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Functional Cell Biology and Molecular Pharmacology of ATP Sensitive Potassium Channels

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A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Master of Philosophy.

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Abbreviations

ABC	ATP-binding cassette
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CR(cl)	concentration ratio with 95 % confidence limits
CRC	concentration response curve
EC_{50}	concentration of agent required to produce a 50% relaxation
E_{max}	maximum relaxation response
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IC_{50}	concentration of agent required to produce a 50% inhibition
K^+	potassium ion
K_{ATP}	ATP sensitive potassium channel
K_{ATP}COs	ATP sensitive potassium channel openers
KCO1 or KCO2	potassium channel opener binding sites
Kir	inwardly rectifying potassium channel

L-MMA	N^G-monomethyl-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NIO	L-N^5-(1-iminoethyl)ornithine
L-NNA	N^G-nitro-L-arginine
mRNA	messenger ribonucleic acid
MgADP	magnesium bound nucleotide
NBD	nucleotide binding domain
NO	nitric oxide
NOS	nitric oxide synthase
P1075	pinacidil analogue
PE	phenylephrine
PHHI	persistent hyperinsulinaemic hypoglycemia of infancy
Rb	rubidium
SDS	sodium dodecylsulphate
SUR	sulphonylurea receptor
TMD	transmembrane domain
UDP	uridine diphosphate

Abstract

Adenosine triphosphate (ATP) sensitive potassium channels (K_{ATP}) transduce changes in cellular metabolism into changes in membrane potential. Activation of K_{ATP} channels causes vascular smooth muscle to hyperpolarize. This leads to a relaxation of the pre-contracted muscle. Inhibition of K_{ATP} channels produces membrane depolarisation and reduces the ability of the vascular smooth muscle to hyperpolarize and thereby relax.

K_{ATP} channels are inwardly rectifying potassium channels (Kir), which are inhibited by ATP and stimulated by magnesium bound nucleotides. The K_{ATP} channel is an octomeric combination of two different protein subunits. The pore is formed by four Kir6.2 subunits, each of which is associated with a regulatory sulphonylurea receptor (SUR) subunit. K_{ATP} channels are found in a diverse range of tissue and are composed of different Kir and SUR subunits. In smooth muscle, Kir6.2 is the pore forming subunit and SUR2B is the associated regulatory subunit.

A structurally diverse group of agents called K_{ATP} channel openers (K_{ATP}COs), have affinity for the SUR of K_{ATP} channels, causing a hyperpolarisation of the cell membrane and a decrease in excitability. K_{ATP} channel openers have a host of potential therapeutic targets, which range from urinary incontinence to obesity.

The K_{ATP}COs used in this study can be placed into two categories. The K_{ATP}COs typified by pinacidil are sensitive to L-arginine analogues whereas the K_{ATP}COs typified by cromakalim are insensitive to L-arginine analogues. This sensitivity appears to be independent of nitric oxide synthase (NOS) action as the vasorelaxant and Rb efflux responses to pinacidil are insensitive to the NOS inhibitor L-N^5-(1-iminoethyl) ornithine (L-NIO).

Pinacidil and cromakalim are believed to have a degree of commonality in how they interact with the K_{ATP}CO binding site on SUR2B. Some parts of pinacidil though are believed to interact with the area of the K_{ATP}CO binding site that recognises L-arginine analogues. In contrast, cromakalim is not believed to interact with this particular region of the K_{ATP}CO binding site, which explains the insensitivity of cromakalim to L-NAME.

1 Introduction

Potassium channels are a diverse group of ion channels, which have a crucial role in the control of cell excitability. Cells in a resting state have an intracellular potassium ion (K^+) concentration that is twenty five times higher than the external K^+ concentration. When a potassium channel opens, an efflux of positive charge and an outward current results. The efflux of K^+ is a mechanism to recover, maintain or enhance the resting membrane potential. The opening of K^+ channels enables the membrane potential of the cell to move towards the equilibrium potential for potassium. The equilibrium potential for potassium can be defined as the membrane potential produced when the concentration of potassium ions across the membrane are in equilibrium.

1.1 Adenosine Triphosphate Sensitive Potassium Channels

Adenosine triphosphate (ATP) sensitive potassium channels (K_{ATP}) are inwardly rectifying potassium channels (Kir), which are inhibited by ATP and stimulated by magnesium bound nucleotides, such as MgATP, adenosine diphosphate (MgADP), guanosine triphosphate (MgGTP), guanosine diphosphate (MgGDP) and uridine diphosphate (MgUDP). Studies have shown that K_{ATP} channels are octomeric combinations of two distinct types of protein subunit. The pore is formed of four Kir6.1 or Kir6.2 subunits, each of which is associated with a regulatory sulphonylurea receptor (SUR) subunit. Kir6.1 and Kir6.2 have two transmembrane domains connected by a pore loop, and belong to the Kir superfamily (Figure 1) (Ashcroft and Gribble, 2000b; Clement *et al.*, 1997; Shyng and Nichols, 1997; Inagaki *et al.*, 1995). Kir channels were first recognised in 1949. At present, seven subfamilies of Kir exist and are designated as Kir1.0 to Kir7.0 (Doupnik *et al.*, 1995). Functional expression of the Kir family showed that members exhibit either strong or weak voltage dependent rectification. K_{ATP} channels are weak rectifiers. Kir channels are essential in the control of resting membrane potential, coupling cellular metabolism to cellular excitability.

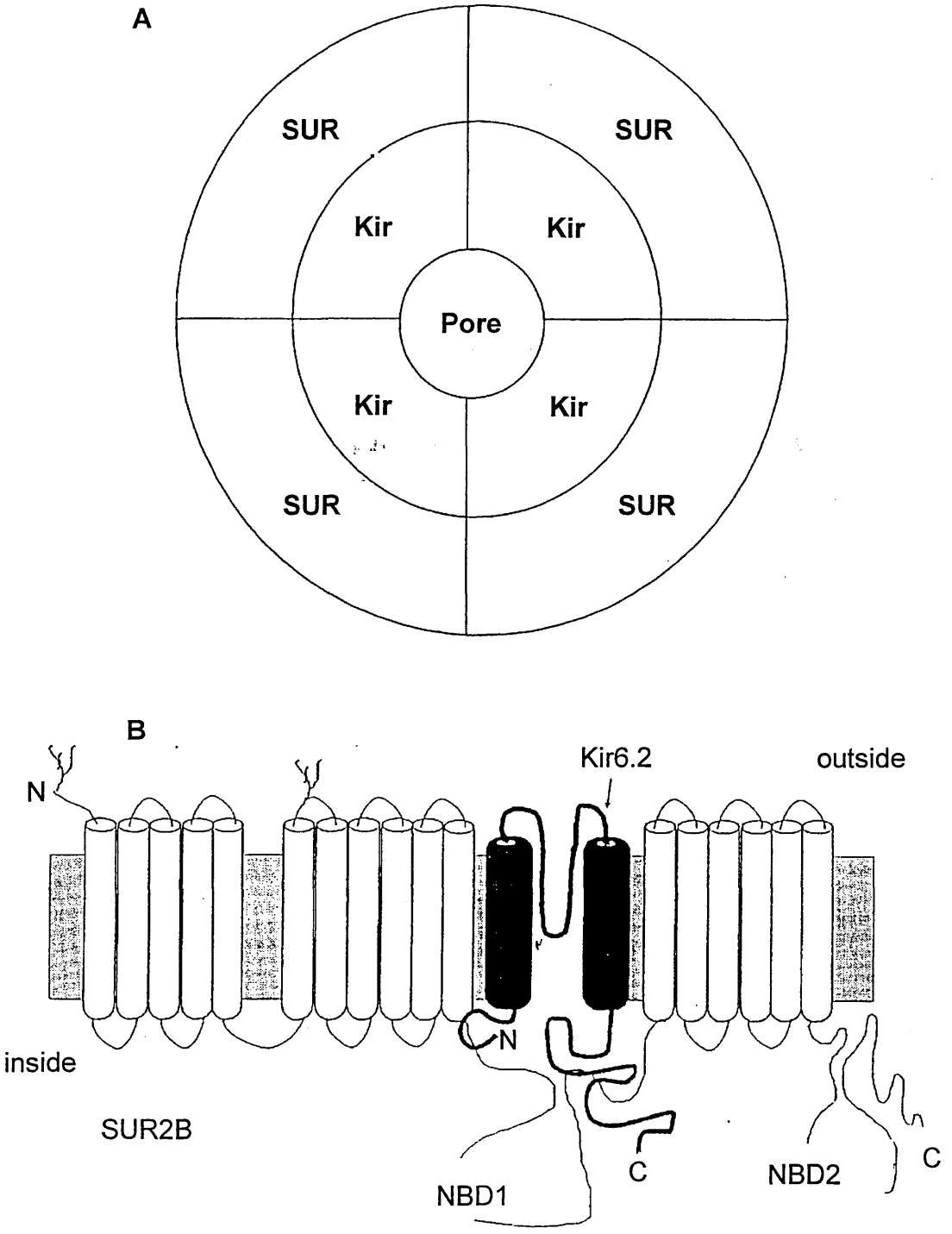


Figure 1. The structure of a K_{ATP} channel. **A** shows the pore surrounded by four inwardly rectifying potassium channel (Kir) subunits and four sulphonylurea receptor (SUR) subunits from a vertical perspective. **B** shows a Kir subunit surrounded by a SUR subunit. In addition, two nucleotide binding domains (NBDs) are also visible.

The second subunit is referred to as the SUR because the purification and the cloning of the founding member of this family was based on its affinity for the sulphonylurea, glibenclamide (Aguilar-Bryan *et al.*, 1995). SURs are members of the ATP-binding cassette (ABC) or transport ATPase superfamily. SURs have seventeen, highly hydrophobic, transmembrane domains (TMDs) that are arranged in three groups, two groups of six and one group of five TMDs. The SUR has two intracellular loops that contain consensus sequences, which are required for nucleotide binding and hydrolysis. These sequences are called the nucleotide binding domains (NBDs) (Tusnady *et al.*, 1997; Aguilar-Bryan *et al.*, 1995). Within each NBD is a conserved region of around 200 amino acids consisting of a Walker A motif, a Walker B motif and a conserved linker sequence. A conserved lysine residue in the Walker A motif is required for ATP hydrolysis and an aspartate residue in the Walker B motif is involved in coordinating the magnesium ion of the magnesium nucleotides. The Walker A and B motifs are separated by the ABC signature motif. The signature motif distinguishes ABC transporters from other nucleotide triphosphate binding proteins, such as kinases, which also contain the Walker sequences (Theodoulou, 2000).

Endoplasmic reticulum retention signals are masked during the assembly of the SUR/Kir complex, which results in only fully assembled channels being able to reach the plasma membrane. An -RKQ- (-Arginine, Lysine, Glutamine-) motif can function to keep incompletely assembled K_{ATP} channels from reaching the cell membrane. Changing the motif to -AAA- (-Alanine, Alanine, Alanine-) allowed the surface expression of either the SUR or the Kir in the absence of their partner subunit. This shows that control is maintained over the assembly of K_{ATP} channels (Zerangue *et al.*, 1999).

1.2 Types of SUR and K_{ATP} Channel Location

K_{ATP} channels in different tissues have a pre-dominance of certain combinations of Kir and SUR subunits. Two different genes encoding SUR1 and SUR2 have

been identified and further diversity is created by alternative splicing of SUR2 mRNA (Chutkow *et al.*, 1996; Inagaki *et al.*, 1996; Isomoto *et al.*, 1996). SUR1 and SUR2 have a 67% sequence homology. A splice site in SUR2, distal to the Walker A and B motifs in NBD2, alters the C-terminal 42 amino acids forming splice variants which are known as SUR2A and SUR2B. SUR2A and SUR2B have a 98% sequence homology. The 42 amino acid segment of SUR plays a critical role in the differential activation of K_{ATP} channels by the potassium channel openers diazoxide, pinacidil, cromakalim and nicorandil (Matsuoka *et al.*, 2000). Although Kir6.1 or Kir6.2 coassemble with different SUR isoforms to form heteromultimeric K_{ATP} channels, it is not believed that Kir6.1 or Kir6.2 heteromultimerise with each other (Seharaseyon *et al.*, 2000).

1.3 ATP Sensitive Potassium Channel Openers (K_{ATP}COs)

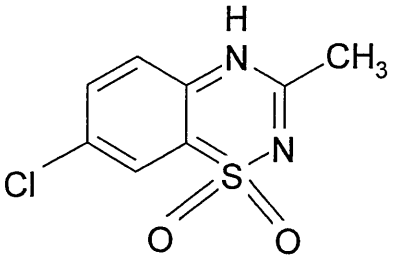
The term "potassium channel openers" was introduced to describe a group of novel synthetic molecules, which were typified by cromakalim that act by stimulation of ion flux through K^+ channels (Hamilton and Weston, 1989; Hamilton *et al.*, 1986). Hamilton *et al.* (1986) reported that cromakalim evoked smooth muscle relaxant effects by the opening of K^+ channels in cell membranes. K_{ATP} channel opening properties have now been demonstrated in a diverse range of synthetic chemical structures and endogenous substances.

The opening of K_{ATP} channels by K_{ATP}COs causes hyperpolarisation of the cell membrane and a pharmacological means of decreasing the excitability of cells. This is thought to have potential in the treatment of diseases such as hypertension, asthma, angina and urinary incontinence (Lawson, 1996). K_{ATP}COs such as pinacidil have shown that they can reduce elevated blood pressure in patients. The mechanism of this effect is an actively mediated decrease in peripheral resistance. The beneficial antihypertensive effects of pinacidil are also accompanied with the adverse effect of a weight gain in approximately 30% of patients (Carlsen *et al.*, 1983).

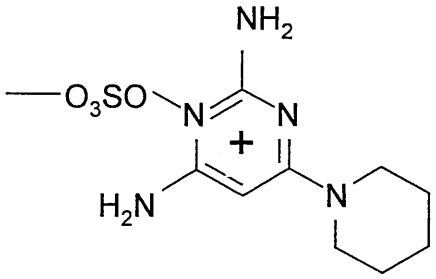
Classification of K_{ATP}COs has been defined as a consequence of their ability to stimulate K^+ flux through the K_{ATP} channel and their sensitivity to blockade by sulphonylureas. The sulphonylurea group of drugs was developed as a result of a chance observation that a sulphonamide derivative (used to treat typhoid) resulted in a marked lowering of blood glucose (Rang *et al.*, 1999). The principal action of sulphonylureas is on the K_{ATP} channels in β cells of the islets of Langerhans. The sulphonylureas reduce the potassium permeability of β cells by blocking K_{ATP} channels. This in turn causes membrane depolarisation, Ca^{2+} entry into the cell and insulin secretion. Sulphonylureas, such as glibenclamide and tolbutamide, have been used to treat non-insulin dependent diabetes mellitus (NIDDM) since 1955, but can stimulate appetite and cause weight gain (Rang *et al.*, 1999, Gopalakrishnan *et al.*, 1993, Ashcroft and Ashcroft, 1990).

K_{ATP} channel opening properties have been demonstrated in a diverse range of synthetic chemical structures (benzothiadiazines [*e.g.*, diazoxide], pyrimidines [*e.g.*, minoxidil], pyridylcyanoguanidines [*e.g.*, pinacidil], nicotinamides [*e.g.*, nicorandil], benzopyrans [*e.g.*, cromakalim] and carbothiamides [*e.g.*, RP 49356]) (Figure 2) (Edwards and Weston, 1990: 1993). Common chemical structural features between benzopyrans, pyridylcyanoguanidines and carbothiamides have been described. Atwal (1992) believed that there was a requirement for the K_{ATP}COs to have a hydrophobic group, an electron deficient aromatic ring and a hydrogen bonding site. This suggests that all K_{ATP}COs may interact with their site(s) of action in the same way as each other. An alternative pharmacophore model of the K_{ATP}COs shows four common regions (Koga *et al.*, 1993). Two of the regions represent areas of lipophilic interaction and the other two are hydrogen bonding regions.

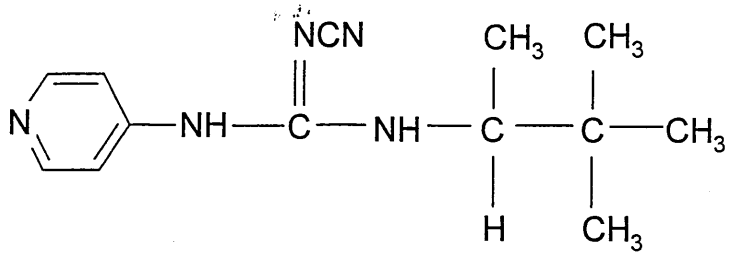
In contrast to the suggestion that all K_{ATP}COs interact with the K_{ATP} channel in the same way, it is known that the K_{ATP}COs have heterogeneous pharmacologies For example, pinacidil, cromakalim and nicorandil open K_{ATP} channels in vascular smooth muscle and cardiac cells, but have no effect on pancreatic β cells.



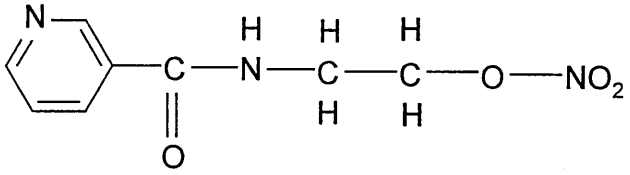
Diazoxide



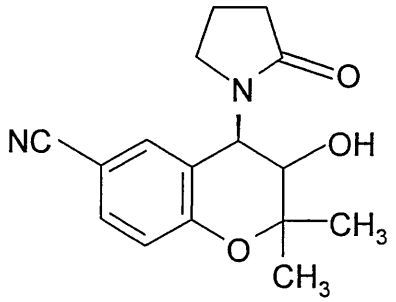
Minoxidil sulphate



Pinacidil



Nicorandil



Cromakalim

Figure 2. Structure of K_{ATP}COs.

Diazoxide can open K_{ATP} channels in pancreatic β cells and vascular smooth muscle, but fails to open K_{ATP} channels in cardiac cells (Inagaki *et al*, 1995: 1996; Isomoto *et al.*, 1996). This suggests that the pharmacology of the K_{ATP} channels as characterised by the K_{ATP}COs is tissue dependent and indicative of multiple subtypes (Lawson, 2000).

1.4 Effect of Different Subunit Combinations

The specific properties of K_{ATP} channels are determined by the SUR and Kir subunit composition (Figure 3). The combination of subunits SUR1 and Kir6.2 form the pancreatic β cell type of K_{ATP} channel (Aguilar-Bryan *et al.*, 1995). The combination controls insulin secretion by regulating the β cell membrane potential. SUR1/Kir6.2 is activated by diazoxide (EC_{50} 20 - 100 μM), but is insensitive to pinacidil, nicorandil or cromakalim. SUR1/Kir6.2 is sensitive to both glibenclamide and ATP (IC_{50} around 1 nM and 10 μM respectively, Inagaki *et al.*, 1995). Mutations in either of the subunits causes loss of β cell K_{ATP} channel activity which causes the condition, familial hyperinsulinism, of which the most common form is Persistent Hyperinsulinaemic Hypoglycaemia of Infancy (PHHI) (Aynsley-Green *et al.*, 2000). Cloning and expression of only SUR1, without the Kir subunit, demonstrated that SUR1 was sufficient to cause high affinity sulphonylurea binding, but not channel activity (Aguilar-Bryan *et al.*, 1995).

In cardiac and skeletal muscle, the SUR2A/Kir6.2 complex forms the K_{ATP} channel. The SUR2A/Kir6.2 combination has been shown to be insensitive to diazoxide, but is activated by pinacadil (EC_{50} 65 μM), nicorandil (EC_{50} 100 - 300 μM) and cromakalim (EC_{50} 30 - 100 μM). SUR2A/Kir6.2 is sensitive to both glibenclamide and ATP (IC_{50} 6 nM and 100 μM respectively) (Inagaki *et al.*, 1996; Findlay, 1992).

In vascular smooth muscle, the SUR2B/Kir6.2 complex forms the K_{ATP} channel. The SUR2B/Kir6.2 combination is activated by pinacidil (EC_{50} 0.6 μM), nicorandil (EC_{50} 10 μM), cromakalim (EC_{50} 2 μM) and diazoxide (EC_{50} 37 μM).

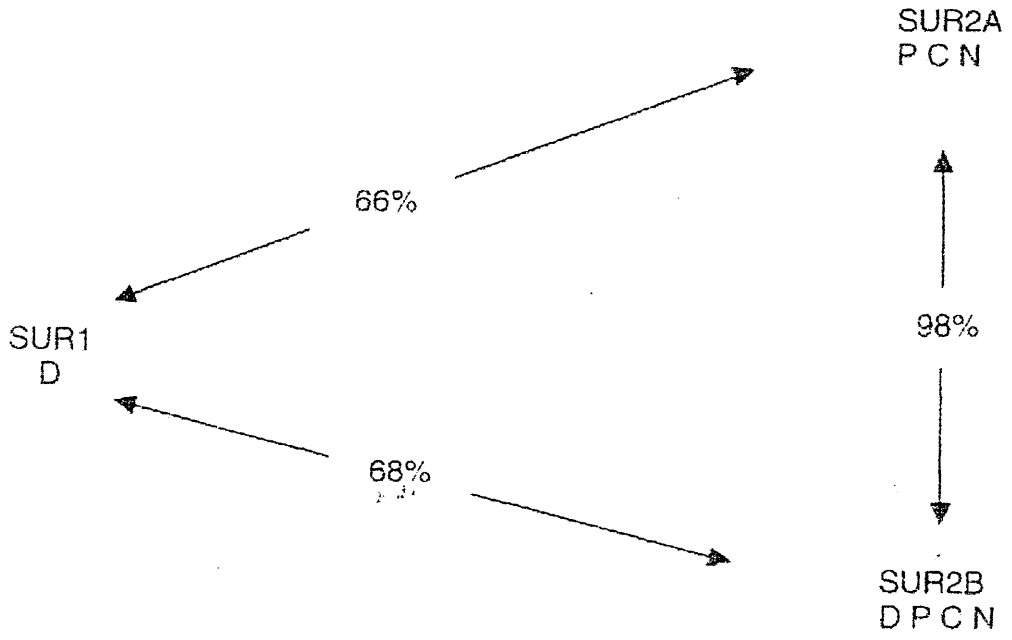


Figure 3. A summarised K_{ATP}CO pharmacology of the sulphonylurea receptor (SUR) subtypes. Activation of the different SUR subtypes is indicated by D, P, C and N for diazoxide, pinacidil, cromakalim and nicorandil, respectively. Percentage values represent the identity levels of the different SUR isoforms (Lawson, 2000).

SUR2B/Kir6.2 is sensitive to both glibenclamide and ATP (IC_{50} 100 nM and 53 μM respectively) (Wellman and Quayle, 1997; Inagaki *et al.*, 1996; Isomoto *et al.*, 1996).

1.5 The K_{ATP}CO Binding Site

K_{ATP}COs have been shown to interact with the SUR subunit of the K_{ATP} channel. For example, the pinacidil analogue [^{3}H] P1075 can bind to cells that express SUR2B in the absence of Kir6.2 (Schwanstecher *et al.*, 1998; Quast *et al.*, 1993). In addition, Kir6.2 is not activated by K_{ATP}COs when expressed in the absence of SUR but becomes activated when co-expressed with the SUR (John *et al.*, 1998).

Uhde *et al.* (1999) examined the diversity of the SUR subunits of K_{ATP} channels. By exchanging domains between SUR1 and SUR2B, and using [^{3}H] P1075, two regions in the second set of transmembrane domains were identified as being crucial for K_{ATP}CO binding. The regions identified as being crucial for K_{ATP}CO binding can be found in the intracellular loop connecting transmembrane domains 13 and 14 (Thr1059-Leu1087) and the domain preceding NBD2 (Arg1218-Asn1320) (Figure 4). It is believed that these regions interact in the formation of the K_{ATP}CO binding pocket. Nevertheless, as TMDs 1-13 are required for full K_{ATP}CO affinity, additional regions may also be involved (Uhde *et al.*, 1999).

Babenko *et al.* (2000) showed that two separate regions in SUR are necessary to determine the selective effects of diazoxide versus cromakalim or pinacidil. TMDs 6-11 and NBD1 of SUR1 were found to control responsiveness to diazoxide, whereas TMDs 12-17 of SUR2 confers sensitivity to cromakalim and pinacidil in chimeric human SUR1-SUR2A/Kir6.2 K_{ATP} channels. These results complement the study by D'Hahan *et al.* (1999), which showed that the transfer of TMDs 12-17 from rat SUR2A into hamster SUR1 is sufficient to allow the cromakalim analogue SR47063 to activate the chimeric SUR/Kir6.2 channels.

