligands to inhibit $I_{K(V)}$ and $I_{KCO}$.

In the presence of okadaic acid, both the induction of $I_{KCO}$ and the inhibition of $I_{K(V)}$ by levcromakalim were decreased as discussed in Chapter 3. Subsequent addition of phentolamine markedly reduced $I_{K(V)}$, and thus the ability of phentolamine to inhibit $I_{K(V)}$ appears unaffected by the inclusion of okadaic acid. Reversal of $I_{KCO}$ by phentolamine, however was affected by phosphatase inhibition. $I_{KCO}$ was inhibited by phentolamine between -70mV and +10mV, however, at potentials positive to +10mV the current level remained markedly elevated. The finding that $I_{KCO}$, over the negative potential range, was reduced by phentolamine suggests that in the presence of okadaic acid, phentolamine is still able to inhibit this current. It thus appears that the ability of phentolamine to inhibit $I_{K(V)}$ and $I_{KCO}$ is unaffected by the inhibition of phosphatase activity and therefore suggests that dephosphorylation does not play a major role in the mechanism of action of phentolamine.

The observation that, at potentials positive to +10mV, the total non-inactivating current remained elevated, following inhibition of $I_{KCO}$ by phentolamine, remains unclear. The pronounced outward rectification of this current suggests the activation of a voltage-dependent channel, possibly $BK_{Ca}$. This however remains unclarified and further experiments are required to identify

the mechanism by which agents such as phentolamine are able to inhibit K-currents.

### 4.4.5. General discussion

The data obtained in the present study collectively suggest that modulation of K-channel function may arise as a result of activation of the IGR site. All the imidazoline- / guanidine-based ligands tested in the present study were capable of modulation of K-channel function. Definitive proof of the involvement of K-channels in the physiological role of the IGR site awaits the development of selective and specific agents targeted at this site.

Further the ability of five out of the six ligands tested to inhibit both $I_{KCO}$ and $I_{K(V)}$ provides further support for the view that $I_{K(V)}$ and $I_{KCO}$ are actually carried by the same underlying channel which can exist in either voltage-dependent ($K_V$) or voltage-independent ($K_{ATP}$) states.

# Chapter 5:

# General discussion

## 5.1. The action of guanabenz and idazoxan on the rat aorta

The data presented in Chapter 2 suggest that the vasorelaxant properties of guanabenz arise from its ability to antagonise the effects of noradrenaline. Two anomalies from this chapter may be explained using the information obtained in Chapters 3 and 4.

Firstly, the guanabenz-induced leftward shift in the dose-response curve to KCl in the rat aorta (Figure 2.18) may arise from the ability of guanabenz to block K-channels. In Chapter 4, guanabenz was shown to inhibit both the delayed rectifier channel, $I_{K(V)}$ and the Induction of $I_{KCO}$. Blockade of K-channels may not directly cause contraction of the aorta, but could render this tissue more susceptible to a depolarising stimulus by shifting the resting membrane potential closer to the threshold potential for the opening of voltage-operated calcium channels (VOCCs). If this is the case then lower concentrations of KCl would be required in the presence of guanabenz to cause the depolarisation required to open the VOCCs and this would be observed as a leftward shift in the dose-response curve to KCl. Alternatively, the leftward shift in the KCl dose-response curve caused by guanabenz may result from the activation of excitatory post-junctional $\alpha_2$-adrenoceptors again moving the resting membrane potential closer to the threshold potential for the opening of VOCCs. It thus seems possible that both the blockade of K-channels and the activation of the post-junctional $\alpha_2$-adrenoceptors could contribute to the increase in tissue sensitivity to KCl.

It is also possible to suggest two putative mechanisms that may underlie the ability of idazoxan to induce phasic contractile activity in the rat aorta. Idazoxan is an imidazoline-based $\alpha_2$-adrenoceptor antagonist and based upon this property it would not be expected to possess any spasmogenic effects. Okumura *et al.* (1992) reported that idazoxan also increased the spontaneous myogenic activity of rat portal vein, a feature common to other imidazoline / guanidine derivatives (see Chapter 4). The data presented in Chapter 4 indicates that imidazoline- / guanidine-based agents are able to inhibit K-channels. This feature of imidazoline compounds may therefore extend to

include idazoxan and thus the contractile effects of this agent in rat aorta may be attributed to the closure of K-channels. As outlined above, K-channel blockade could exert a depolarising influence on the tissue and therefore cause contraction as was observed with TEA.