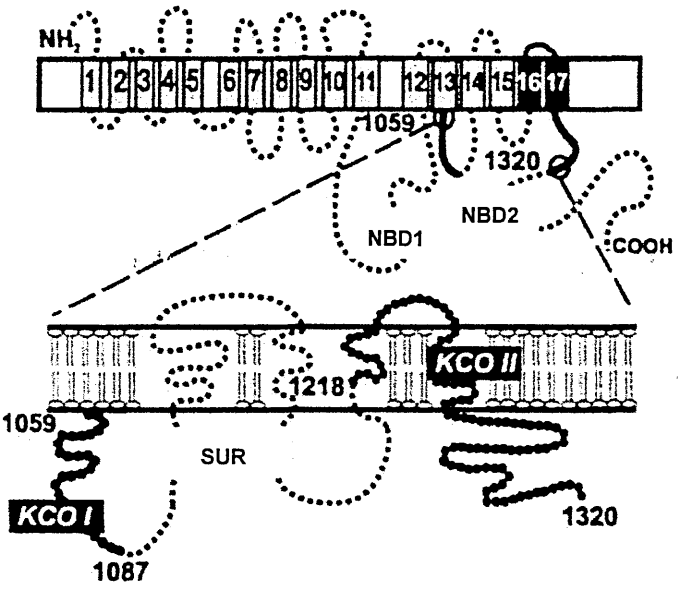


Figure 4. Putative transmembrane topologies of the regions essential for K_{ATP}CO (KCO I and II), nucleotide (NBD) and sulphonylurea binding (SUR). Adapted from Uhde *et al.*, (1999).

Babenko *et al.* (2000) also complements the observations made by Uhde *et al.* (1999) that smaller segments, Thr1059-Leu1087 (KCO1) and Arg1218-Asn1320 (KCO2) of rat SUR2 confer specific [^{3}H] P1075 binding when placed in a hamster SUR1 background (Figure 4). The results suggest that the TMDs 12-17 are essential in forming a K_{ATP}CO binding pocket. In addition, the Babenko *et al.* (2000) study shows that stimulation by diazoxide involves domains of SUR1 other than TMDs 12-17. The demonstration that stimulation by diazoxide involves domains of SUR1 other than TMDs 12-17 suggests that these domains either couple diazoxide binding at TMDs 12-17, or at another segment, to the Kir gating mechanism or form an additional K_{ATP}CO binding site. Consistent with such a coupling mechanism, diazoxide can displace [^{3}H] P1075 from SUR2B and [^{3}H] glibenclamide from SUR1 (Schwanstecher *et al.*, 1998). The results are compatible with a minimal model in which different classes of K_{ATP}COs, including diazoxide, occupy the same site in TMDs 12-17 and that this site in SUR1 is in close proximity to the sulphonylurea binding site (SUR). The proximity of these sites suggests that negative allosteric interactions may be occurring. Therefore multiple regions of SUR contribute to coupling K_{ATP}CO occupied sites with the Kir gating machinery. It is also suggested that K_{ATP}COs increase an ATPase activity of SUR and stabilise MgADP on NBD2 of SUR (Ueda *et al.*, 1999). Therefore K_{ATP}CO binding to TMD 12-17 and cooperative binding of ATP and MgADP to NBD1 and NBD2 respectively, may influence each other allosterically. This would explain the need for MgATP and intact NBDs for K_{ATP}CO binding and the modulation of this binding by MgADP (Babenko *et al.*, 2000; Hambrock *et al.*, 1999; Schwanstecher *et al.*, 1998).

Although 67% of the primary sequence of amino acids in SUR1 and SUR2A are identical, SUR1 and SUR2A possess around 300 non-matching amino acids throughout their sequences. The pancreatic isoform, SUR1 and the cardiac muscle isoform SUR2A along with the cromakalim analogue, SR47063 were used to establish which of the 300 non-matching amino acids between SUR1 and SUR2A are responsible for the observed differences in terms of potassium

channel opener pharmacology (Moreau *et al.*, 2000). The K_{ATP}COs, pinacidil, cromakalim and nicorandil act on SUR2 isoforms, but with the exception of diazoxide, the SUR1 isoform is insensitive to K_{ATP}COs. Matched chimeras incorporating progressively smaller fragments of SUR2A in a SUR1 background led to the finding that the phenotype of SUR2A could be carried over to SUR1 through single residue substitutions. The two SUR residues that were intimately involved in the activation of K_{ATP} channels by K_{ATP}COs were found to be located in the last TMD of SUR at positions 1249 and 1253 of SUR2A and at positions 1286 and 1290 of SUR1. The residues are Leucine and Threonine in opener-sensitive isoforms SUR2A and SUR2B and Threonine and Methionine in SUR1. SUR1 mutants with the two residues from SUR2A acquired responsiveness to SR47063. The transfer of the two residues from SUR1 into SUR2A did not completely remove the responses to SR47063, which suggests that other regions of SUR2A are also important in cromakalim binding.

The two residues are believed to form part of the binding site for K_{ATP}COs (Moreau *et al.*, 2000). The residues are one α helix turn away from each other and are therefore positioned adjacently on the same hydrophilic face of the last TMD17 (Moreau *et al.*, 2000). Three other residues in TMD17 differ between SUR1 and SUR2A, but helical wheel projections predict these three residues lie on the opposite face of the helix. The transfer of these other three residues into SUR2A from SUR1 or from SUR1 into SUR2A failed to modify the K_{ATP}CO phenotypes of SUR1 or SUR2A. As SUR2B is identical to SUR2A, apart from 42 amino acids at the C-terminal end (Isomoto *et al.*, 1996), it seems likely that opener affinity is modulated by direct interactions between the C-terminal end and the neighbouring TMD17 (Moreau *et al.*, 2000).

The stoichiometry of K_{ATP}CO action was examined by analysing the [^{3}H] P1075 sensitivity of channels coassembled from SUR1 and a chimeric construct derived from SUR1 and SUR2B, which had a high KCO affinity (Gross *et al.*, 1999). Each K_{ATP} channel has four SURs for the binding of sulphonylureas and K_{ATP}COs

(Figure 1). When the action of [^{3}H] P1075 is examined on the coassembled SUR1 and SUR2B channels, the occupation of one SUR subunit appears to be sufficient for channel activation. Concentration activation curves for complementary DNA (cDNA) ratios of 1:1 or 1:10 resembled those for channel opening resulting from interaction with a single site. Models for activation requiring the occupation of two, three or four sites did not correspond with the obtained data. Therefore, K_{ATP} channel activation appears to be mediated by the interaction of a single SUR subunit with a K_{ATP}CO (Gross *et al.*, 1999).

1.6 Function and Regulation of K_{ATP} Channels

The activity of K_{ATP} channels is affected by nucleotide levels, thus these channels link membrane conductance to metabolism (Cook & Hales, 1984). An example of the coupling of metabolism to electrical activity is pancreatic β cells. Under normal conditions, pancreatic β cell K_{ATP} channels stay open to maintain the membrane potential. When the blood glucose level increases, glucose is transported into the pancreatic β cell. The glucose is then metabolised, which increases the levels of ATP and decreases the concentration of MgADP in the pancreatic β cell. The increase in the ATP:ADP ratio closes the K_{ATP} channel, thereby depolarising the pancreatic β cell membrane. The depolarisation leads to the opening of Ca^{2+} channels, allowing Ca^{2+} influx into the β cells. The rise in the intracellular concentration of Ca^{2+} triggers insulin release (Misler *et al.*, 1986).

In vascular smooth muscle, which is the basis of this project, the opening of K_{ATP} channels by a K_{ATP}CO, leads to an increase in the negativity of the resting membrane potential (hyperpolarisation) toward the equilibrium potential for potassium together with an outward current of K^+ (Hamilton and Weston, 1989). This change in membrane potential is followed by a reduction in the levels of free intracellular Ca^{2+}. The hyperpolarisation caused by the K^+ efflux, closes the voltage activated Ca^{2+} channel, preventing Ca^{2+} from entering the cytosol, hence a reduction in the intracellular Ca^{2+} levels occur. At low calcium concentrations, very few of the calcium binding sites on troponin are occupied, and therefore

cross bridge activity is blocked by tropomyosin. Following the application of the α_1-adrenoceptor agonist, phenylephrine, there is a rapid increase in intracellular calcium levels, which allows calcium to bind to troponin, removing the blocking effect of tropomyosin, which allows cross-bridge cycling to occur. Other mechanisms may also contribute to the effects of K_{ATP}COs. For example, in rabbit tracheal smooth muscle cells, cromakalim reduced the uptake into and the release of $^{45}Ca^{2+}$ from the endoplasmic reticulum (Chopra *et al.*, 1992).

1.7 The Effects of Magnesium Bound Nucleotides, ATP and ADP

The nucleotides ATP and ADP bind to an intracellular site on Kir6.2, and cause the K_{ATP} channel to close (Babenko *et al.*, 1999). The photoaffinity analogue of ATP, 8-azido-[γ-^{32}P]ATP, was shown to directly label COS-7 cells transiently expressing Kir6.2. This labelling was reduced by competition with ATP. The related subunit Kir4.1, which is not inhibited by ATP, is not labelled (Tanabe *et al.*, 1999). Further evidence of the direct interaction of ATP with Kir6.2 is provided by the observation that mutations in Kir6.2 that reduce the inhibitory effect of ATP on channel activity also reduce the photoaffinity labelling (Tanabe *et al.*, 1999).

To understand the roles of the two NBDs of SUR in the regulation of K_{ATP} channels, point mutations were introduced in the consensus sequence of the Walker A or B motif of each NBD of SUR1 and characterised ATP binding and ADP or MgADP antagonism to it (Ueda *et al.*, 1997). SUR1 was photolabelled with 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP in the presence or absence of Mg^{2+}. NBD1 mutations impaired ATP binding but NBD2 mutations did not impair ATP binding. MgADP antagonised ATP binding, and the NBD2 mutation reduced MgADP antagonism. These results show that SUR1 binds ATP at NBD1 even in the absence of Mg^{2+}, and that MgADP, through binding at NBD2, antagonises the Mg^{2+} independent high affinity ATP binding at NBD1 (Gribble *et al.*, 1997, Ueda *et al.*, 1997).

Ueda *et al.* (1999) has proposed a model of nucleotide activation of the K_{ATP} channel through the SUR1 subunit. Channel activation is induced when SUR1 binds ATP in NBD1 and MgADP in NBD2. When the intracellular MgADP concentration decreases, MgADP dissociates from NBD2. MgADP dissociation from NBD2 leads to instability of ATP binding at NBD1, allowing the release of ATP. This dissociation of ATP from NBD1 may be involved in channel inactivation of the K_{ATP} channels. The model suggests that the intracellular concentration of MgADP is the primary factor determining the nucleotide activation of the K_{ATP} channel.

The nucleotide binding properties of the NBD1 and NBD2 are different between SUR2A and SUR2B. The affinity of NBD1 of SUR2B for ATP is higher than that of SUR2A (K_i 51 and 110 μM respectively). The affinity of NBD2 of SUR2B for MgADP is higher than that of SUR2A (K_i 67 and 170 μM respectively) (Matsuo *et al.*, 2000). As SUR2A and SUR2B share the same amino acid sequence except for their C-terminal 42 amino acids, this C-terminal region is thought to affect the nucleotide binding properties of NBD1 and NBD2 (Matsuo *et al.*, 2000). Babenko *et al.* (1999) has reported that these C-terminal 42 amino acids specify the effect of ATP on gating. The C-terminal regions may therefore alter the sensitivity of the K_{ATP} channel to inhibitory ATP through affecting affinities of the NBDs of SUR2A and SUR2B to ATP (Matsuo *et al.*, 1999: 2000).

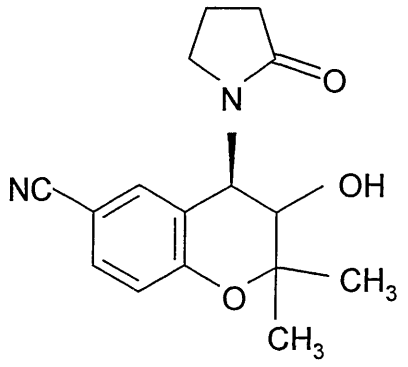
For a potassium channel opener to bind to SUR2A and SUR2B, Mg^{2+} and ATP are required in the low micromolar range (EC_{50} 3 – 5 μM respectively) (Hambrock *et al.*, 1998: 1999; Schwanstecher *et al.*, 1998). In addition, it has been shown that non-hydrolysable ATP analogues such as methylene-ADP or α,β-methylene-ATP do not support K_{ATP}CO binding to SUR2B (Hambrock *et al.*, 1999; Schwanstecher *et al.*, 1998; Dickinson *et al.*, 1997). In the presence of MgATP, micromolar concentrations of MgADP formed by ATPase activity, increased [^{3}H] P1075 binding to SUR2A, but inhibited [^{3}H] P1075 binding to SUR2B. Hambrock *et al.*, (1999) speculates that the carboxyl terminal of SUR2A

and SUR2B folds back to affect the interaction of MgADP with the [^{3}H] P1075 binding site. The carboxyl terminus may also form part of the binding site for K_{ATP}COs such as [^{3}H] P1075 (Hambrock *et al.*, 1999).

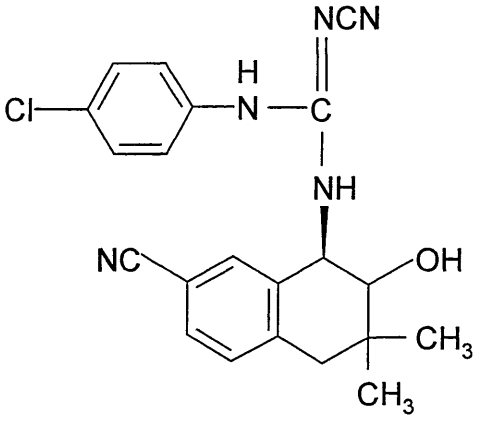
1.8 Second Generation K_{ATP}COs

K_{ATP}COs are recognised as having a therapeutic potential in the control of cell excitability (Lawson, 1996). Although attempts have been made to propose common pharmacophoric features for K_{ATP}COs, the heterogeneous pharmacology due to the existence of SUR isoforms and the chemical diversity of the K_{ATP}COs suggests variation between the different K_{ATP}CO binding sites. A disadvantage with the first generation K_{ATP}COs is that they can cause significant side effects such as severe headaches and fluid retention (Atwal, 1994). A greater understanding of the K_{ATP}CO pharmacophore for each SUR isoform will provide an insight into the K_{ATP}CO binding sites. This knowledge should then lead to the production of rationally designed molecules, which offer highly selective modulation of distinct K_{ATP}CO channel subtypes (Lawson and Dunne, 2001, Atwal *et al.*, 1993).

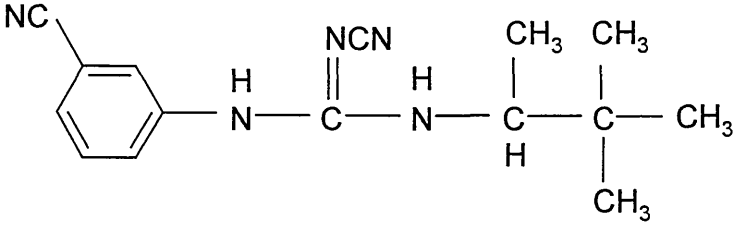
Development of second generation K_{ATP}COs through rational design and the identification of new chemical families with K_{ATP}CO properties have provided agents with cardiac greater tissue selectivity (Atwal *et al.*, 1993). Combining the structural features of cromakalim and pinacidil has led to the synthesis of compounds, which selectively exhibit either the vascular or cardiac properties of these agents (BMS-180448 – cardiac selective and BMS-182264 – vascular selective) (Figure 5, Atwal *et al.*, 1993). This study not only allows the identification of tissue selectivity by uncoupling the smooth muscle relaxation from cardiac actions within the pharmacophore of K_{ATP}COs, but also indicates that modifications of existing structures can provide the desired selective profiles required (Lawson, 2000).



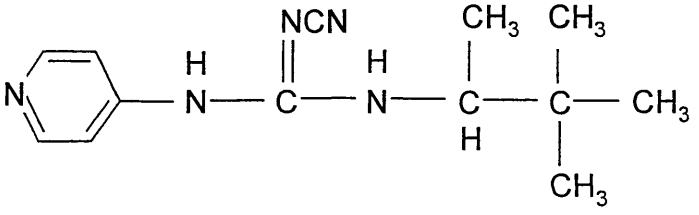
Cromakalim



BMS-180448
Cardiac Selective



BMS-182264
Vascular Selective



Pinacidil

Figure 5. Tissue selective K_{ATP}COs. Compounds combining the structural features of cromakalim and pinacidil have allowed the separation of vascular smooth muscle relaxation from the cardiac actions of these K_{ATP}COs (Atwal *et al.*, 1993).

New chemical groups of K_{ATP}COs such as the anilide tertiary compound, ZD6169, have been shown to display urinary bladder relaxant activity, but without hypotensive side effects (Howe *et al.*, 1995).

In this project, second generation K_{ATP}COs, which have been rationally designed from first generation K_{ATP}COs, have been used. These compounds may enable a greater understanding of the K_{ATP}CO pharmacophore and the K_{ATP}CO binding site in vascular smooth muscle to be obtained.

A series of thienylcyanoguanidine derivatives, which possess different substituents on the thienyl ring have been studied. The thienylcyanoguanidine K_{ATP}COs have been evaluated for smooth muscle relaxation activity and are believed to bind to the K_{ATP}CO binding site in the same way as pinacidil (Yoshiizumi *et al.*, 1997, 2000). The way in which these thienylcyanoguanidine derivatives interact with K_{ATP}CO binding site in rat isolated aorta will be examined by the use of L-arginine analogues. If the thienylcyanoguanidine derivatives interact with the K_{ATP}CO binding site in the same way, then it would be reasonable to expect that the L-arginine analogues would be able to either modify all or none of the vasorelaxant responses to these K_{ATP}COs.

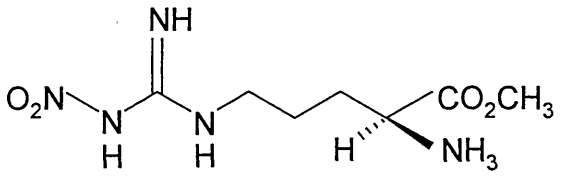
1.9 Effect of L-Arginine Analogues on K_{ATP}CO Induced Vasorelaxations

Vasodilatory responses to the K_{ATP}COs pinacidil and cromakalim have been shown to be attenuated by L-arginine analogues (Maczewski and Beresewicz, 1997). In Langendorff perfused rat hearts, N^G-nitro-L-arginine (L-NNA) reduced the basal coronary flow by 44% and N^G-monomethyl-L-arginine (L-MMA) reduced the basal coronary flow by 43%. The pinacidil concentration response curve was displaced to the right by both L-MMA and L-NNA (Maczewski and Beresewicz, 1997). In rat aorta derived from Sprague-Dawley rats, the vasorelaxant responses to the benzopyran K_{ATP}CO, BRL-38227 were selectively attenuated by the peptide endothelin-1 (ET-1) in contrast to the vasorelaxant responses to

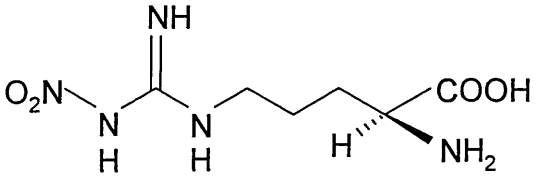
pinacidil and RP-49356 (thioformamide) that were insensitive to ET-1 Lawson et al., 1993). The nitric oxide synthase (NOS) inhibitory properties of the L-arginine analogues supported endothelium-derived nitric oxide (NO) playing a role in K_{ATP}CO induced vasodilations (Janigro *et al.*, 1997; Lawson *et al.*, 1993).

In the endothelium, the L-arginine analogues, such as N^G-nitro-L-arginine methyl ester (L-NAME), L-MMA or L-NNA (Figure 6), act by competing with L-arginine at the active site of NOS. NOS can catalyse the conversion of L-arginine to L-citrulline, and in the process produce NO (Anggard, 1994). If NO is produced by the endothelium, it diffuses to the vascular smooth muscle where it activates guanylate cyclase. The activation of guanylate cyclase, by a series of poorly defined events, then leads to a decrease in intracellular calcium and smooth muscle relaxation (Hamilton *et al.*, 1999; Hobbs *et al.*, 1999; Nichols *et al.*, 1996).

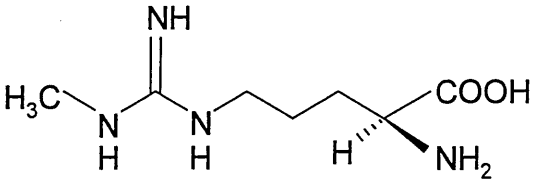
L-NAME has also been suggested to inhibit K_{ATP}CO vasodilation by directly blocking K_{ATP} channels (Kontos and Wei, 1996). Studies to determine the mechanism by which L-arginine analogues modify the vasorelaxant effects of K_{ATP}COs have demonstrated that L-NAME can differentiate between structurally different K_{ATP}COs in the absence of a functional endothelium (Carr and Lawson, 1999). This indicates that the endothelium and endothelial NOS are not involved in modifying the vasorelaxant responses to K_{ATP}COs. These findings therefore suggest that K_{ATP}COs do not possess a common pharmacophore and may interact with the K_{ATP}CO binding site on the K_{ATP} channel in a different manner.



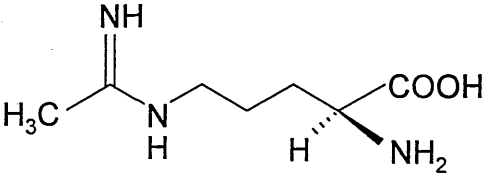
L-NAME



L-NNA



L-MMA



L-NIO

Figure 6. The structures of the L-arginine (L-NAME, L-NNA and L-MMA) and the L-ornithine (L-NIO) analogues, which will be used in this project.

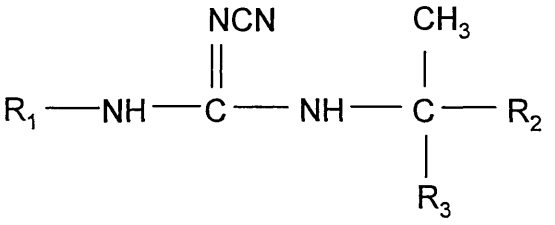
1.10 Aims of the Project

The aim of the project is to gain an insight into the K_{ATP}CO pharmacophore and the K_{ATP}CO binding site on the SUR2B. An improved understanding of both the K_{ATP}CO pharmacophore and the K_{ATP}CO binding site may allow the development of more specific molecules, which could offer selectivity to distinct K_{ATP} channel subtypes to be produced. The chemical diversity of K_{ATP}COs has suggested the involvement of multiple sites of action on the target membrane (Lawson, 1996).

K_{ATP}CO pharmacophore and the K_{ATP}CO binding site

L-NAME can selectively attenuate the vasorelaxant response to the cyanoguanidine K_{ATP}CO, pinacidil, in contrast to the benzopyran K_{ATP}CO, cromakalim in rat isolated aorta devoid of a functional endothelium (Carr and Lawson, 1999). To examine this difference in L-NAME sensitivity, a series of thienylcyanoguanidine K_{ATP}COs, which are structurally similar to pinacidil, have been studied (Table 1) (Yoshiizumi *et al.*, 1997). The thienylcyanoguanidine K_{ATP}COs are believed to interact with the K_{ATP} channel in the same way as pinacidil (Yoshiizumi *et al.*, 1997). The way in which these thienylcyanoguanidine derivatives interact with K_{ATP}CO binding site in rat isolated aorta was examined by the use of the L-arginine analogues, L-NAME, L-MMA and L-NNA. If the thienylcyanoguanidine derivatives interact with the K_{ATP}CO binding site in the same way, then it would be reasonable to expect that the L-arginine analogues would be able to either modify all or none of the vasorelaxant responses to these K_{ATP}COs. If differences in the vasorelaxant responses to the thienylcyanoguanidine K_{ATP}COs were observed, then this would provide clues as to how the thienylcyanoguanidine K_{ATP}COs interact with the K_{ATP}CO binding site and an insight into the cyanoguanidine K_{ATP}CO pharmacophore.

In contrast to pinacidil, L-NAME failed to modify the vasorelaxant responses to cromakalim. Other benzopyran K_{ATP}COs such as DY-9708, symakalim, bimakalim and SKP-450 were used to examine whether all benzopyran K_{ATP}COs



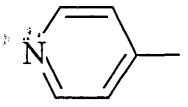
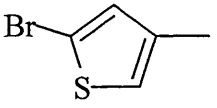
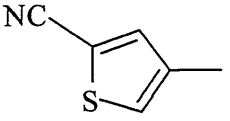
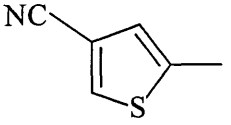
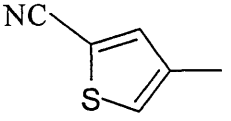
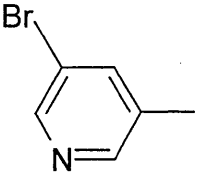
Compound	R_1	R_2	R_3
Pinacidil		-C(CH_3)	-H
KB-R10101		-CH_2.CH_3	-CH_3
KB-R6844		-CH_2.CH_3	-CH_3
KB-R10757		-CH_2.CH_3	-CH_3
KB-R6907		-C(CH_3)$_3$	-H
KB-R10758		-CH_2.CH_3	-CH_3

Table 1. A comparison of a series of thienylcyanoguanidine K_{ATP}COs with pinacidil and KB-R10758. The skeleton structure is shown above the table. The substitutions to the structure are shown at positions R_1, R_2 and R_3.

interact with the K_{ATP}CO binding site on SUR2B in the same way. An insight into the benzopyran K_{ATP}CO pharmacophore should therefore be obtained.

Involvement of Nitric Oxide

The L-arginine analogues L-NAME, L-MMA and L-NNA inhibit nitric oxide synthase (NOS) activity. The effect of endothelium on the vasorelaxant responses to cyanoguanidine K_{ATP}COs in rat isolated aorta was examined in the presence of other NOS and guanylate cyclase inhibitors (L-N^5-(1-iminoethyl)ornithine (L-NIO) and methylene blue respectively). This study was used to establish whether the sensitivity of the cyanoguanidine K_{ATP}CO induced vasorelaxations to L-arginine analogues such as L-NAME is due to the inhibition of NOS, or whether the L-arginine analogues are having an effect elsewhere in the vascular smooth muscle.

Mechanisms behind the responses

To examine the mechanism behind the modification of the vasorelaxant responses to pinacidil by L-NAME, a rubidium efflux assay was used. The rubidium efflux assay, studies the efflux of rubidium ions from the vascular smooth muscle as a direct consequence of K_{ATP}CO application. The rubidium efflux assay is used to trace the efflux of K^+ and establish whether L-NAME is having its effect at the level of the K_{ATP} channel or whether it may be affecting an alternative mechanism. A combination of these studies will provide valuable information into the functional cell biology of K_{ATP} channels in vascular smooth muscle.

2 Methods

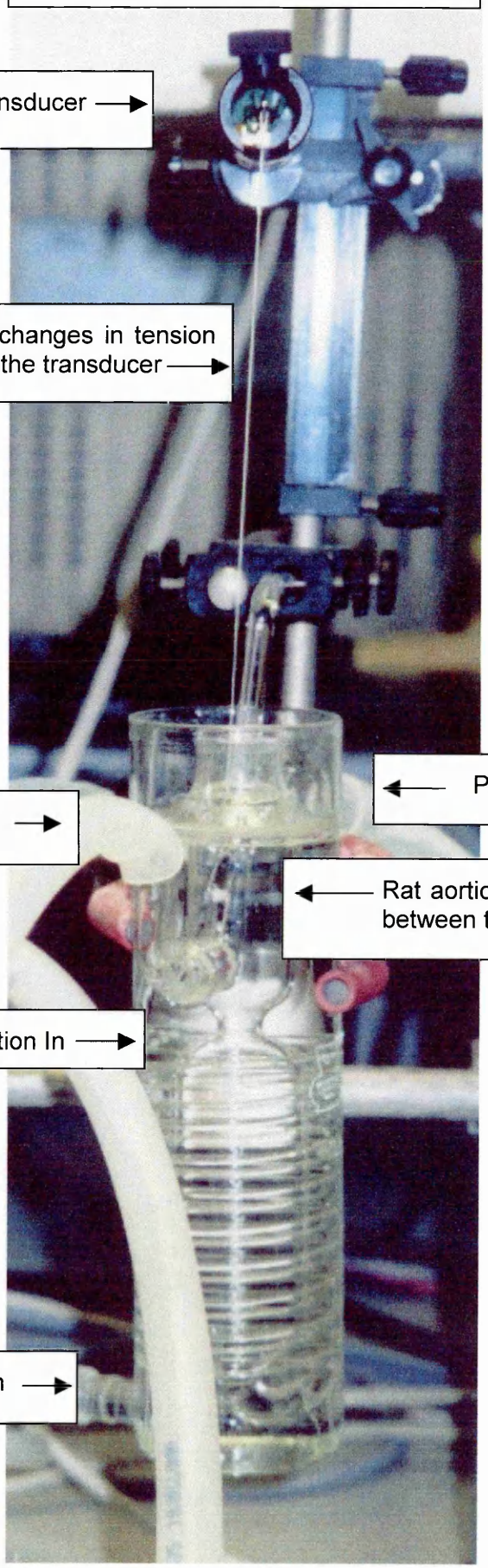
2.1 Vasorelaxation Studies on Pre-contracted Rat Aorta

The thoracic aorta was dissected from male Wistar rats weighing between 200 to 250 g. The removed piece of aorta came from the section between the aortic arch and the diaphragm. Once dissected, the connective tissue was carefully removed from the aorta in Kreb's bicarbonate solution (composition in mM: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 1.2, glucose 10.0 and $NaHCO_3$ 25.0). Four equal rings, each measuring approximately 4 mm, were then cut from the thoracic aorta. The aortic rings were either denuded of functional endothelium by gently rubbing the intima using a pair of forceps, or the endothelium was left intact.

The aortic rings were individually suspended in organ baths (20 mls) containing Kreb's bicarbonate solution maintained at 37 ± 1°C, and gassed with 95% O_2 and 5% CO_2. An initial resting tension of 2 g was applied to the aortic rings. During an equilibration period of 80 - 100 minutes, the tension in the aortic rings was on occasion seen to either slightly contract or relax. When this occurred, the tension was adjusted back to the original 2 g of tension level after the bathing medium had been replaced with fresh Kreb's bicarbonate solution at 20 minute intervals. The tension in the aortic rings was continuously recorded isometrically via FSG-01/20 force-displacement transducers (Linton Instrumentation) (Figure 7) and displayed on a Gould (8000) chart recorder.

When equilibrium had been reached, the aortic rings were maximally contracted by the addition of phenylephrine (1 μM) to the bathing medium. Once the contraction due to phenylephrine had reached a plateau, the integrity of the endothelium was determined by the subsequent addition of acetylcholine (1 μM). The failure of acetylcholine to produce a relaxation showed that the endothelium was non-functional. If functional endothelium was still present, then a relaxation

Figure 7. Isolated Organ Bath



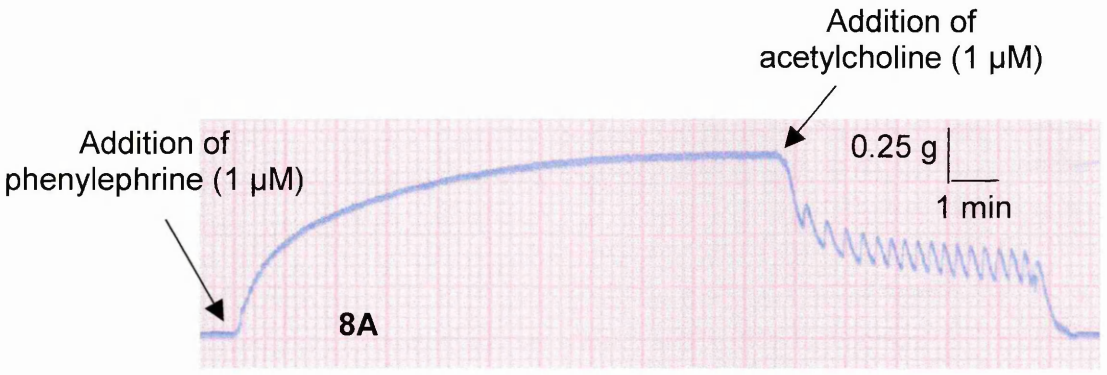
occurred (Figure 8). Following the confirmation of the presence or absence of the functional endothelium, the bathing medium was replaced with fresh Krebs bicarbonate solution, until the tension in the preparations had returned to pre-contractile levels.

2.2 Experimental Protocol

Once the tension had returned to pre-contractile levels, a pre-treatment was applied to the aortic rings for 30 minutes. Table 2 shows the pre-treatments that were applied to the aortic rings in the study. The concentrations relate to the final concentration of the compound in the bathing medium.

Ten minutes after the addition of pre-treatment, the aortic rings were contracted with phenylephrine (1 μM). A 100% contraction is achieved when the aortic ring has been maximally contracted by phenylephrine (1 μM) and the contraction has reached a plateau during the 20 minute period prior to the application of the K_{ATP}COs. Thirty minutes after the addition of the pre-treatment to the bathing medium, cumulative concentrations of K_{ATP}COs were added to the baths. The range of final concentrations of the K_{ATP}COs in the bathing medium, are recorded in Table 3.

In the following experiments, only one concentration response curve (either control or test) was obtained per aortic ring preparation and only one ring preparation per animal was exposed to a particular treatment regime.



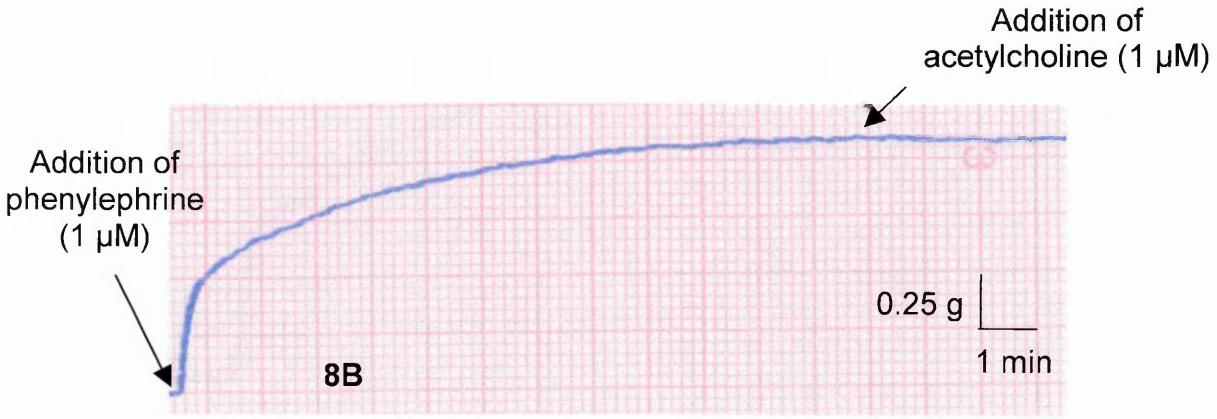


Figure 8. The effect of phenylephrine and acetylcholine on aortic rings, with (A) and without (B) a functional endothelium.

Pre-Treatment	Concentration of pre-treatment in bathing medium (μM)
L-arginine analogues	
N^G-nitro-L-arginine methyl ester (L-NAME)	30, 100 or 300
N^G-monomethyl-L-arginine (L-MMA)	10, 30 or 100
N^G-nitro-L-arginine (L-NNA)	10, 30 or 100
L-ornithine analogue	
L-N^5-(1-iminoethyl) ornithine (L-NIO)	100
Other	
Methylene Blue	10

Table 2. The concentrations of pre-treatments used in the vasorelaxation study.

$K_{ATP}CO$	Concentration range of $K_{ATP}CO$ used (μM)
Pinacidil	0.05 - 25.6
Cromakalim	0.05 - 3.2
KB-R6844	0.05 - 25.6
KB-R6907	0.05 - 25.6
KB-R10101	0.05 - 12.8
KB-R10757	0.05 - 25.6
KB-R10758	0.001 - 0.256
DY-9708	0.004 - 0.256
Bimakalim	0.004 - 0.256
Symakalim	0.004 - 0.256
SKP-450	0.0001 - 0.064

Table 3. The concentration ranges of the $K_{ATP}COs$ in the bathing medium used in the vasorelaxation studies. The cyanoguanidine $K_{ATP}COs$ are in *italics* and the benzopyran $K_{ATP}COs$ are in **bold**.

In each study, the lowest concentration of $K_{ATP}CO$ was initially added to the bathing medium. Once the relaxation produced as a consequence of adding the $K_{ATP}CO$ had reached a plateau, the next concentration of $K_{ATP}CO$ was added (Figure 9). The end of the concentration range was determined either when the addition of the proceeding concentration of $K_{ATP}CO$ failed to produce further relaxation, hence the concentration response curve had reached a plateau or when 100% relaxation had been reached. A 100% relaxation is noted when the level of contraction in the isolated aortic rings has returned to baseline levels. The $K_{ATP}COs$ were added hemilogarithmically, which means the concentration of $K_{ATP}CO$ in the bathing medium doubled with each addition of $K_{ATP}CO$. The only alteration to this procedure occurred in areas of the concentration response curve where a large relaxation (50%) was recorded with the doubling of the concentration of $K_{ATP}CO$. When this was noticed, additional, smaller increases in $K_{ATP}COs$ were made in future experiments, to ensure that smaller relaxations were obtained.

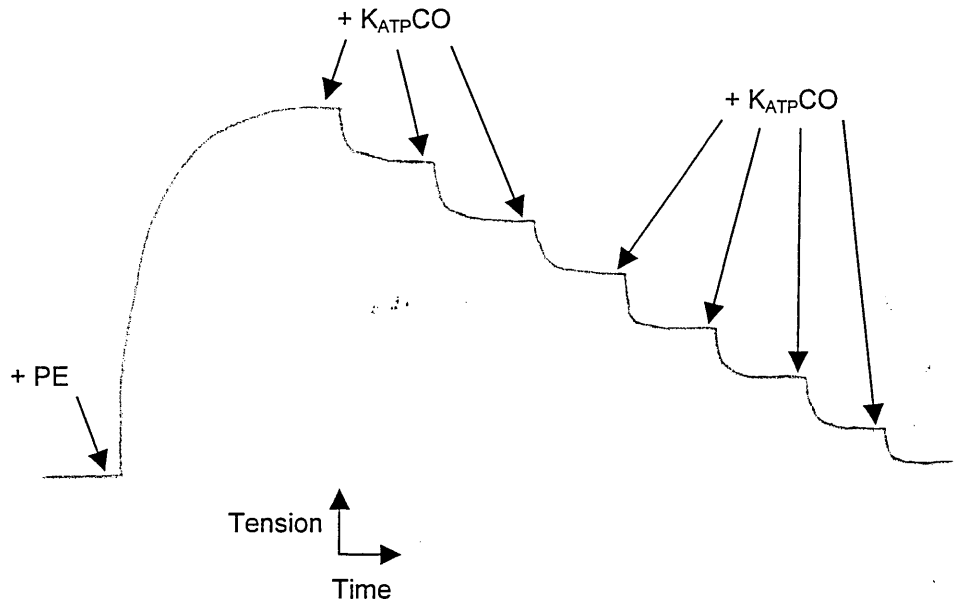


Figure 9. A trace of a typical concentration dependent relaxation to a $K_{ATP}CO$ in rat isolated aorta. The trace shows that a contraction occurs after the addition of phenylephrine (PE). Once the phenylephrine-induced contraction has reached a plateau, cumulative concentrations of a $K_{ATP}CO$ are added to the bathing medium. The resulting relaxations are used to construct concentration-response curves to the $K_{ATP}CO$.

2.3 Introduction to Rubidium Efflux Assay

A non-radioactive rubidium efflux assay for functional analysis of ion channels has been used (Terstappen, 1999). The tissue is loaded with rubidium ions (Rb^+), a tracer for potassium ions (K^+), and after channel activation, Rb^+ distribution between intracellular and extracellular space is determined by atomic emission spectroscopy. The relative amount of Rb^+ in the supernatant is a direct measure of channel activity.

The advantages of this technique are that it is a safe and fast method for analysis of ion channels with conductivity for rubidium. As rubidium efflux is a direct measure of channel activity, the method is reliable and not prone to disturbances as is the case with indirect methods such as fluorescence-based procedures. The method also avoids the use of high-energetic radioisotopes like ^{86}Rb, which represent severe hazards and need special safety precautions. In addition, such isotopes are expensive and their disposal is problematic.

The following study determines the effects of L-NAME and L-NIO on the pinacidil and cromakalim induced Rb efflux in rat isolated aorta. The data in the Rb efflux study is expressed in the form of the percentage Rb efflux in relation to the amount of Rb taken up by the aortic section devoid of endothelium during the incubation period. The control efflux is produced in the absence of pinacidil or cromakalim stimulation.

2.4 Rubidium Efflux Experimental Protocol

The rubidium efflux method used was modified from Terstappen (1999). The aorta was cut into 12 ring sections measuring 2 mm each. The 12 aortic sections were randomly placed into 12 individual wells on a 24 well plate. Each well contained 0.5 ml of Kreb's bicarbonate solution (as described in section 2.1) containing RbCl (5.4 mM) in place of KCl. The Kreb's bicarbonate solution containing RbCl, had a pH of 7.4 and was gassed for 30 minutes with 95% O_2

and 5% CO_2 prior to being placed into the wells. The plate was placed in an incubator at 37 ± 1 °C for 4 hours. L-NAME (10-100 µM), L-NIO (100 µM), glibenclamide (1 or 10 µM), ouabain (10 mM) or vehicle (control) was added for the final 30 minutes of the 4 hour incubation. The concentrations of the above compounds represent the concentrations found in each individual well of the plate. At the end of the 4 hour incubation period, the plate was placed in a water bath at 37 ± 1 °C.

The individual wells containing the aortic section, firstly had the RbCl solution removed by pipette. The aortic ring section was then washed three times with 1.5 ml of warmed Kreb's bicarbonate solution (pH 7.4) containing KCl (4.6 mM) in place of RbCl and the relevant pre-treatment of L-NAME, L-NIO, glibenclamide or ouabain. Immediately after the last Kreb's bicarbonate solution wash had been removed, 0.5 ml of Kreb's bicarbonate solution (control) or 0.5 ml of Kreb's bicarbonate solution containing either pinacidil (up to 50 µM) or cromakalim (up to 1.2 µM), and the relevant pre-treatment where required, was added to the aortic ring section for up to 8 minutes. The supernatant was then removed and added to 4.5 ml of distilled water in a bijou. The aortic ring section was then exposed to 0.5 ml of 10 % sodium dodecylsulphate (SDS) for 18 hours, after which, the lysate was removed and added to 4.5 ml of distilled water in a bijou.

The amount of rubidium present in the supernatant and lysate samples was determined by atomic emission spectroscopy at a wavelength of 780 nm. The atomic emission spectrometer was calibrated using a 2 µg/ml rubidium chloride standard. Once the wavelength of 780 nm had been set, the samples were sprayed into the acetylene flame of the spectrometer for analysis. The readings with the spectrometer offer a linear relationship with respect to rubidium concentration. Distilled water was introduced into the spectrometer between samples to ensure that each reading was entirely due to the rubidium that was present in the sample. If collected samples were not immediately analysed for

rubidium by atomic emission spectroscopy, then they were stored at 4°C without any negative effects on rubidium analysis (Terstappen, 1999).

2.5 Data Analysis

2.5.1 Vasorelaxation Studies on Pre-contracted Rat Aorta

Responses to the K_{ATP}COs after the respective treatments were compared with responses from time matched control preparations from the same animal. Concentration-response curves were constructed with the data expressed as mean ± standard error of the mean (s.e.m.) of n determinations. The concentration-response curves are expressed as the log concentration of K_{ATP}CO across the x axis and the % relaxation of the pre-contracted isolated rat aorta on the y axis. Differences between the paired data points, which formed the test and control concentration response curves, were evaluated by an unpaired t-test. When P was less than 0.05, the two values were considered to be statistically different. The E_{max} (maximum relaxation response (%) where 100% = return to pre-contraction baseline) and EC_{50} (concentration of compound required to relax the pre-contracted aorta by 50% in comparison with the control maximum) values were calculated for individual concentration-response curves. Mean ± s.e.m. values for E_{max} and EC_{50} were calculated from the individual values. Concentration ratios (CR) were initially calculated by comparing the EC_{50} values from individual test and control curves.

$$CR = \text{Test } EC_{50} / \text{Control } EC_{50}$$

Mean concentration ratios with 95% confidence limits (CR(cl)) were then determined from the individual concentration ratios.

The n value relates to the number of different animals used in each experiment. Control Hill co-efficients were calculated using the GraphPad software (Prism 3.0).

2.5.2 Rb Efflux Studies

The % Rb efflux in each test and control experiment, was calculated using the following formula.

$$\% \text{ Efflux} = \frac{\text{Rb Content of Supernatant}}{\text{(Rb Content of Supernatant + Lysate)}} \times 100$$

The mean ± s.e.m. for n determinations (number of aortic sections per test or control group) were statistically evaluated by an unpaired t-test. When P was less than 0.05, the two means were considered to be statistically different.

2.6 Reagents

Potassium chloride (KCl), magnesium sulphate ($MgSO_4.7H_20$), sodium hydrogen carbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), rubidium chloride (RbCl) and potassium dihydrogen orthophosphate (KH_2PO_4) were supplied by BDH, Poole, UK. Ouabain, dimethyl sulphoxide (DMSO), pinacidil, N^G-nitro-l-arginine methyl ester hydrochloride (L-NAME), L-phenylephrine hydrochloride, N^G-monomethyl-l-arginine hydrochloride (L-MMA) and acetylcholine chloride were supplied by Sigma, St. Louis, USA. Sodium chloride (NaCl) was supplied by Prime Chemicals, Rotherham, UK. N^G-nitro-l-arginine hydrochloride (L-NNA) and L-N^5-(1-Iminoethyl)-ornithine dihydrochloride (L-NIO) was supplied by Calbiochem-Novabiochem Corporation, San Diego, USA. Glibenclamide and cromakalim

were supplied by Roche Bioscience, Palo Alto, USA. D(+)-glucose anhydrous was supplied by Prolabo, Fontenay, France. KB-R10101, KB-R6844, KB-R6907, KB-R10757 and KB-R10758 were kindly donated by Dr K Yoshiizumi of Kanebo Ltd, Osaka, Japan. DY-9708 was kindly donated by Daiichi Pharmaceutical Company, Tokyo, Japan. SKP-450 was kindly donated by the Korean Research Institute of Chemical Technology, Seoul, South Korea. Bimakalim, and symakalim were kindly donated by Merck KgaA, Darmstadt, Germany.

L-NAME, L-MMA, L-NNA, L-NIO and ouabain (10 mM) stock solutions were freshly made by being dissolved in Kreb's bicarbonate solution when required. Bimakalim, cromakalim, DY-9708, KB-R10101, KB-R6844, KB-R6907, KB-R10757, KB-R10758, pinacidil, SKP-450, symakalim and glibenclamide were dissolved in dimethyl sulphoxide and stored as a stock solution of 10 mM at -20°C, before being diluted in Kreb's bicarbonate solution when required.

3 – 6 Results

In rat isolated aorta devoid of (lack of relaxant response to acetylcholine (1 μM)) or with a functional endothelium, phenylephrine (1 μM) evoked a contraction (0.75 g – 1.6 g) that maintained a stable plateau for at least 120 minutes in control preparations. All of the K_{ATP}COs used in this project produced concentration-related relaxations of phenylephrine contracted rat aorta.

3.1 Intra and Inter-rat Variability

To examine intra-rat variability, a piece of rat aorta from one animal was divided into four ring sections and randomly suspended into four organ baths. Pinacidil (0.1 – 25.6 μM) produced concentration related relaxations of the phenylephrine contracted aortic ring sections (Figure 10A). Figure 10A shows that the shapes of the four concentration response curves were similar. The mean EC_{50} value to pinacidil from the four sections was 0.60 ± 0.07 μM. The EC_{50} values to pinacidil show that there is minimal variation between pieces of rat isolated aorta from the same animal.

Pinacidil (0.1 - 25.6 μM) produced concentration related relaxations of phenylephrine contracted rat aorta. A mean concentration response curve to pinacidil was derived from seventeen individual concentration response curves to pinacidil from seventeen different rats (Figure 10B). The mean EC_{50} value to pinacidil was 0.54 ± 0.06 μM. Once more, when comparisons are made between the same or different animals, the variability between the EC_{50} values to pinacidil is low.