An alternative mechanism for the spasmogenic effect of idazoxan may be suggested from the work of Satake *et al.* (1984). These authors reported that phentolamine induced phasic, contractile activity in guinea-pig detrusor muscle. These phasic contractions were blocked by cyclo-oxygenase inhibitors and thus these authors suggested that this ability of phentolamine was due to the production of arachidonic acid metabolites, possibly prostaglandins. In these experiments, the spontaneous rhythmic contractions normally associated with guinea-pig detrusor muscle were prevented by maintaining the tissue at 32°C. Considering the structural homology between idazoxan and phentolamine (both are imidazoline-based adrenoceptor antagonists, see Table 1.2 and Figure 4.1 for structures) it is possible that the phasic contractions evoked by idazoxan in rat aorta are also due to the production of arachidonic acid metabolites. However, the ability of phentolamine to induce this phasic activity was not shared by another imidazoline derivative, clonidine, and thus this does not appear to be a property common to all imidazoline-based compounds.

The ability of idazoxan to induce rhythmic contractile activity in rat aorta therefore may be due either to production of arachidonic acid metabolites or to K-channel closure. Alternatively, a different mechanism may underlie this contractile activity and further experiments are clearly required to determine the nature of this response.

## 5.2. Do $K_V$ and $K_{ATP}$ exist as a single channel ?

The ability of the five imidazoline- / guanidine-based agents to inhibit both the induction of $I_{KCO}$ by levcromakalim and the delayed rectifier current, $I_{K(V)}$, as demonstrated in Chapter 4, raises the possibility that the channels underlying these two currents may be functionally linked. This was first suggested by Beech and Bolton (1989) who proposed that the K-channel openers act by interfering with the inactivation gate of the delayed rectifier channel and initiate its conversion into a channel carrying a non-inactivating current. In addition to the ability of phentolamine, cirazoline, clonidine, antazoline and guanabenz all to inhibit both $I_{KCO}$ and $I_{K(V)}$, further evidence in accordance with this view can be obtained from Chapter 3.

The ability of the K-channel openers simultaneously to induce $I_{KCO}$ and inhibit $I_{K(V)}$ is consistent with the theory that these agents work by converting the channel underlying $I_{K(V)}$ ($K_V$) into that channel which underlies $I_{KCO}$ ($K_{ATP}$).

The observation that in the presence of either a raised intracellular ATP or the phosphatase inhibitor, okadaic acid, the induction of $I_{KCO}$ *and* the inhibition of $I_{K(V)}$ by levcromakalim are reduced suggests that a conversion from $K_V$ to $K_{ATP}$ could occur via dephosphorylation of the $K_V$ channel. By raising intracellular ATP, the probability of phosphorylation will be increased (because of the increase in available ATP as fuel) and the dynamic balance between the rate of phosphorylation and dephosphorylation will shift in favour of phosphorylation. By addition of okadaic acid, the natural rate of dephosphorylation of $K_V$ will be reduced and therefore the potential for dephosphorylation in the dynamic phosphorylation / dephosphorylation balance will be decreased. In both of these situations therefore, the likelihood of dephosphorylation of $K_V$ will be reduced relative to the potential for phosphorylation. The possibility that the K-channel openers could cause dephosphorylation of both $K_{ATP}$ and $K_V$ is suggested in Chapter 3 and has also been speculated by other workers (Kozlowski *et al.*, 1989; Noack *et al.*, 1992d). These data are therefore consistent with the view that levcromakalim may cause conversion from $K_V$ to $K_{ATP}$ via dephosphorylation. Furthermore, Edwards *et al.* (1993) have reported that the effects of

levcromakalim can be mimicked by procedures designed to reduce channel phosphorylation, both by inhibition of kinase activity and by dephosphorylating agents such as butanedione monoxime.

Evidence already in the literature also indicates that modification of $K_V$ represents a feasible mechanism of action of the K-channel openers. The Na-channel activator, N-bromoacetamide to is able to remove the ability of voltage-dependent K-channels to inactivate (Matteson & Carmeliet, 1988) in a manner similar to its actions on the voltage-dependent Na-channel. Furthermore, upon metabolic exhaustion of skeletal muscle fibres, voltage-dependent currents lose the ability to inactivate (Fink & Wettwer, 1978). These reports further strengthen the supposition that under dephosphorylating conditions (eg. metabolic exhaustion and a presumed decrease in ATP) the ability of voltage-dependent K-channels to inactivate is removed and that these will then adopt the profile of voltage-independent channels.