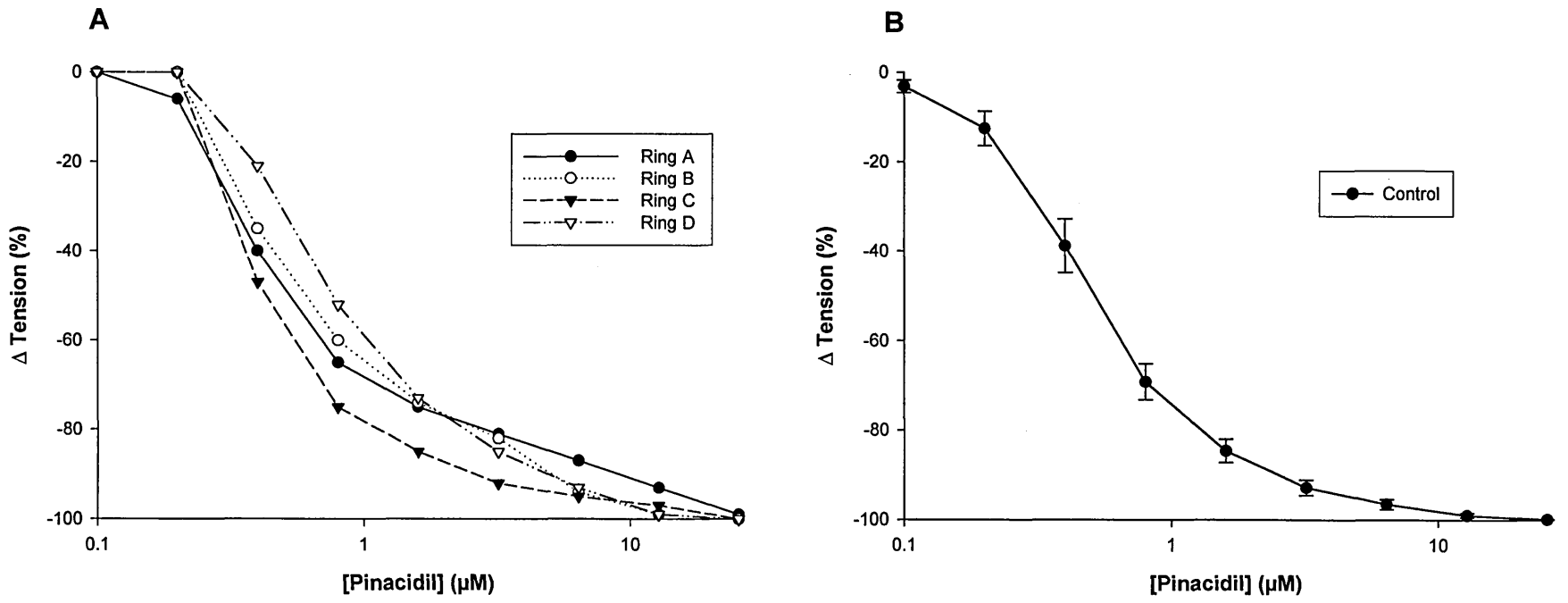


Figure 10. (A) Effect of different sections (A, B, C and D) from the same rat on the vasorelaxant responses to pinacidil in aortic rings devoid of endothelium. Cumulative concentration-response curves to pinacidil were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to fresh Kreb's bicarbonate solution.

(B) A mean concentration response curve to pinacidil. Seventeen concentration response curves to pinacidil from seventeen different rats were combined to produce this mean concentration response curve. Data are mean values ± s.e.m.

3.2 Effect of L-NAME and L-NIO on the Vasorelaxant Responses to the K_{ATP}COs Pinacidil and Cromakalim

The effects of L-NAME on the vasorelaxant responses to the cyanoguanidine K_{ATP}CO, pinacidil, and the benzopyran K_{ATP}CO, cromakalim, were determined in contracted rat isolated aorta devoid of endothelium. Exposure of the endothelium denuded rat aorta preparations to L-NAME (30, 100 or 300 μM) did not modify the base-line tension. Pinacidil (0.1 - 25.6 μM) and cromakalim (0.05 - 3.2 μM) produced concentration related relaxations of phenylephrine contracted rat aorta (Pinacidil EC_{50} 0.43 ± 0.08 μM and Cromakalim EC_{50} 0.15 ± 0.08 μM, E_{max} 100% and 84 ± 6%, n=7/group respectively; Figure 11). The presence of L-NAME (100 μM), significantly ($P<0.05$) displaced to the right of the control, the CRC to pinacidil (EC_{50} 1.66 ± 0.14 μM, CR(cl) 5.05 (3.56-6.54), n=7), without modifying the maximal response. Likewise, L-NAME (300 μM), significantly ($P<0.05$) displaced to the right of the control, the CRC to pinacidil (EC_{50} 1.75 ± 0.08 μM, CR(cl) 5.24 (3.45-7.01), n=7), without modifying the maximal response. L-NAME (30 μM), however failed to modify the CRC to pinacidil (Control EC_{50} 0.58 ± 0.10 μM, L-NAME 30 μM EC_{50} 0.92 ± 0.21 μM, n=6; Figure 12). As the displacement of the CRC to pinacidil by L-NAME (300 μM) was not greater than that observed with L-NAME (100 μM), L-NAME (100 μM) was used in the rest of the study. In contrast, the vasorelaxant responses to cromakalim in rat isolated aortic rings were not modified by L-NAME (100 μM) (EC_{50} 0.20 ± 0.07 μM, n=7).

Exposure of the endothelium denuded rat aorta preparations to L-NIO (100 μM) did not modify the base-line tension. L-NIO (100 μM) failed to modify the CRC to pinacidil (Control EC_{50} 0.50 ± 0.10 μM, L-NIO 0.73 ± 0.23 μM, n=6; Figure 13).

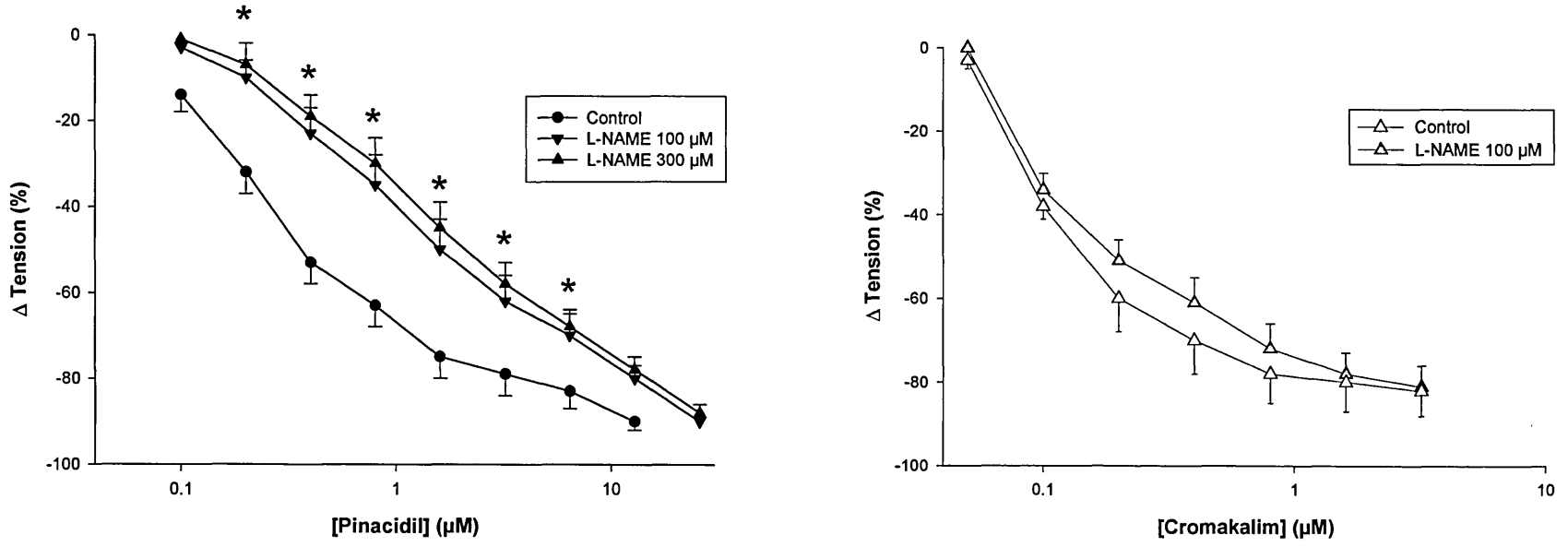


Figure 11. Effects of L-NAME on the vasorelaxant responses to pinacidil and cromakalim in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to pinacidil or cromakalim were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-NAME (100 or 300 μM). Relaxations are expressed as a percentage of the contractile response just prior to the addition of $K_{ATP}CO$. The * indicates responses in the presence of L-NAME (100 and 300 μM) that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with respect to the control for L-NAME (100 μM or 300 μM) against pinacidil are CR(cl) 5.05(3.56-6.54) and 5.24(3.45-7.01) respectively. Data are mean values ± s.e.m. (n=7/group).

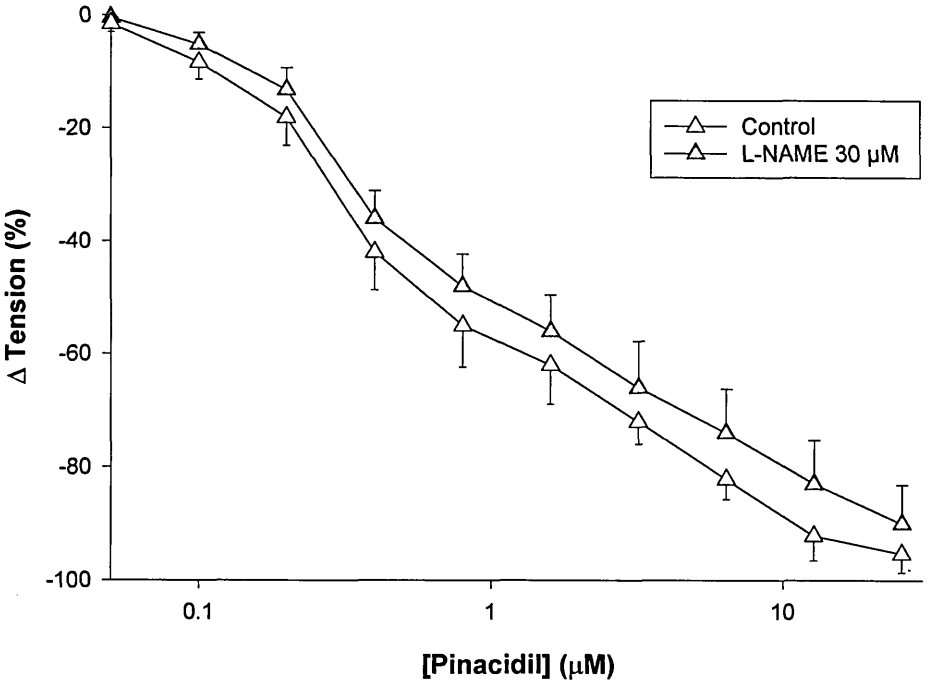


Figure 12. Effect of L-NAME (30 μM) on the vasorelaxant responses to pinacidil in rat isolated aorta devoid of endothelium. A cumulative concentration-response curve to pinacidil was constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-NAME (30 μM). Relaxations are expressed as a percentage of the contractile response just prior to application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=6).

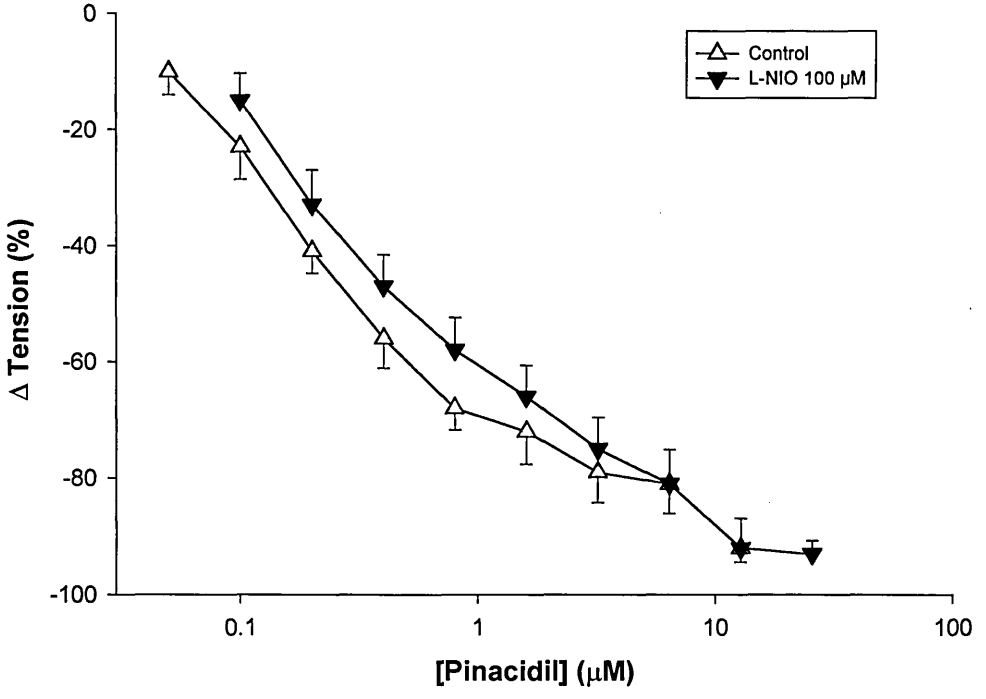


Figure 13. Effects of L-NIO on the vasorelaxant responses to pinacidil in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to pinacidil were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either control or L-NIO. Relaxations are expressed as a percentage of the contractile responses just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=6).

3.3 Effect of L-MMA and L-NNA on the Vasorelaxant Responses to the Cyanoguanidine K_{ATP}CO Pinacidil

Exposure of the rat isolated aorta devoid of endothelium to L-MMA (100 µM) or L-NNA (100 µM) did not modify the base-line tension. Pinacidil (0.1 - 25.6 µM) produced a concentration related relaxation of phenylephrine contracted rat aorta (EC_{50} 0.41 ± 0.08 µM, E_{max} 100 %, n=6, Control Hill co-efficient 1.14 ± 0.25; Figure 14). L-MMA (100 µM) and L-NNA (100 µM) significantly displaced ($P<0.05$) the CRC to pinacidil to the right of the control curve without modifying the maximal response (L-MMA EC_{50} 0.79 ± 0.13 µM, CR(cl) 2.15 (1.03-3.26) and L-NNA EC_{50} 0.63 ± 0.08 µM, CR(cl) 1.65 (1.15-2.15), n=6, respectively). In contrast, lower concentrations of L-MMA (10 and 30 µM) failed to modify the CRC to pinacidil (Control EC_{50} 0.49 ± 0.08 µM, L-MMA 10 µM EC_{50} 0.43 ± 0.09 µM, L-MMA 30 µM EC_{50} 0.42 ± 0.05 µM, n=4; Figure 15). In addition, L-NNA (10 and 30 µM) also failed to modify the CRC to pinacidil (Control EC_{50} 0.30 ± 0.07 µM, L-NNA 10 µM 0.34 ± 0.06 µM, L-NNA 30 µM 0.32 ± 0.08 µM, n=4; Figure 15).

L-MMA (100 µM) or L-NNA (100 µM) produced a significant displacement of the CRC to pinacidil. Lower concentrations of either L-MMA or L-NNA failed to modify the CRC to pinacidil. In view of these observations, L-MMA (100 µM) was used in the rest of the study.

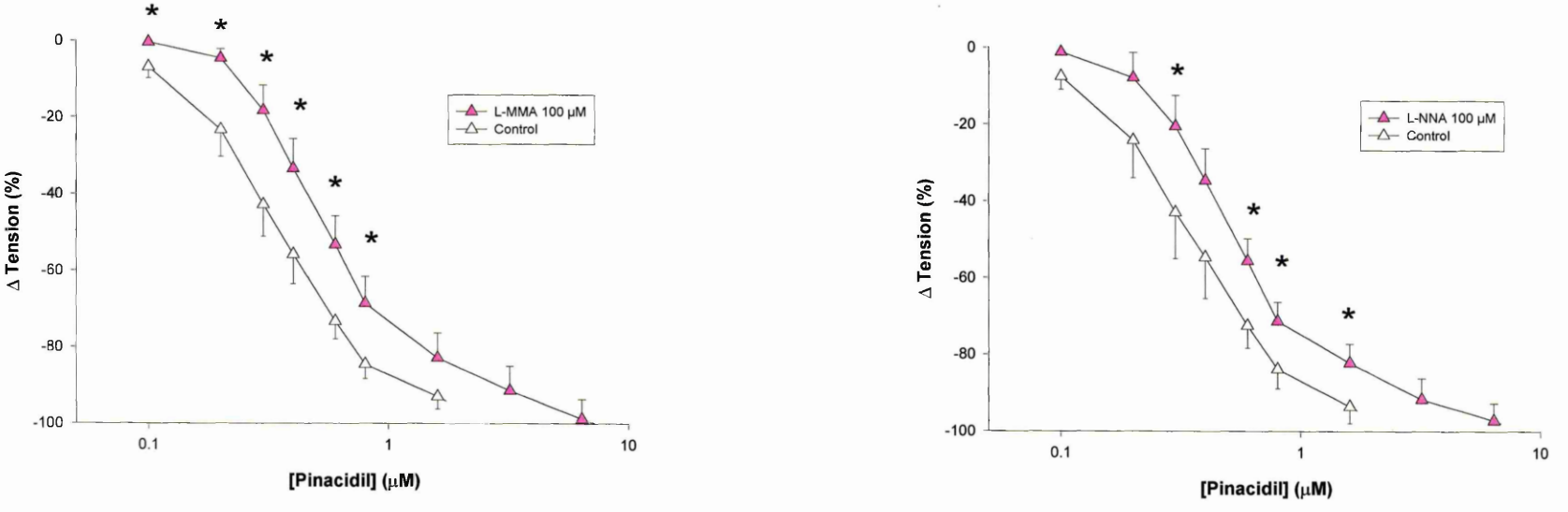


Figure 14. Effects of L-MMA and L-NNA on the vasorelaxant responses to pinacidil in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to pinacidil were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to either solvent, L-MMA (100 µM) or L-NNA (100 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=6/group). The * indicates responses in the presence of the L-MMA and L-NNA that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with respect to the control for L-MMA and L-NNA against pinacidil are CR(cl) 2.15 (1.03-3.26) and 1.65 (1.15-2.15) respectively.

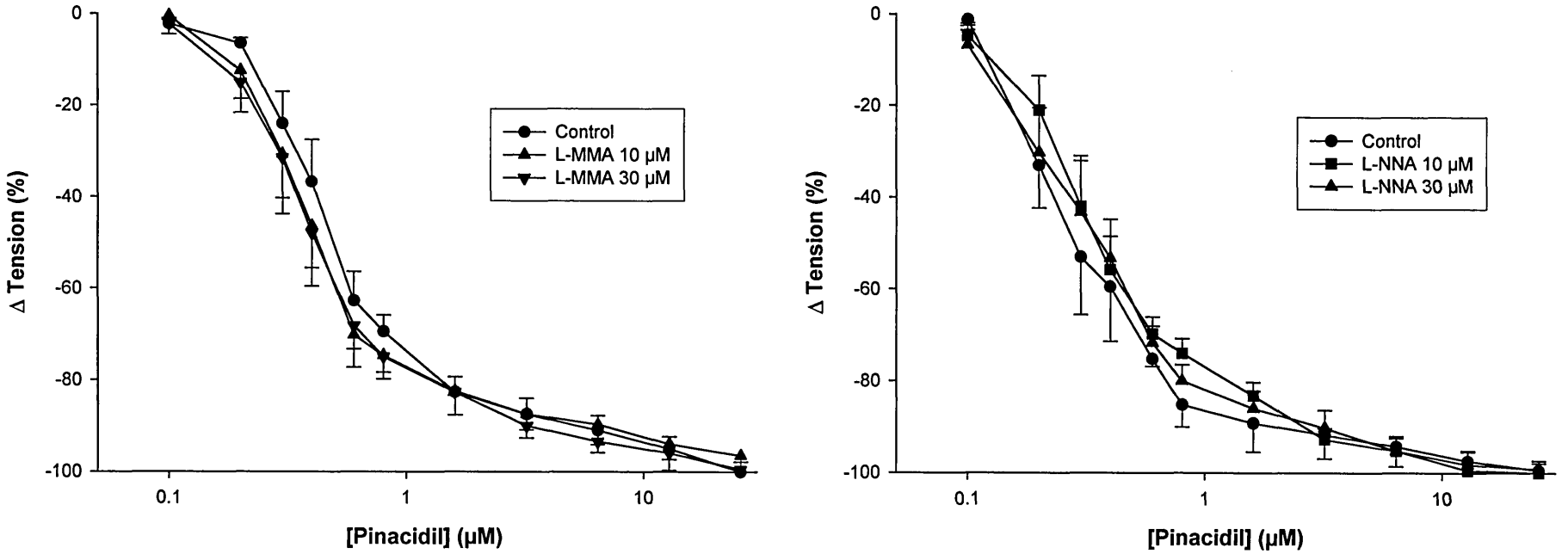


Figure 15. Effects of L-MMA (10 and 30 μM) and L-NNA (10 and 30 μM) on the vasorelaxant responses to pinacidil in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to pinacidil were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent, L-MMA (10 or 30 μM) or L-NNA (10 or 30 μM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=4/group).

4 Pinacidil or Cromakalim Induced Rubidium (Rb) Efflux

4.1 Effect of Pinacidil on Rb Efflux

Pinacidil (50 μM) was used to induce a Rb efflux in rat aorta, devoid of endothelium, that had been divided into twelve sections. The application of pinacidil (50 μM) to the aortic sections increased the Rb efflux relative to the control for the first minute (Figure 16). For longer time periods, the pinacidil induced Rb efflux was lower than the efflux produced after one minute, but still higher than that seen in the control sections.

In view of this, a period of one minute was allowed to elapse between pinacidil application and supernatant removal in subsequent studies. Pinacidil concentrations of 1, 5 and 35 μM, significantly ($P<0.05$) increased the Rb efflux in relation to the control efflux (control 4.83 ± 0.38%, 1 μM 8.55 ± 1.15%, 5 μM 7.10 ± 0.49% and 35 μM 8.48 ± 1.43%, n=8; Figure 17). Statistical analysis showed that Rb effluxes produced as a result of pinacidil stimulation were not significantly different from each other at any of the concentrations of pinacidil used.

An examination of time points less than a minute showed that there was no significant increase in the Rb efflux produced 45 seconds after exposure to pinacidil (1 μM) compared to the control Rb efflux. A significantly increased efflux ($P<0.05$) between the test and control efflux values was only observed when 60 seconds were allowed to elapse between pinacidil (1 μM) application and supernatant removal (control 7.15 ± 0.68%, pinacidil 45 seconds 9.34 ± 1.26%, pinacidil 60 seconds 13.28 ± 1.60%, n=8; Figure 18).

Smaller pinacidil concentrations (less than 1 μM) could also produce a significantly increased Rb efflux relative to the control efflux after a 60 second exposure period. Pinacidil (0.8 μM) produced a significantly increased Rb

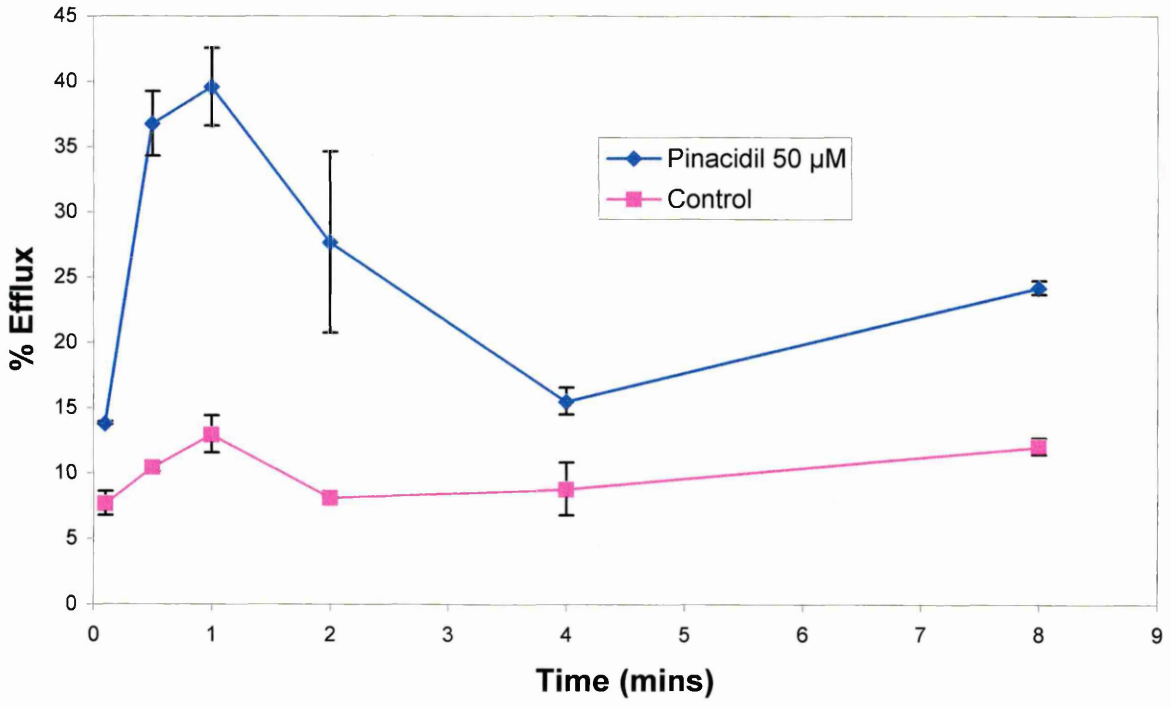


Figure 16. Pinacidil (50 µM) induced Rb efflux in rat isolated aortic sections devoid of endothelium over an eight minute time period. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=3/group).