The conductance of the channel underlying the action of the K-channel openers in the present study was calculated to be 10.5pS at 0mV under quasi-physiological K-conditions. This correlates well with the calculations of other authors for the conductance of this channel (Noack *et al.*, 1992a; Kajioka *et al.*, 1990; Langton *et al.*, 1993). The delayed rectifier channel, $K_V$, is also a low conductance channel with reports of the unitary conductance values varying between 12.7pS (Boyle *et al.*, 1992) and 5pS (Beech & Bolton, 1989). Thus it appears that both $I_{K(V)}$ and $I_{KCO}$ are carried by channels with similar unitary conductances. These findings are therefore also consistent with the view that $K_{ATP}$ may be a modified form of the $K_V$ channel.

Evidence which is apparently not consistent with the $K_V$-$K_{ATP}$ conversion hypothesis is the lack of inhibitory effect of glibenclamide on the delayed rectifier current. In the experiments presented in Chapter 3, $I_{KCO}$ generated by the K-channel openers was fully inhibited by glibenclamide whereas the accompanying inhibition of $I_{K(V)}$ was only partially reversed. This may be explained by the ability of glibenclamide to increase intracellular ATP levels via an increase in glycolysis (Caro, 1990 see section 3.4.7). Such an increase in

intracellular ATP could immediately close the $K_{ATP}$ form of the channel via binding to the channel inhibitory site (a mechanism not requiring hydrolysis of ATP - see Edwards & Weston, 1993). However, the rephosphorylation back to the $K_V$ form via the raised intracellular ATP (and requiring ATP hydrolysis) could be a slower process. It is therefore possible that upon prolonged exposure to glibenclamide a full reversal of the K-channel opener-induced inhibition of $I_{K(V)}$ would be observed.

Guanethidine inhibited the induction of $I_{KCO}$ by levcromakalim but it was without effect on the delayed rectifier channel. Thus, guanethidine possessed a slightly different profile to the other IGR site ligands tested, all of which inhibited both $I_{KCO}$ and $I_{K(V)}$. As speculated in Chapter 4, this may arise from the ability of guanethidine only to inhibit the $K_{ATP}$ form of the channel and that in the fully phosphorylated state ($K_V$), guanethidine is unable to inhibit the channel.

In general, therefore, the evidence presented in Chapters 3 and 4 is consistent with the view that the K-channel openers cause dephosphorylation of the delayed rectifier channel and thus convert it into a channel that will open independently of membrane potential. However, it is also possible that the $K_{ATP}$ channel does exist as an entity distinct from $K_V$ and that the K-channel openers are able to modulate the two channels by a similar mechanism. A possible direct displacement (by the K-channel openers) of the ATP that maintains the $K_{ATP}$ channel closed under normal conditions would cause this channel to open (Thuringer & Escande, 1989). In addition, interaction of the K-channel opener with an ATP binding site on, say the kinase responsible for phosphorylating $K_V$ would reduce the phosphorylation of $K_V$ and thus induce its closure (Noack *et al.*, 1992d). By this mechanism, the K-channel openers could 'open' $K_{ATP}$ and simultaneously inhibit $K_V$.

The mechanism of action of the K-channel openers therefore remains a point of controversy. It appears that $I_{KCO}$ is carried by an ATP-dependent channel but whether this exists as a substate of the delayed rectifier channel clearly requires further investigation. A definitive demonstration of the mechanisms underlying the induction of $I_{KCO}$ by the K-channel openers will

inevitably come from techniques employing a molecular approach to this problem.

If $K_{ATP}$ does exist as a separate entity from $K_V$ then the cloning and full sequencing of the $K_{ATP}$ channel protein should be possible. To date only a single ATP-sensitive K-channel has been cloned (Ho *et al.*, 1993) but this does not possess the pharmacological characteristics consistent with the channel activated by levcromakalim. The cloning of a glibenclamide- and levcromakalim-sensitive K-channel with an amino-acid sequence that differs from that of the delayed rectifier channel would thus indicate that the $K_{ATP}$ channel and $K_V$ channels exist as separate entities.

Conversely, if $K_{ATP}$ does exist as a partially dephosphorylated form of $K_V$ then it should be possible to generate $I_{KCO}$ currents in cells which contain only delayed rectifier channels. Expression of only delayed rectifier channels, in cell lines, and the subsequent analysis of the effects of the K-channel openers on these cells could thus indicate whether these agents are able to modify the gating of voltage-dependent channels.

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