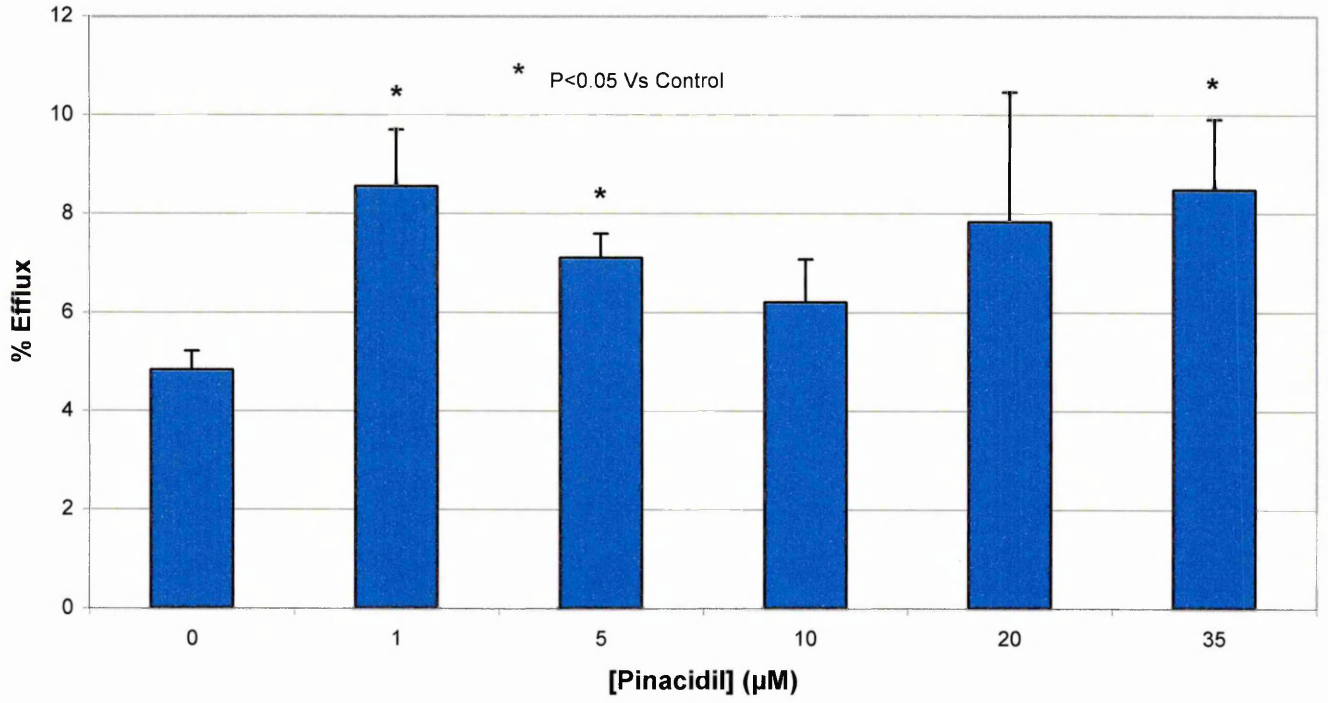


Figure 17. Effect of different pinacidil concentrations on the Rb efflux in rat isolated aortic sections devoid of endothelium. The * indicates an efflux which is significantly different (P<0.05) from the control efflux. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=8/group).

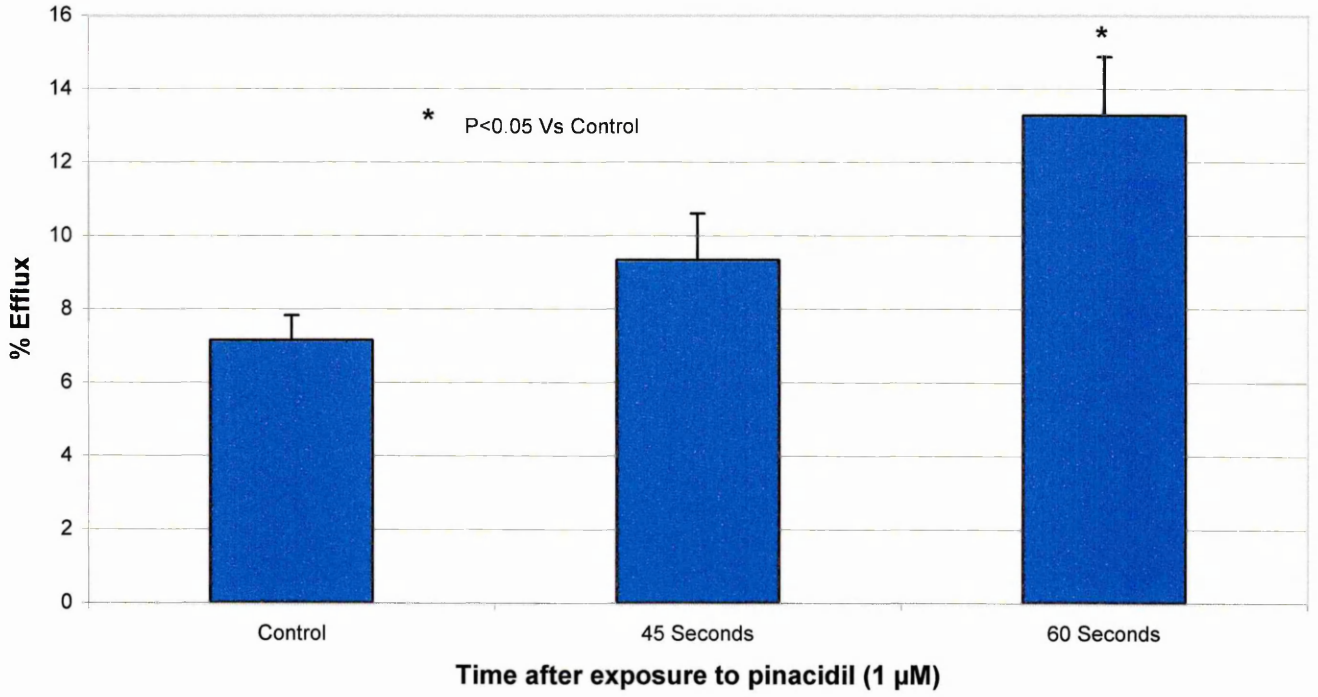


Figure 18. Effect of time on the Rb efflux responses to pinacidil in rat isolated aortic sections devoid of endothelium. The * indicates an efflux which is significantly different (P<0.05) from the control efflux. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=8/group).

efflux ($P<0.05$) in relation to the control efflux (control 8.48 ± 0.97%, 0.8 µM 16.76 ± 2.56%, n=8; Figure 19). There was no significant difference between Rb efflux induced by pinacidil 0.4 and 0.6 µM and the control efflux.

Rat isolated aorta exposed to pinacidil (0.8 µM) for 60 seconds has been shown to produce a significantly increased Rb efflux relative to the control efflux. The subsequent studies therefore examine the effect of L-NAME, L-NIO, cromakalim and glibenclamide on the Rb efflux produced as a consequence of exposing rat isolated aorta to pinacidil (0.8 µM) for 60 seconds.

4.2 Effect of L-NAME on Pinacidil Induced Rb Efflux

Pinacidil (0.8 µM) caused a statistically significant ($P<0.05$) increase in Rb efflux relative to the control efflux (control 7.46 ± 0.69%, pinacidil 0.8 µM 10.95 ± 1.53%, n=16; Figure 20). L-NAME (100 µM) alone failed to modify the Rb efflux relative to the control (L-NAME 100 µM, 6.28 ± 0.69%). L-NAME (100 µM) and pinacidil (0.8 µM) produced a Rb efflux which was significantly lower ($P<0.05$) than the efflux induced by pinacidil alone (L-NAME and pinacidil 6.64 ± 0.69%).

As L-NAME (100 µM) alone failed to modify the Rb efflux relative to the control efflux, it was assumed that lower concentrations of L-NAME would also fail to modify the Rb efflux. The effect of a range of smaller L-NAME concentrations (10, 30, 50 and 75 µM) on the pinacidil induced Rb efflux showed that when rat isolated aorta was exposed to L-NAME (75 µM) and pinacidil (0.8 µM) for 60 seconds, a significantly lower ($P<0.05$) Rb efflux was recorded in relation to the pinacidil (0.8 µM) induced Rb efflux (L-NAME 75 µM and pinacidil 0.8 µM 6.17 ± 0.27%). Lower concentrations of L-NAME failed to modify the Rb efflux from that induced by pinacidil alone. As observed previously, pinacidil (0.8 µM) significantly increased ($P<0.05$) the Rb efflux relative to the control (control 6.82 ± 0.69%, pinacidil 0.8 µM 11.45 ± 1.60%, n=6; Figure 21).

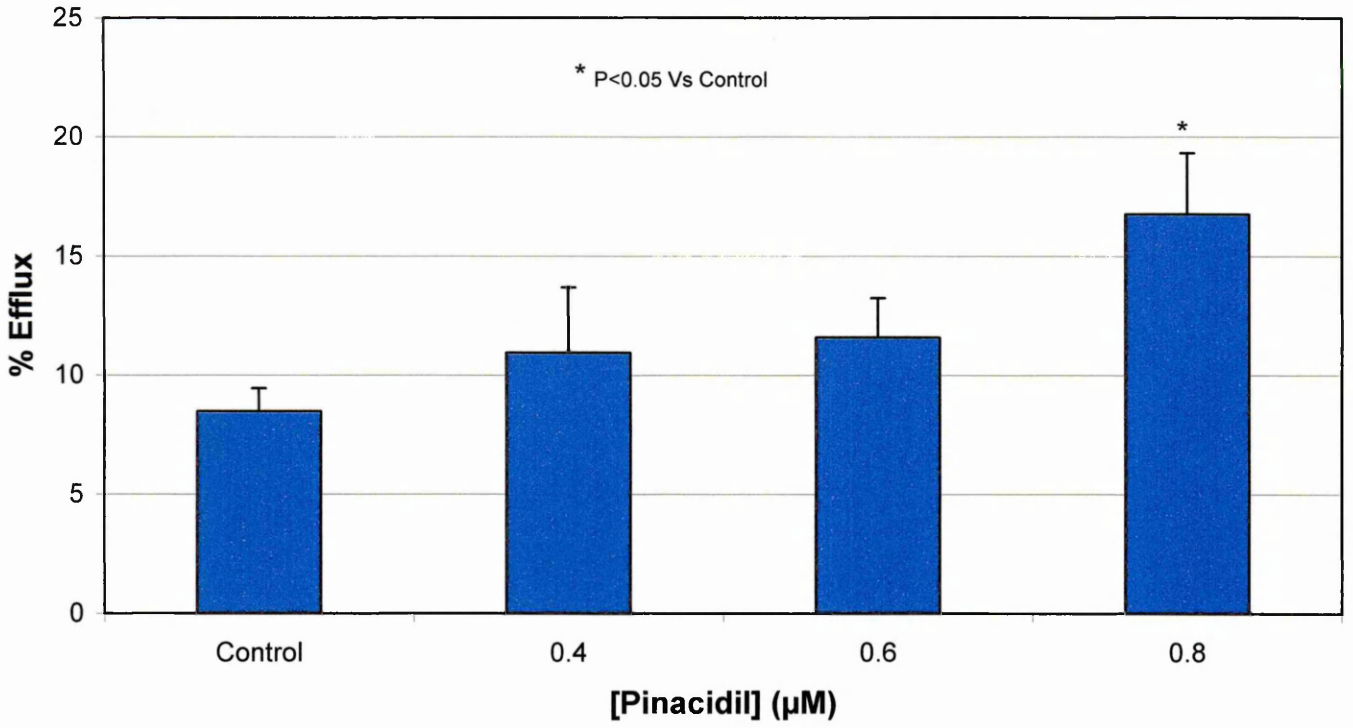


Figure 19. Effects of different pinacidil concentrations on Rb efflux in rat isolated aortic sections devoid of endothelium. The * indicates a significant difference ($P<0.05$) in Rb efflux in comparison with the control Rb efflux value. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=8/group).

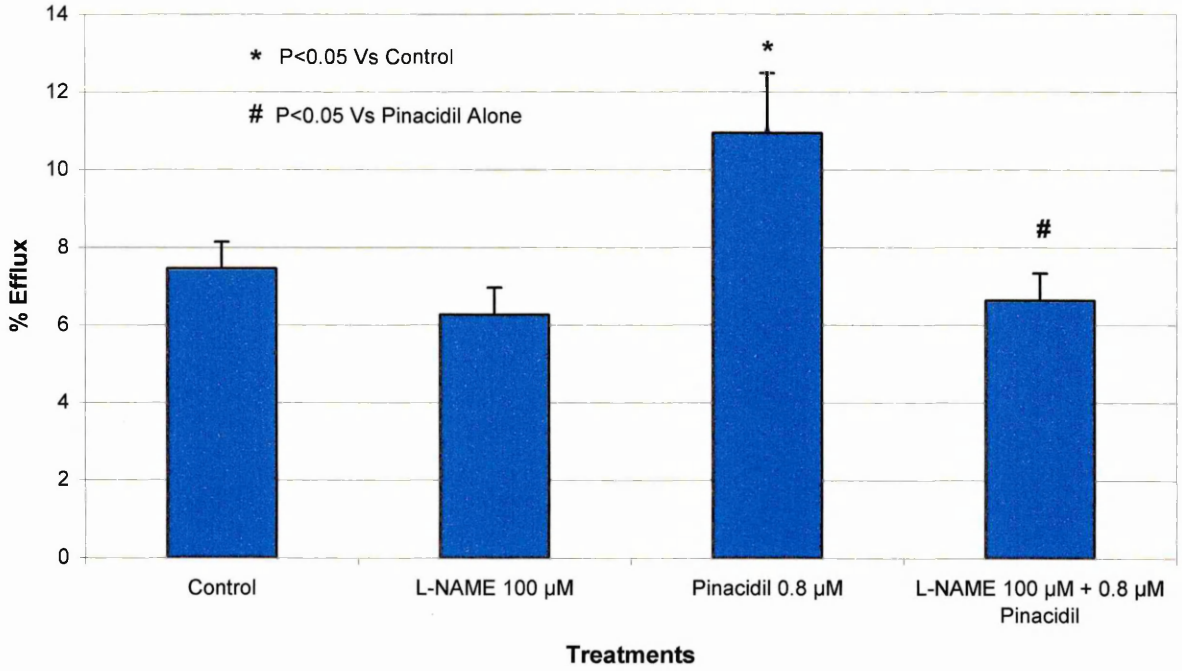


Figure 20. Effects of L-NAME on the Rb efflux responses to pinacidil in rat isolated aortic sections devoid of endothelium. The sections were exposed to L-NAME for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl. The symbols * and # indicate a significant difference ($P<0.05$) in Rb efflux in comparison with the control or pinacidil alone Rb efflux values respectively. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=16/group).

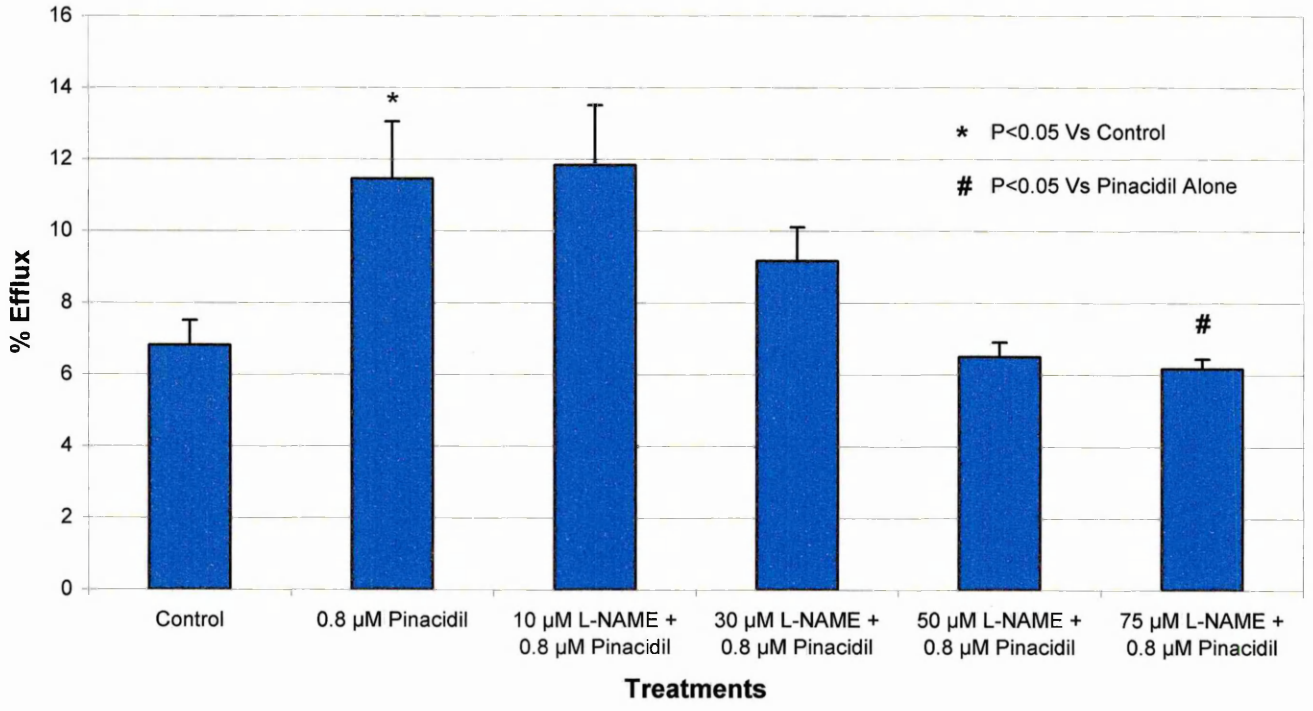


Figure 21. Effects of L-NAME on the Rb efflux responses to pinacidil in rat isolated aortic sections devoid of endothelium. The sections were exposed to L-NAME for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl and for the duration of the experiment with pinacidil. The symbols * and # indicate a significant difference ($P<0.05$) in Rb efflux in comparison with the control and pinacidil alone Rb efflux values respectively. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=6/group).

4.3 Effect of L-NIO on Pinacidil Induced Rb Efflux

In contrast to L-NAME, the pinacidil induced Rb efflux was not modified by L-NIO. Pinacidil (0.8 μM) significantly increased ($P<0.05$) the Rb efflux in relation to the control Rb efflux (control 6.04 ± 0.69%, pinacidil alone 9.69 ± 1.14%, n=11; Figure 22). L-NIO (100 μM) alone, failed to modify the Rb efflux from the control Rb efflux (L-NIO alone 5.42 ± 0.66%). L-NIO (100 μM) and pinacidil (0.8 μM), failed to modify the Rb efflux from either the control or the pinacidil induced Rb efflux (L-NIO 100 μM and pinacidil 0.8 μM 7.58 ± 0.61%).

These studies have shown that L-NAME (75 and 100 μM) but not L-NIO (100 μM) can significantly reduce ($P<0.05$) the pinacidil induced Rb efflux. Further studies could investigate the effect of larger L-NAME and L-NIO concentrations on the pinacidil induced Rb efflux.

4.4 Effect of Glibenclamide on Pinacidil Induced Rb Efflux

Pinacidil alone significantly increased ($P<0.05$) the Rb efflux relative to the control (control 3.56 ± 0.39%, pinacidil 0.8 μM 7.73 ± 1.02%, n=11; Figure 23A). Glibenclamide (10 μM) significantly lowered ($P<0.05$) the pinacidil induced Rb efflux in relation to the efflux stimulated by pinacidil alone (glibenclamide 10 μM and pinacidil 0.8 μM 4.97 ± 0.40%). In contrast, glibenclamide (1 μM) failed to modify the pinacidil induced Rb efflux (glibenclamide 1 μM and pinacidil 0.8 μM 5.84 ± 0.83%). Glibenclamide (1 and 10 μM) alone also did not modify the Rb efflux relative to the control (glibenclamide 1 μM 5.34 ± 0.91%, glibenclamide 10 μM 4.76 ± 0.35%).

4.5 Effect of Ouabain on Pinacidil Induced Rb Efflux

Although the pinacidil induced Rb efflux was higher than the control efflux, the effluxes were not significantly different (control 5.73 ± 1.03%, pinacidil 0.8 μM 8.39 ± 1.67%, n=7; Figure 23B). Ouabain alone (10 mM) failed to modify the Rb efflux relative to the control efflux (8.00 ± 0.52%). Ouabain (10 mM) also failed to modify the pinacidil induced Rb efflux (Ouabain 10 mM and pinacidil 0.8 μM 8.13 ± 0.91%). In summary, the pinacidil induced Rb efflux was shown to be modified by glibenclamide (10 μM) but not by ouabain.

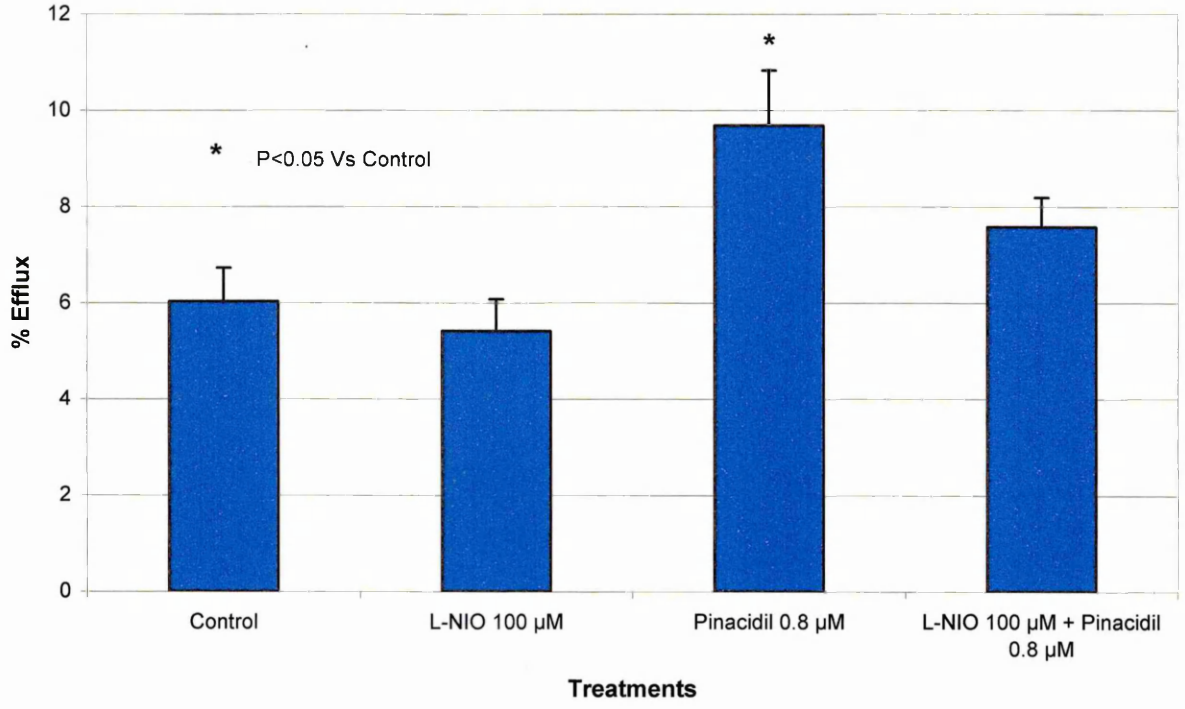


Figure 22. Effects of L-NIO on the Rb efflux responses to pinacidil in rat isolated aortic sections devoid of endothelium. The sections were exposed to L-NIO for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl and for the duration of the experiment with pinacidil. The * indicates a significant difference ($P<0.05$) in comparison with the control Rb efflux value. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=11/group).

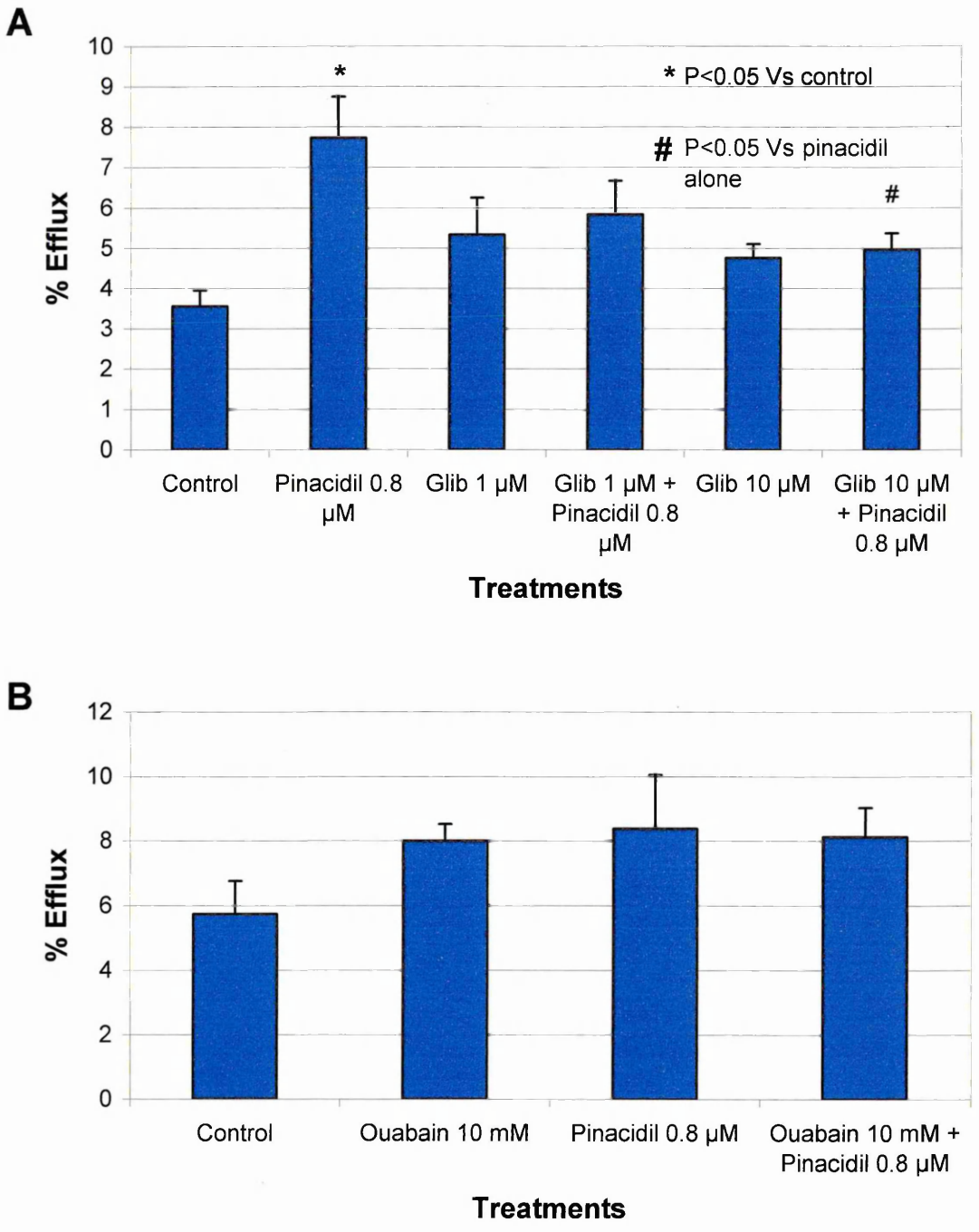


Figure 23. Effects of glibenclamide (**A**) and ouabain (**B**) on the Rb efflux responses to pinacidil in rat isolated aortic sections devoid of endothelium. The sections were exposed to glibenclamide or ouabain for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl and for the duration of the experiment with pinacidil. The symbols * and # indicate a significant difference ($P<0.05$) in Rb efflux in comparison with the control and pinacidil alone efflux values respectively. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=11 and 7/group) respectively.

4.6 Effect of Cromakalim on Rb Efflux

Cromakalim (1.2 μM) significantly increased ($P<0.05$) the Rb efflux in relation to the control efflux (control 5.06 ± 0.94%, cromakalim 1.2 μM 10.37 ± 0.47%, n=8; Figure 24). Cromakalim (0.4 and 0.8 μM) failed to modify the Rb efflux relative to the control (0.4 μM 7.50 ± 0.89% and 0.8 μM 7.44 ± 1.37%). As cromakalim (1.2 μM) produced a significantly increased Rb efflux in comparison to the control Rb efflux, the following studies examined the effects of L-NAME, glibenclamide and ouabain on a cromakalim (1.2 μM) induced Rb efflux over a 60 second time period.

4.7 Effect of L-NAME on Cromakalim Induced Rb Efflux

Cromakalim (1.2 μM) alone produced a significantly larger ($P<0.05$) Rb efflux in comparison to the control efflux (control 6.01 ± 1.13%, cromakalim alone 10.90 ± 2.43%, n=9; Figure 25). L-NAME alone, failed to modify the Rb efflux from the control efflux (L-NAME 5.49 ± 0.35%). In contrast to the pinacidil study, L-NAME failed to modify the cromakalim induced Rb efflux from the Rb efflux induced by the presence of cromakalim alone (L-NAME 100 μM and cromakalim 1.2 μM 10.94 ± 1.96%). The Rb efflux induced by cromakalim and L-NAME was significantly larger ($P<0.05$) than the control efflux. L-NAME is therefore able to modify the pinacidil but not the cromakalim induced Rb efflux.

4.8 The Effect of Glibenclamide on Cromakalim Induced Rb Efflux

The presence of cromakalim (1.2 μM) alone produced a significantly higher ($P<0.05$) Rb efflux in comparison with the control efflux (control 5.73 ± 0.45%, cromakalim 8.55 ± 1.42%, n=12; Figure 26A). Glibenclamide (1 μM) significantly reduced ($P<0.05$) the cromakalim induced Rb efflux (glibenclamide 1 μM and cromakalim 5.32 ± 0.79%). Glibenclamide (10 μM) failed to modify the cromakalim induced Rb efflux (glibenclamide 10 μM and cromakalim 5.85 ± 0.80%). Glibenclamide (1 and 10 μM) alone also failed to modify the Rb efflux in relation to the control efflux (glibenclamide 1 μM 5.38 ± 0.74%, glibenclamide 10 μM 5.27 ± 0.48%).

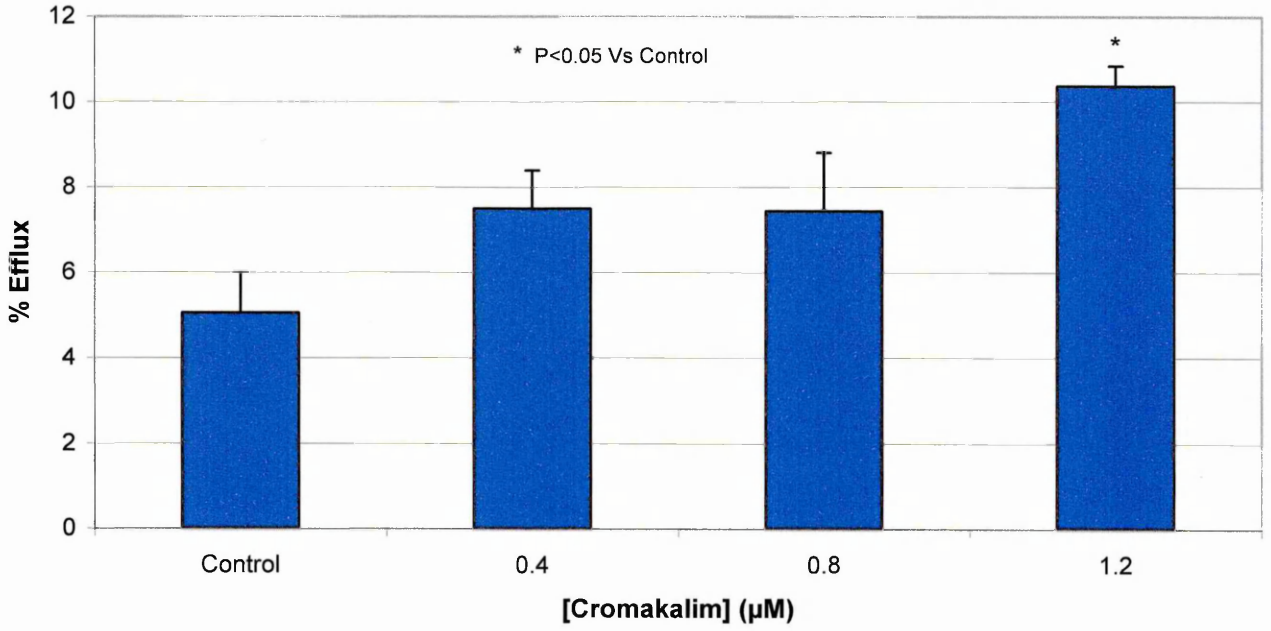


Figure 24. Effects of cromakalim on Rb efflux in rat isolated aortic sections devoid of endothelium. The * indicates a significant difference ($P<0.05$) in Rb efflux in comparison with the control Rb efflux value. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=8/group).

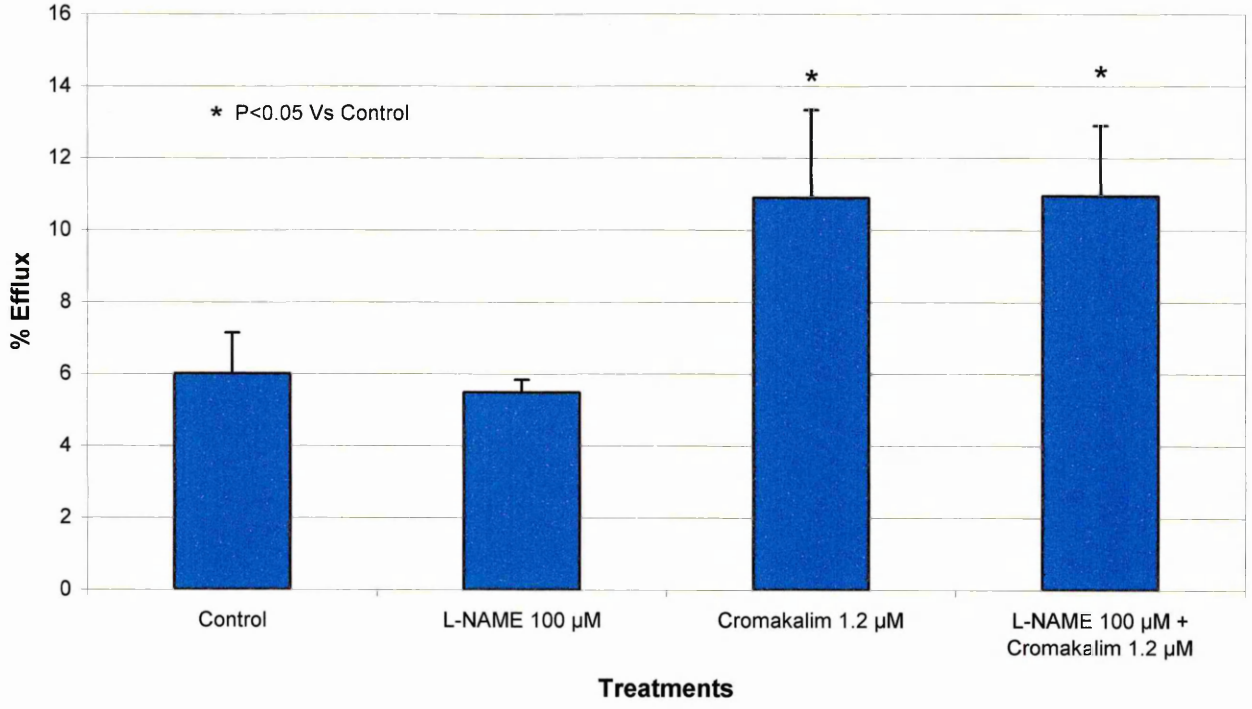


Figure 25. Effect of L-NAME and cromakalim on the Rb efflux in rat isolated aortic sections devoid of endothelium. The sections were exposed to L-NAME for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl and for the duration of the experiment with cromakalim. The * indicates a significant difference ($P<0.05$) in Rb efflux in comparison with the control Rb efflux value. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=9/group).

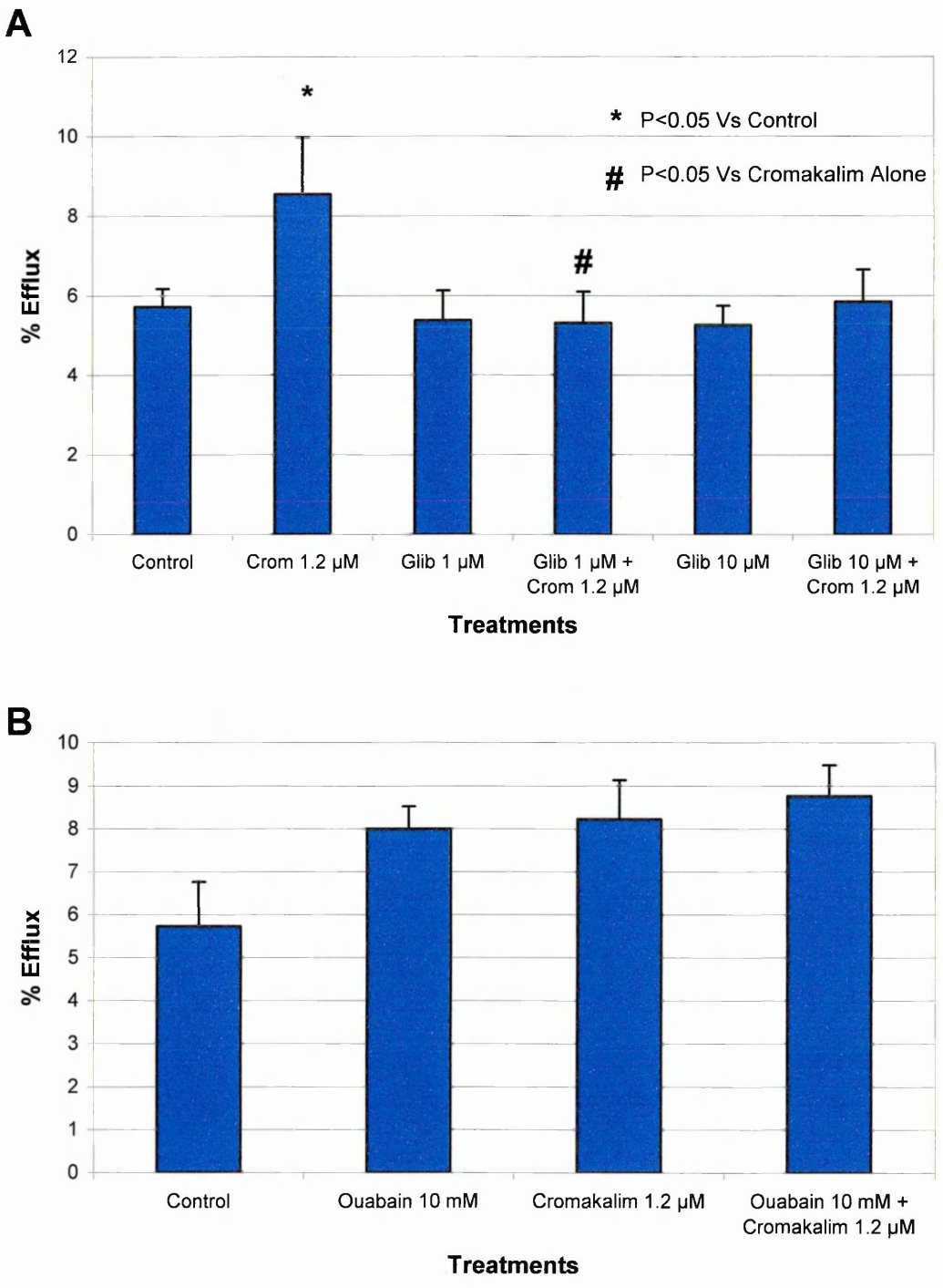


Figure 26. Effect of glibenclamide (1 and 10 µM) (**A**) and ouabain (**B**) on the Rb efflux responses to cromakalim in rat isolated aortic sections devoid of endothelium. The sections were exposed to glibenclamide or ouabain for the final 30 minutes of incubation in the Kreb's bicarbonate solution containing RbCl and for the duration of the experiment with cromakalim. The symbols * and # indicate a significant difference ($P<0.05$) in Rb efflux in comparison with the control or cromakalim alone Rb efflux values respectively. The % efflux values ± s.e.m. are derived from the Rb content of the supernatant and lysate fractions (n=12 and 7 /group) respectively.

4.9 The Effect of Ouabain on Cromakalim Induced Rb Efflux

Although the cromakalim induced Rb efflux was higher than the control efflux, the effluxes were not significantly different (control 5.73 ± 1.03%, cromakalim 8.23 ± 0.91%, n=7; Figure 26B). Ouabain (10 mM) alone failed to modify the Rb efflux from the control efflux (ouabain 8.00 ± 0.52%). Ouabain also failed to modify the cromakalim induced Rb efflux (ouabain and cromakalim 8.77 ± 0.72%). In summary, the cromakalim induced Rb efflux was shown to be modified by glibenclamide (1 μM) but not by ouabain.

5 Cyanoguanidine K_{ATP}COs

Pinacidil-induced vasorelaxations in rat isolated aorta have been shown to be sensitive to the L-arginine analogue, L-NAME (100 and 300 μM). In contrast, cromakalim-induced vasorelaxations have been shown to be insensitive to L-NAME (100 μM). In contrast, the pinacidil-induced vasorelaxations are not modified by the L-ornithine analogue, L-NIO (100 μM). However, two other L-arginine analogues, L-MMA (100 μM) and L-NNA (100 μM), have been shown to modify the vasorelaxant responses to pinacidil. The sensitivity of other cyanoguanidine K_{ATP}COs to L-arginine analogues is explored in this study.

5.1 Vasorelaxant Responses to Cyanoguanidine K_{ATP}COs

The exposure of endothelium denuded rat aortic preparations to L-MMA (100 μM), L-NIO (100 μM) or methylene blue (10 μM) did not modify the base line tension.

Name of K_{ATP}CO	Concentration Range (μM)
KB-R6844	0.05 – 25.6
KB-R6907	0.05 – 25.6
KB-R10757	0.05 – 25.6
KB-R10101	0.05 – 12.8
KB-R10758	0.001 – 0.256

Table 4. Concentration ranges of the cyanoguanidine K_{ATP}COs required to produce a concentration related relaxation of phenylephrine contracted rat aorta, devoid of an endothelium.

KB-R6844

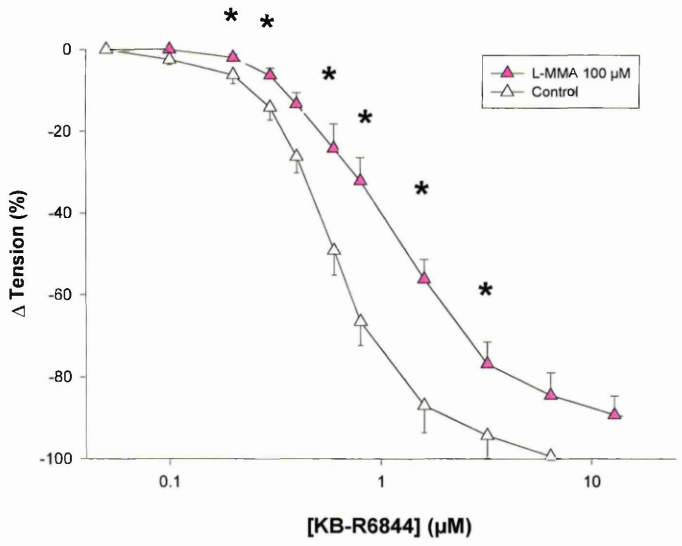
L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R6844 to the right of the control curve, without modifying the maximal response (Control EC_{50} 0.66 ± 0.05 μM, L-MMA EC_{50} 1.68 ± 0.29 μM, CR(cl) 2.67 (1.50-3.85), E_{max} 100 %, n=7, Hill co-efficient 1.93 ± 0.21) (Figure 27). In the presence of L-MMA (100 μM) a significantly ($P<0.05$) larger concentration of KB-R6844 was required to produce the same degree of relaxation as seen in the control sections between KB-R6844 concentrations ranging from 0.2 to 3.2 μM. L-NIO (100 μM) failed to modify the CRC to KB-R6844 (Control EC_{50} 1.13 ± 0.16 μM, L-NIO EC_{50} 1.35 ± 0.28 μM, n=5; Figure 27). Methylene blue (10 μM) also failed to modify the CRC to KB-R6844 (Control EC_{50} 1.20 ± 0.17, Methylene Blue EC_{50} 1.24 ± 0.16 μM, n=4; Figure 27).

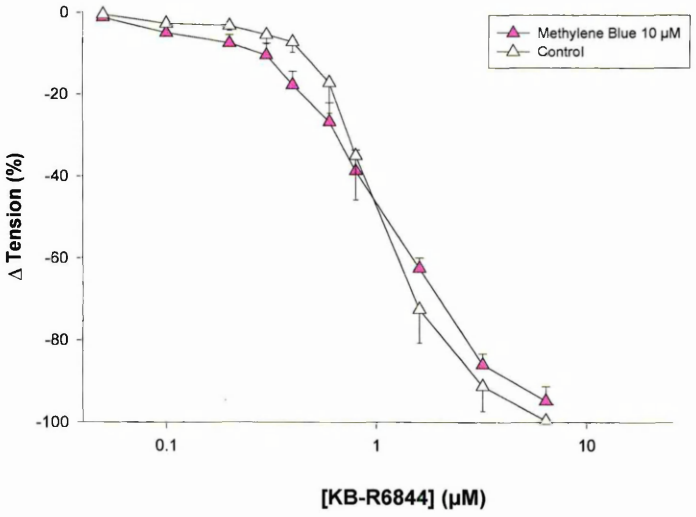
KB-R6907

L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R6907 to the right of the control curve, without modifying the maximal response (Control EC_{50} 2.28 ± 0.08 μM, L-MMA EC_{50} 7.48 ± 1.79 μM, CR(cl) 3.22 (1.66-4.77), E_{max} = 100 %, n=5, Control Hill co-efficient 1.35 ± 0.17; Figure 28). In the presence of L-MMA (100 μM), a significantly ($P<0.05$) larger concentration of KB-R6907 was required to produce the same degree of relaxation as seen in the control sections between KB-R6907 concentrations ranging from 0.4 to 12.8 μM. L-NIO (100 μM) failed to modify the CRC to KB-R6907 (Control EC_{50} 2.64 ± 0.35 μM, L-NIO EC_{50} 4.82 ± 1.46 μM, n=5; Figure 28). Methylene blue (10 μM) also failed to modify the CRC to KB-R6907 (Control EC_{50} 2.44 ± 0.59 μM, methylene blue EC_{50} 5.76 ± 1.78 μM, n=4; Figure 28).

KB-R10757

L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R10757 to the right of the control curve, without modifying the maximal response (Control





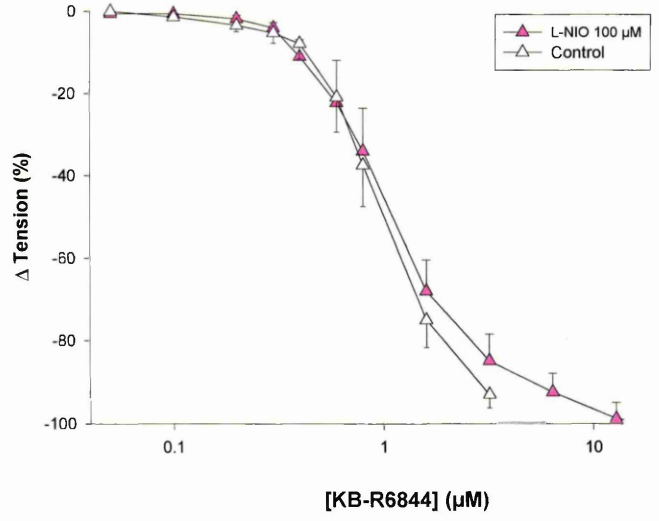
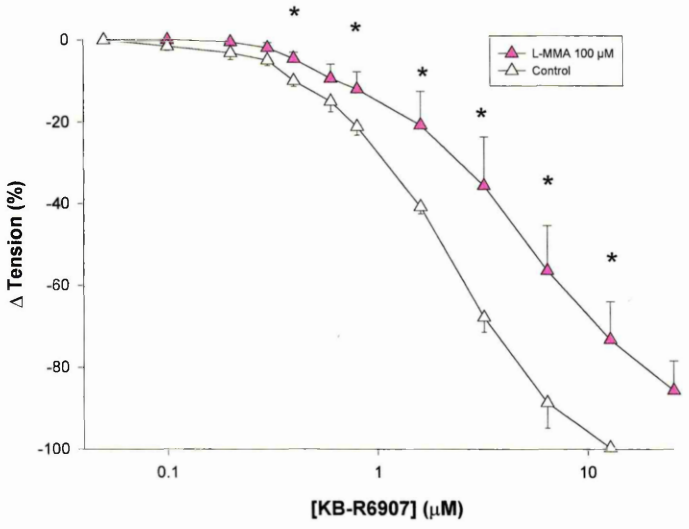
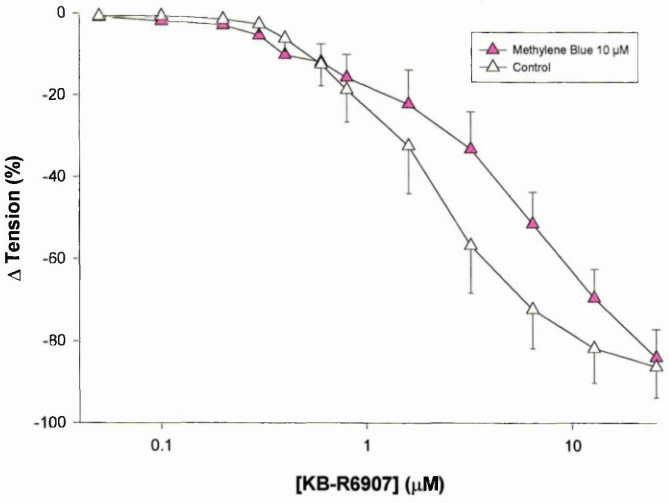


Figure 27. Effects of L-MMA, L-NIO and methylene blue on the vasorelaxant responses to KB-R6844 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to KB-R6844 were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to either solvent, L-MMA (100 µM), L-NIO (100 µM) or methylene blue (10 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=7,5 and 4/group) respectively. The * indicates responses in the presence of L-MMA that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratio with confidence limits with respect to the control for L-MMA against KB-R6844 is CR(cl) 2.67 (1.50-3.85).





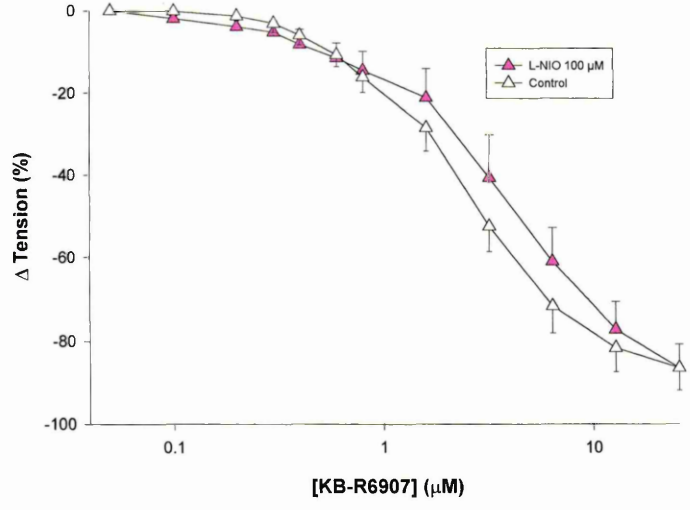


Figure 28. Effect of L-MMA, L-NIO and methylene blue on the vasorelaxant responses to KB-R6907 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to KB-R6907 were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to control, L-MMA (100 µM), L-NIO (100 µM) or methylene blue (10 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=5,5 and 4/group) respectively. The * responses in the presence of L-MMA were significantly different ($P<0.05$) from corresponding control responses. The concentration ratio in respect to the control for KB-R6907 against L-MMA is CR(cl) 3.22 (1.66-4.77).

EC_{50} 1.07 ± 0.30 μM, L-MMA EC_{50} 1.99 ± 0.35 μM, CR(cl) 2.12 (1.32-2.93), E_{max} 100 %, n=5, Control Hill co-efficient 1.45 ± 0.20; Figure 29). In the presence of L-MMA (100 μM), a significantly ($P<0.05$) larger concentration of KB-R10757 was required to produce the same degree of relaxation as seen in the control sections between KB-R10757 concentrations ranging from 0.3 to 1.6 μM. L-NIO (100 μM) failed to modify the CRC to KB-R10757 (Control EC_{50} 1.54 ± 0.36 μM, L-NIO EC_{50} 2.08 ± 0.35 μM, n=5; Figure 29).

KB-R10101 and KB-R10758

In contrast to pinacidil, the relaxant responses to KB-R10101 were not modified by L-MMA (100 μM) (Control EC_{50} 0.16 ± 0.03 μM, L-MMA EC_{50} 0.19 ± 0.05 μM, E_{max} 100%, n=7; Figure 30). In addition, L-MMA (100 μM) also failed to modify the relaxant responses to KB-R10758 (Control EC_{50} 0.0024 ± 0.0003 μM, L-MMA EC_{50} 0.0027 ± 0.0005 μM, E_{max} 100%, n=5, Control Hill co-efficient 2.42 ± 0.72; Figure 30)

L-MMA (100 μM) significantly modified ($P<0.05$) the CRCs to pinacidil, KB-R6844, KB-R6907 and KB-R10757. In contrast, L-MMA (100 μM) failed to modify the CRCs to KB-R10101 and KB-R10758. The NOS inhibitor, L-NIO (100 μM) was unable to modify the CRCs to pinacidil, KB-R6844, KB-R6907 and KB-R10757. In addition, the guanylate cyclase inhibitor, methylene blue (10 μM) failed to modify the CRC to KB-R6844 and KB-R6907.

5.2 Vasorelaxant Responses to Cyanoguanidine K_{ATP}COs in Rat Isolated Aorta with a Functional Endothelium

In rat isolated aorta with a functional endothelium (relaxant response to acetylcholine (1 μM)), phenylephrine (1 μM) evoked a contraction (0.75 – 1.6 g) that maintained a stable plateau for at least 120 minutes in control preparations.

Figure 29. Effects of L-MMA and L-NIO on the vasorelaxant responses to KB-R10757 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to KB-R10757 were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent, L-MMA (100 μM) or L-NIO (100 μM). The * indicates responses in the presence of L-MMA that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratio with confidence limits with respect to the control for L-MMA against KB-R10757 is CR(cl) 2.12 (1.32-2.93). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values $\pm$ s.e.m. (n=5 /group).

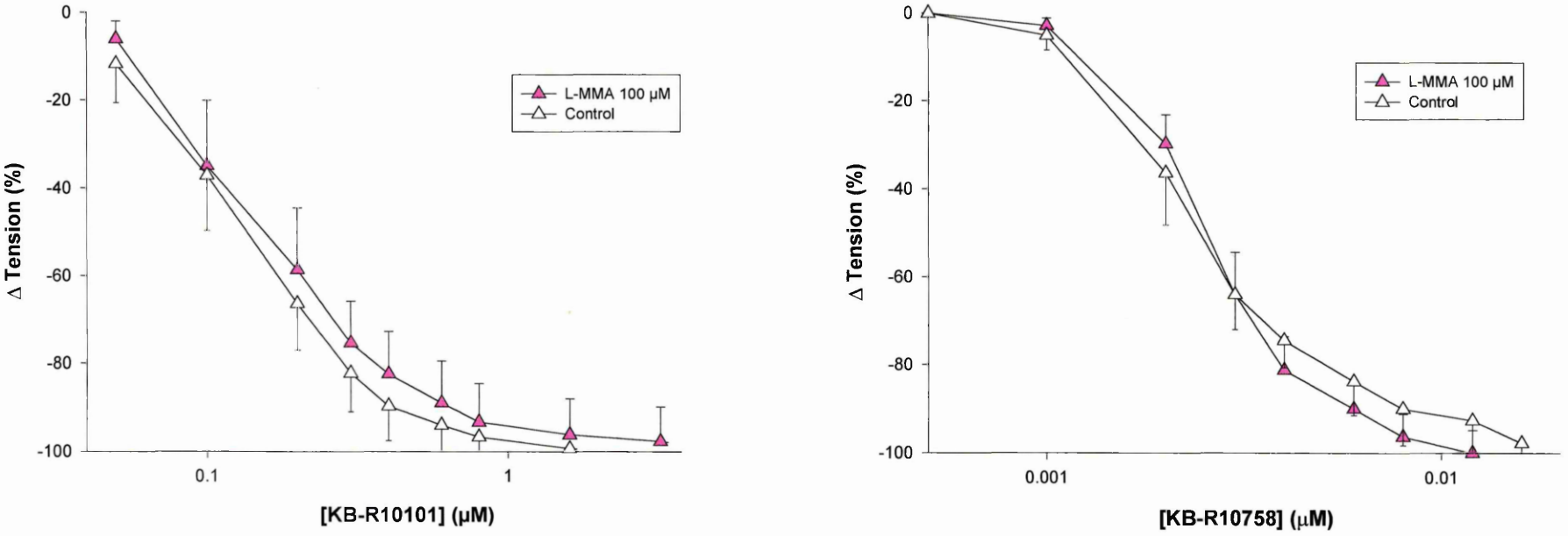


Figure 30. Effects of L-MMA on the vasorelaxant responses to KB-R10101 and KB-R10758 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to KB-R10101 and KB-R10758 were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-MMA (100 μM). Relaxations are expressed as a percentage of the contractile response prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=7 and 5/group) respectively.

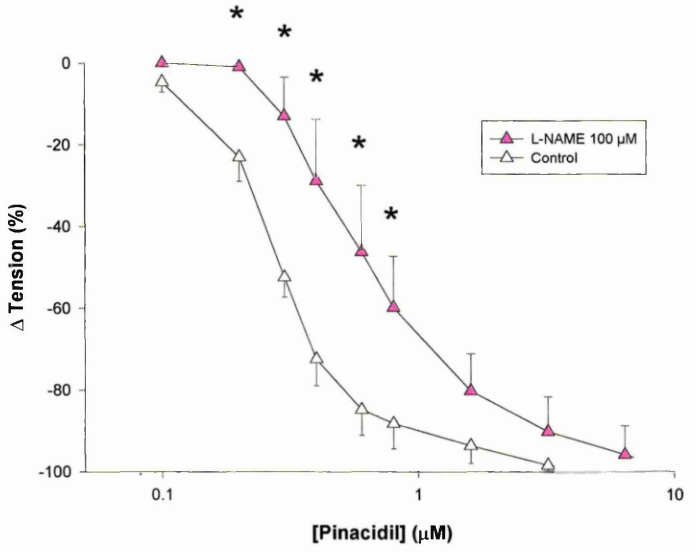
Exposure of rat aorta preparations with a functional endothelium to L-NAME (100 μM), L-MMA (100 μM), L-NNA (100 μM), L-NIO (100 μM) or methylene blue (10 μM) did not modify the base line tension.

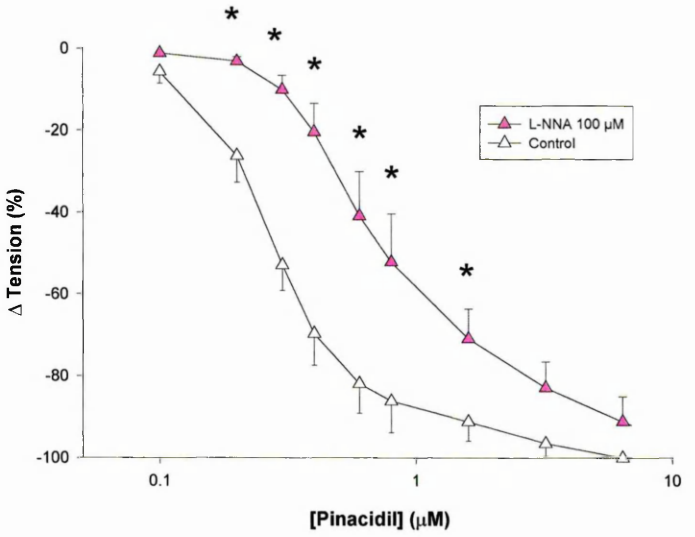
Name of K_{ATP}CO	Concentration Range (μM)
Pinacidil	0.1 – 25.6
KB-R6844	0.05 – 25.6
KB-R6907	0.05 – 25.6
KB-R10758	0.0005 – 0.256

Table 5. Concentration ranges of the cyanoguanidine K_{ATP}COs required to produce a concentration related relaxation of phenylephrine contracted rat aorta with a functional endothelium

Pinacidil

L-NAME (100 μM) significantly displaced the CRC to pinacidil to the right of the control curve, without modifying the maximal response (Control EC_{50} 0.32 ± 0.04 μM, L-NAME EC_{50} 0.87 ± 0.25 μM, CR(cl) 2.69 (1.23-4.15), E_{max} 100 %, n=5; Figure 31). L-MMA (100 μM) significantly displaced the CRC to pinacidil to the right of the control curve without modifying the maximal response (Control EC_{50} 0.33 ± 0.05 μM, L-MMA 0.66 ± 0.11 μM, CR(cl) 2.10 (1.25-2.95), E_{max} 100%, n=4; Figure 31). L-NNA (100 μM) significantly displaced the CRC to pinacidil to the right of the control curve without modifying the maximal response (Control EC_{50} 0.32 ± 0.05 μM, L-NNA 0.89 ± 0.20 μM, CR(cl) 3.03 (1.07-4.98), E_{max} 100%, n=4; Figure 31).





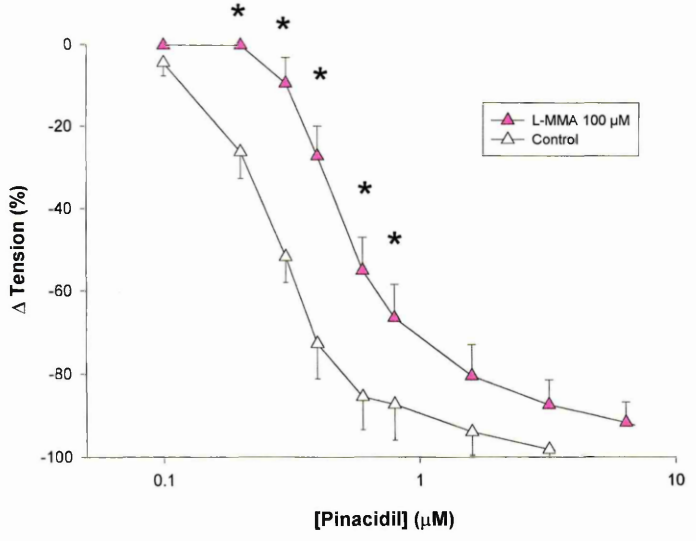


Figure 31. Effects of L-NAME, L-MMA and L-NNA on the vasorelaxant responses to pinacidil in rat isolated aortic rings with endothelium. Cumulative concentration-response curves to pinacidil were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to either solvent, L-NAME (100 µM), L-MMA (100 µM) or L-NNA (100 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=5,4 and 4/group) respectively. The * indicates responses in the presence of L-NAME, L-MMA and L-NNA that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with confidence limits with respect to the control for L-NAME, L-MMA and L-NNA against pinacidil are CR(cl) 2.69 (1.23-4.15), 2.10 (1.25-2.95) and 3.03 (1.07-4.98) respectively.

KB-R6844

L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R6844 without modifying the maximal response (Control EC_{50} 0.39 ± 0.10 μM, L-MMA EC_{50} 1.82 ± 0.70 μM, CR(cl) 4.43 (2.50-6.36), E_{max} 100%, n=5; Figure 32). L-NIO (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R6844 without modifying the maximal response (Control EC_{50} 0.40 ± 0.10 μM, L-NIO EC_{50} 2.13 ± 0.43 μM, CR(cl) 8.46 (1.75-15.18), E_{max} 100 %, n=5; Figure 32). Methylene blue (10 μM) significantly ($P<0.05$) displaced the CRC to KB-R6844 without modifying the maximal response (Control EC_{50} 0.35 ± 0.14 μM, methylene blue EC_{50} 1.66 ± 0.49 μM, CR(cl) 6.26 (1.54-10.97), E_{max} 100 %, n=3; Figure 32).

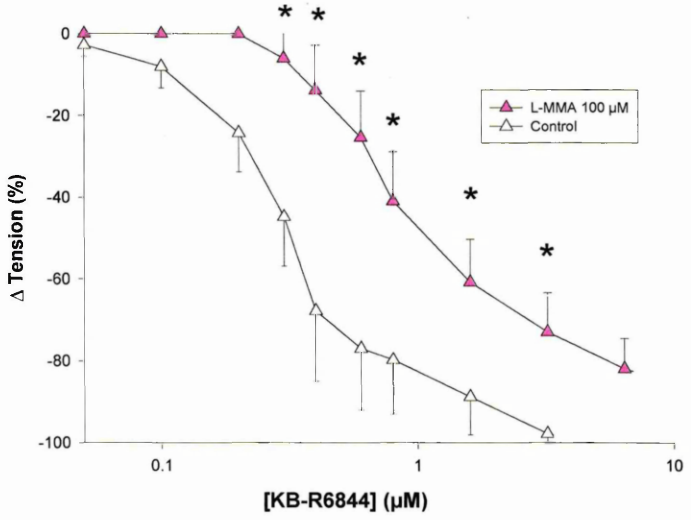
KB-R6907

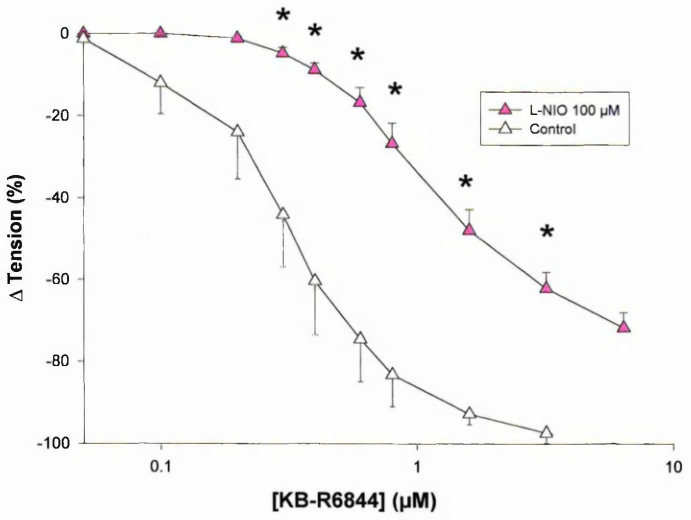
L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to KB-R6907 to the right of the control curve, without modifying the maximal response (Control 0.80 ± 0.32 μM, L-MMA EC_{50} 2.34 ± 0.33 μM, CR(cl) 3.56 (1.87-5.24), E_{max} 100 %, n=5; Figure 33).

KB-R10758

KB-R10758 (0.0005 – 0.256 μM) produced a concentration related relaxation of phenylephrine contracted rat aorta. In contrast, L-MMA (100 μM) failed to modify the CRC to KB-R10758 (Control EC_{50} 0.0020 ± 0.0003 μM, L-MMA EC_{50} 0.0024 ± 0.0005, E_{max} 100 %, n=5; Figure 33).

L-MMA (100 μM) was able to significantly modify the vasorelaxant responses to pinacidil, KB-R6844 and KB-R6907 but not to KB-R10758 in rat isolated aorta with and without a functional endothelium. The differences between the vasorelaxant studies in rat isolated aorta with and without a functional endothelium, were found in relation to L-NIO and methylene blue. In rat





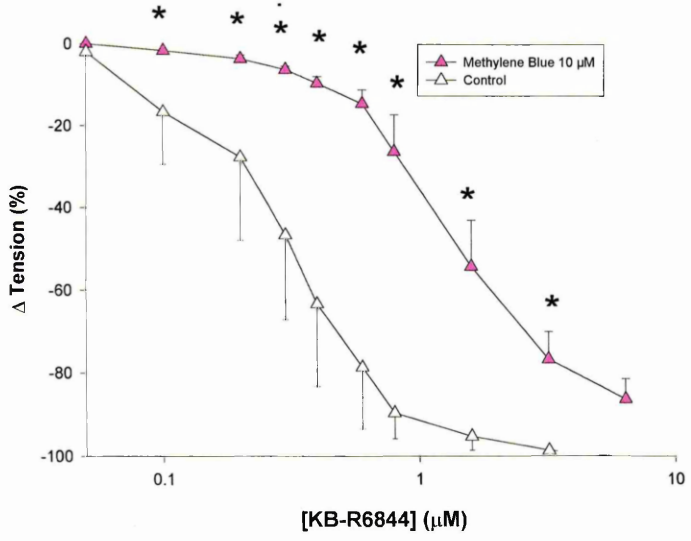


Figure 32. Effects of L-MMA, L-NIO and methylene blue on the vasorelaxant responses to KB-R6844, in rat isolated aortic rings with endothelium. Cumulative concentration-response curves to this compound were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to either solvent, L-MMA (100 µM), L-NIO (100 µM) or methylene blue (10 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=5,5 and 3/group) respectively. The * indicates responses in the presence of L-MMA, L-NIO and methylene blue, that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with confidence limits with respect to the control for L-MMA, L-NIO and methylene blue against KB-R6844 are, CR(cl) 4.43 (2.50-6.36), 8.46 (1.75-15.18) and 6.26 (1.54-10.97) respectively.

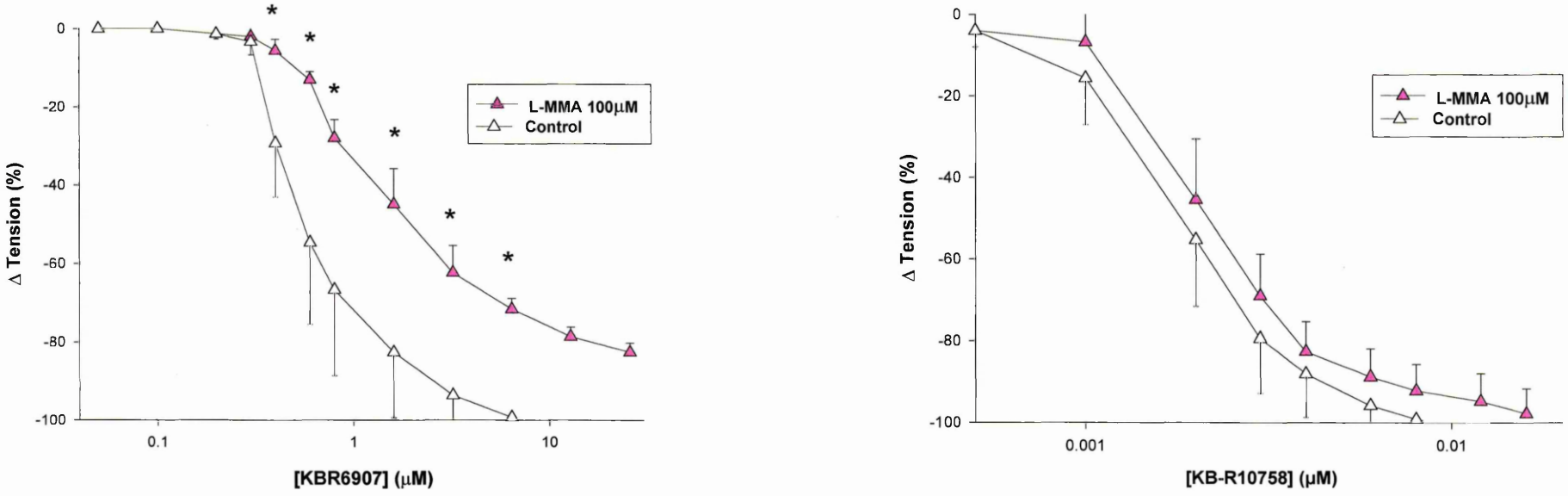


Figure 33. Effects of L-MMA on the vasorelaxant responses to KB-R6907 and KB-R10758 in rat isolated aortic rings with endothelium. Cumulative concentration-response curves to these compounds were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either control or L-MMA (100 μM). Relaxations are expressed as a percentage of the contractile response just prior to the application of K_{ATP}CO. Data are mean values ± s.e.m. (n=5/group). The * responses in the presence of L-MMA were significantly different ($P<0.05$) from corresponding control responses. The concentration ratio in preparations with endothelium, with respect to the control for L-MMA against KB-R6907 is CR(cl) 3.56 (1.87-5.24).

isolated aorta devoid of a functional endothelium, L-NIO and methylene blue failed to modify the vasorelaxant responses to any of the cyanoguanidine K_{ATP}COs. In the presence of a functional endothelium, L-NIO and methylene blue modified the vasorelaxant responses to KB-R6844.

6 Benzopyran K_{ATP}COs

Cromakalim-induced vasorelaxations in rat isolated aorta have been shown to be insensitive to the L-arginine analogue L-NAME. The sensitivity of the benzopyran K_{ATP}COs, DY-9708, SKP-450, bimakalim and symakalim (Figure 34) to L-arginine analogues in contracted rat isolated aorta devoid of endothelium is explored in this study.

6.1 Vasorelaxant Responses to Benzopyran K_{ATP}COs

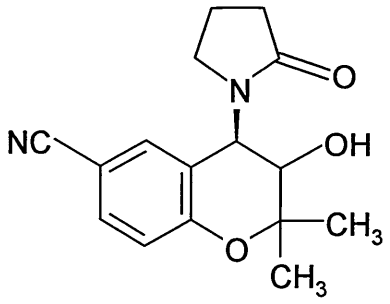
The exposure of endothelium denuded rat aorta preparation to L-NAME (100 or 300 μM), L-MMA (100 μM), L-NNA (100 μM) or L-NIO (100 μM) did not modify the base-line tension.

Name of K_{ATP}CO	Concentration Range
DY-9708	0.004 – 0.256 μM
SKP-450	0.1 – 64 nM
Bimakalim	0.004 – 0.256 μM
Symakalim	0.004 – 0.256 μM

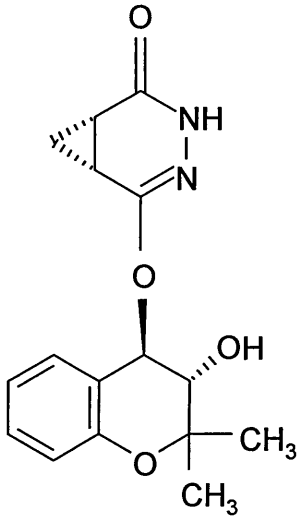
Table 6. Concentration ranges of the benzopyran K_{ATP}COs required to produce a concentration related relaxation of phenylephrine contracted rat aorta.

DY-9708 and SKP-450

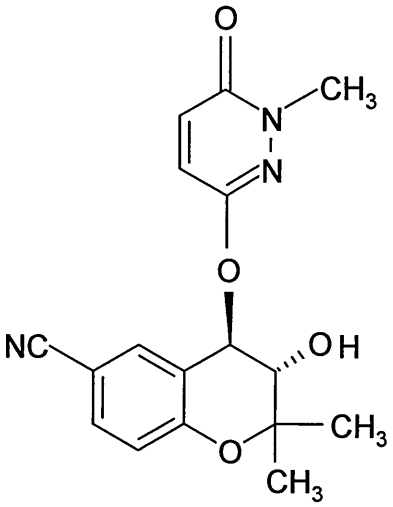
L-NAME (100 μM) significantly ($P<0.05$) displaced the CRC to DY-9708 to the right of the control curve without modifying the maximal response (Control EC_{50} 0.016 ± 0.002 μM, L-NAME EC_{50} 0.026 ± 0.005 μM, CR(cl) 1.93 (1.36-2.51), E_{max} 74 ± 10 %, n=10, Control Hill co-efficient 2.88 ± 0.63; Figure 35). L-NAME (300 μM) significantly ($P<0.05$) displaced the CRC to DY-9708, but failed to increase the displacement produced by L-NAME (100 μM) EC_{50} 0.030 ± 0.006 μM, CR(cl) 2.60(1.13-4.08). In contrast to DY-9708, the



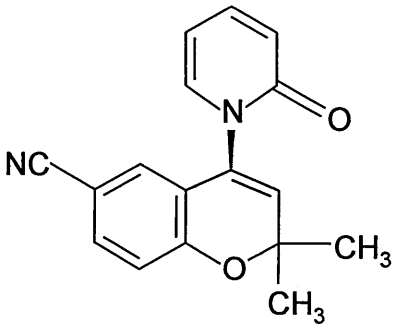
Cromakalim



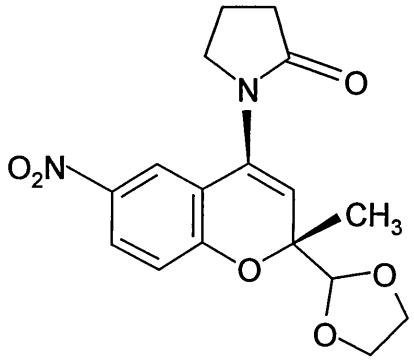
DY-9708



Symakalim



Bimakalim



SKP-450

Figure 34. Structures of the benzopyran K_{ATP}COs studied.

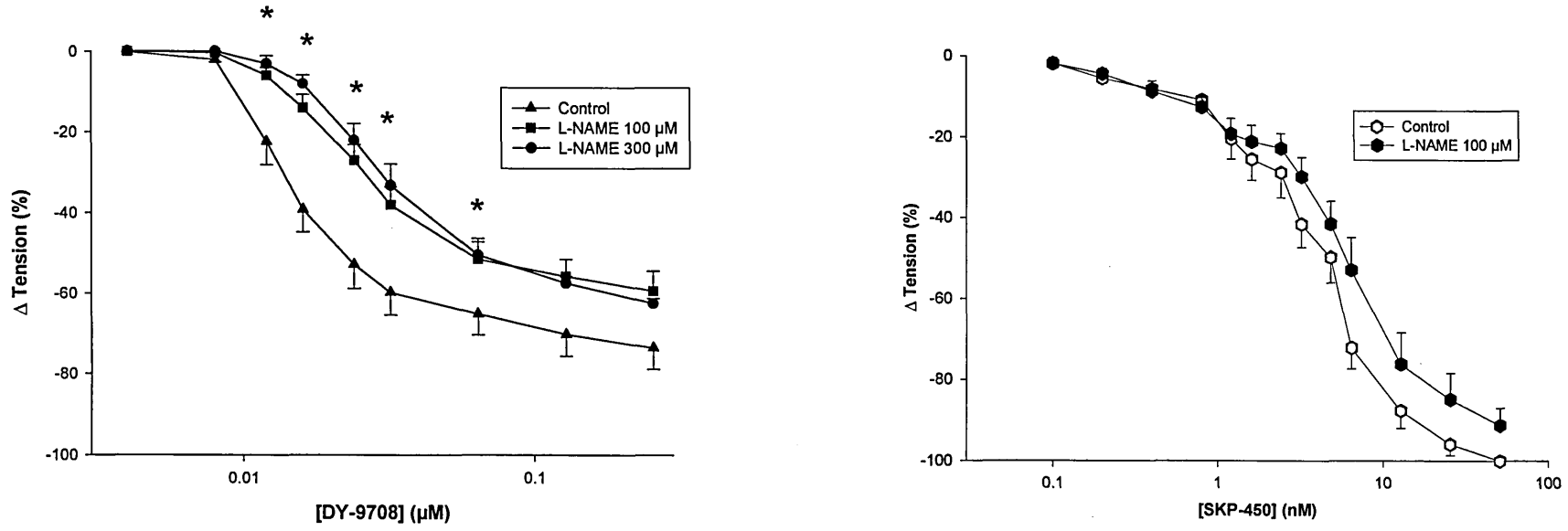


Figure 35. Effects of L-NAME on the vasorelaxant responses to DY-9708 and SKP-450 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-responses curves to DY-9708 and SKP-450 were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-NAME (100 or 300 μM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=10 and 6 per group). The * indicates responses in the presence of L-NAME (100 and 300 μM) that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with respect to the control for L-NAME (100 or 300 μM) against DY-9708 are CR(cl) 1.93 (1.36-2.51) and 2.60 (1.13-4.08) respectively.

relaxant responses to SKP-450 (Figure 35) in rat isolated aorta were not modified by L-NAME (100 μM) (Control EC_{50} 4.1 ± 0.5 nM, L-NAME EC_{50} 7.0 ± 2.9 nM, E_{max} 100%, n=6).

L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to DY-9708 to the right of the control curve without modifying the maximal response (Control EC_{50} 0.016 ± 0.003 μM, L-MMA EC_{50} 0.039 ± 0.007 μM, CR(cl) 2.48 (1.42-3.53), E_{max} 94 ± 5%, n=7; Figure 36). L-NNA (100 μM) significantly ($P<0.05$) displaced the CRC to DY-9708 to the right of the control curve without modifying the maximal response (Control EC_{50} 0.016 ± 0.003 μM, L-NNA EC_{50} 0.038 ± 0.011 μM, CR(cl) 1.56 (1.01-2.11), E_{max} 94 ± 5%, n=7, Figure 36). In contrast, L-NIO (100 μM) failed to modify the CRC to DY-9708 (Control EC_{50} 0.018 ± 0.004 μM, L-NIO EC_{50} 0.022 ± 0.001, n=4, Figure 37).

Bimakalim and Symakalim

L-MMA (100 μM) significantly ($P<0.05$) displaced the CRC to bimakalim to the right of the control curve without modifying the maximal response (Control EC_{50} 0.018 ± 0.003 μM, L-MMA EC_{50} 0.026 ± 0.003 μM, CR(cl) 1.57 (1.09-2.05), E_{max} 100 %, n=6, Hill co-efficient 1.32 ± 0.42). In contrast, L-MMA (100 μM) failed to modify the CRC to symakalim (Control EC_{50} 0.012 ± 0.002 μM, L-MMA EC_{50} 0.015 ± 0.004 μM, E_{max} 100 %, n=6, Hill co-efficient 2.96 ± 0.46; Figure 38).

L-MMA (100 μM) and L-NAME (100 μM) but not L-NIO (100 μM) were able to modify the vasorelaxant responses to DY-9708. L-MMA also modified the vasorelaxant responses to bimakalim but not to symakalim. In contrast, L-NAME was unable to modify the vasorelaxant responses to either cromakalim or SKP-450.

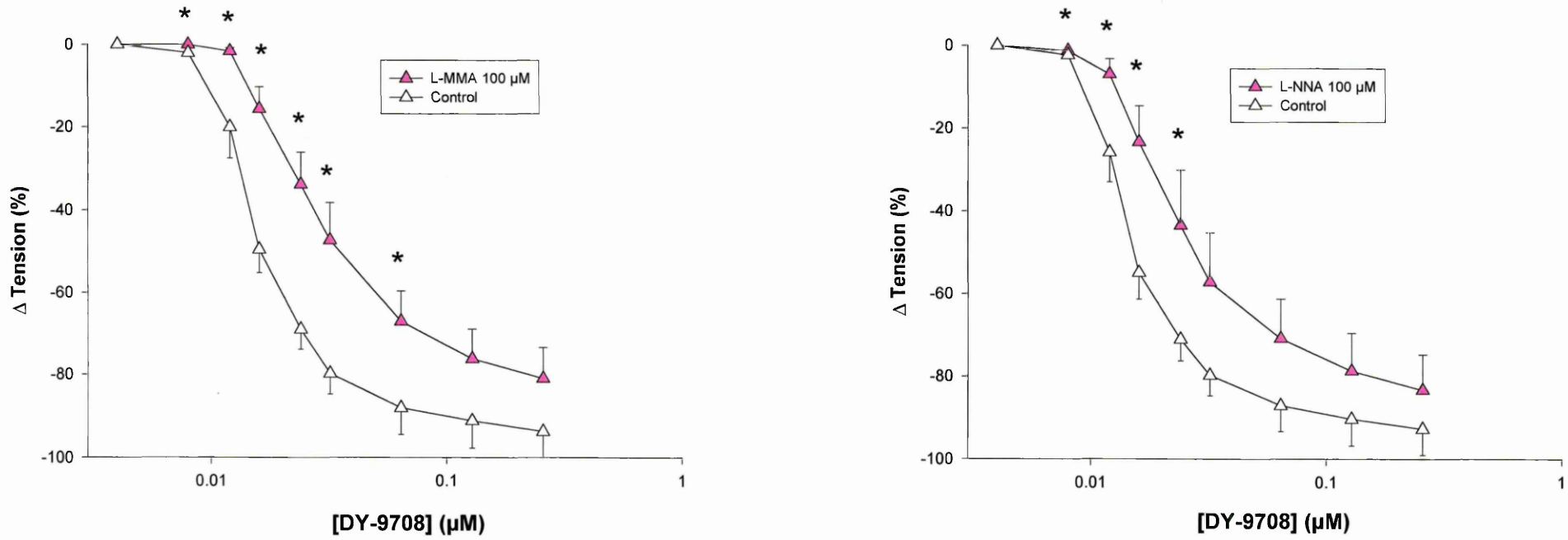


Figure 36. Effects of L-MMA and L-NNA on the vasorelaxant responses to DY-9708 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to DY-9708 were constructed in phenylephrine (1 µM) contracted preparations 30 minutes after exposure to either solvent or L-MMA (100 µM) or L-NNA (100 µM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=7/group). The * indicates responses in the presence of L-MMA and L-NNA that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratios with confidence limits with respect to the control for L-MMA and L-NNA against DY-9708 are CR(cl) 2.48 (1.42-3.53) and CR(cl) 1.56 (1.01-2.11) respectively.

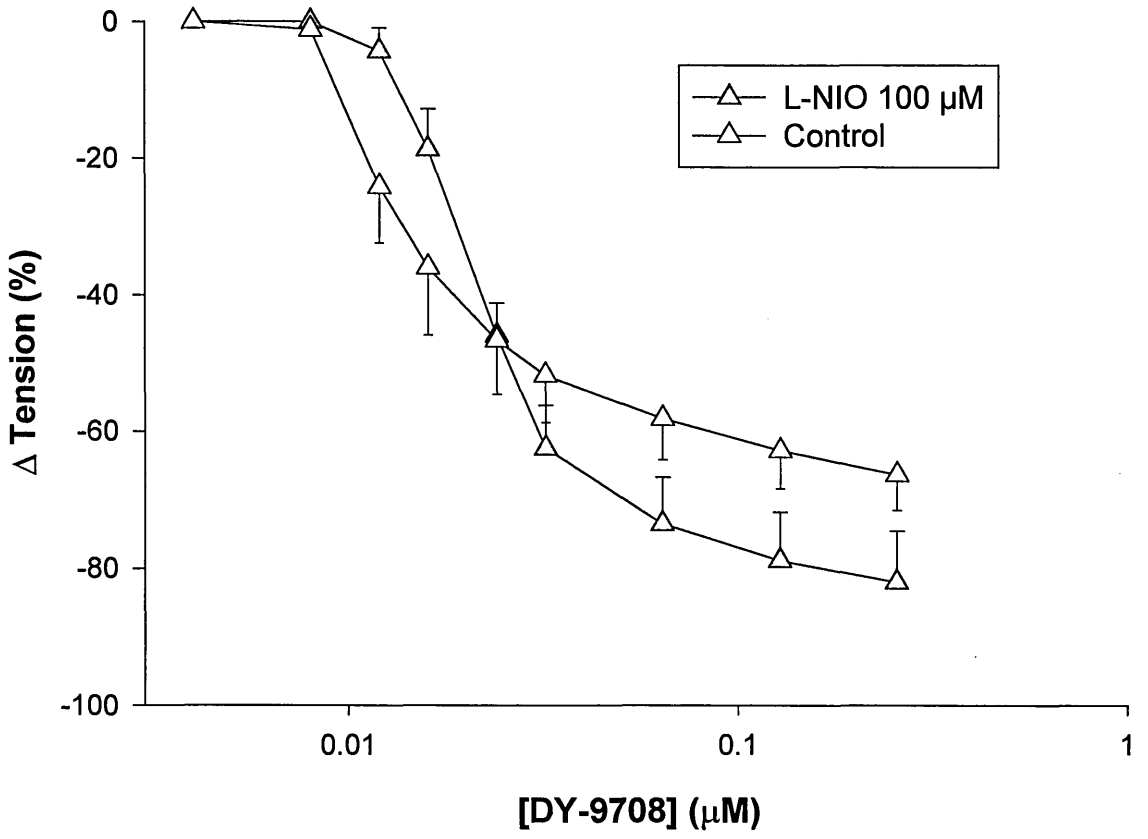


Figure 37. Effects of L-NIO on the vasorelaxant responses to DY-9708 in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to DY-9708 were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-NIO (100 μM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=4/group).

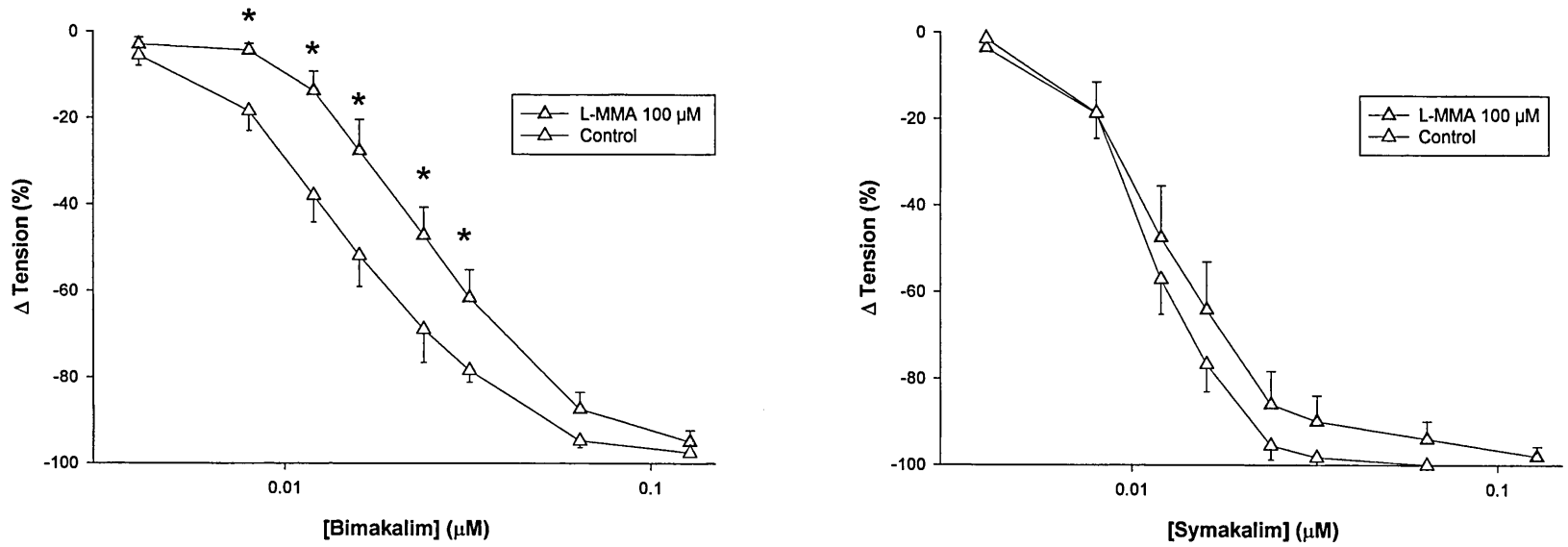


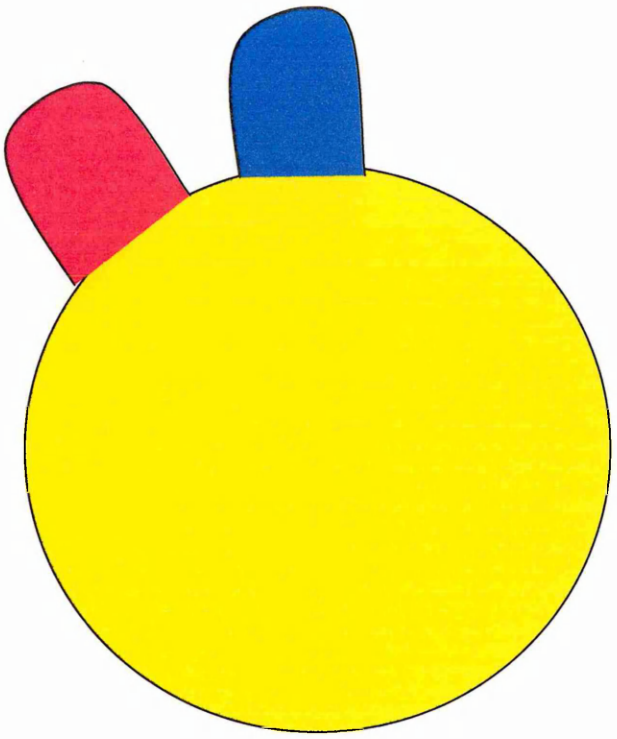
Figure 38. Effects of L-MMA on the vasorelaxant responses to bimakalim and symakalim in rat isolated aortic rings devoid of endothelium. Cumulative concentration-response curves to bimakalim and symakalim were constructed in phenylephrine (1 μM) contracted preparations 30 minutes after exposure to either solvent or L-MMA (100 μM). Relaxations are expressed as a percentage of the contractile response just prior to the application of $K_{ATP}CO$. Data are mean values ± s.e.m. (n=6/group). The * indicates responses that are significantly different ($P<0.05$) from corresponding control responses. The concentration ratio with confidence limits with to the control for L-MMA against bimakalim is CR(cl) 1.57 (1.09-2.05).

7 Discussion

The K_{ATP}COs used in this project can be separated into two groups that are typified by pinacidil and cromakalim. The vasorelaxant responses to the pinacidil group of K_{ATP}COs are sensitive to the L-arginine analogues whereas the vasorelaxant responses to the cromakalim group of K_{ATP}COs are insensitive to L-arginine analogues in rat isolated aorta devoid of a functional endothelium. By inference, it can be suggested that the K_{ATP}COs that are sensitive to L-arginine analogues activate a mechanism that is different to that operated by the K_{ATP}COs that are insensitive to L-arginine analogues. The L-NAME mediated inhibition of the vasorelaxant response to pinacidil was surmountable. In addition, the pinacidil induced Rb efflux is sensitive to L-NAME, whereas L-NAME failed to modify the cromakalim induced Rb efflux. In contrast to L-NAME, the NOS inhibitor L-NIO failed to modify both the vasorelaxant and Rb efflux responses to pinacidil. The vasorelaxant responses to pinacidil were also shown to be sensitive to two other L-arginine analogues, L-MMA and L-NNA.

7.1 Proposed Mechanism to Explain the L-Arginine Sensitivity of the Vasorelaxant and Rb Efflux Resposes to Pinacidil

A possible mechanism to explain the sensitivity of the pinacidil induced vasorelaxant and Rb efflux responses to the L-arginine analogues is shown in Figure 39. Figure 39 suggests that large parts of pinacidil and cromakalim interact with the K_{ATP}CO binding site on SUR2B in a similar manner. The yellow area on the diagram shows this commonality in binding to the K_{ATP}CO binding site. The study though has shown that there are differences in the sensitivity of the K_{ATP}COs to the L-arginine analogues. For example, in the presence of an L-arginine analogue, a larger concentration of pinacidil is required to produce the same degree of relaxation as seen in the control sections. This difference in sensitivity is shown by the blue region in Figure 39. The blue region is the area where some parts of pinacidil interact with the



Region where both cromakalim and pinacidil interact with the $K_{ATP}CO$ binding site.

Region where only cromakalim interacts with the $K_{ATP}CO$ binding site.

Region where pinacidil and L-arginine analogues interact with the $K_{ATP}CO$ binding site.

Figure 39. A diagrammatic representation of how pinacidil, cromakalim and the L-arginine analogues interact with the $K_{ATP}CO$ binding site on SUR2B. The model shows there is a degree of commonality in how pinacidil and cromakalim interact with the $K_{ATP}CO$ binding site (yellow area) but certain parts of the $K_{ATP}CO$ binding site are sensitive to both pinacidil and the L-arginine analogues (blue area).

K_{ATP}CO receptor on SUR2B. This area of the K_{ATP}CO binding site may also be the area where the L-arginine analogues interact with the K_{ATP}CO binding site hence explaining why a larger concentration of pinacidil is required to produce the same degree of relaxation in the presence of the L-arginine analogues. The red area of the diagram shows the area where some parts of cromakalim interact with the K_{ATP}CO binding site. The L-arginine analogues may not interact with this particular area of the K_{ATP}CO binding site explaining why the amount of cromakalim required to produce a relaxation is the same in the presence or absence of the L-arginine analogues.

Support for the model that only some parts of the K_{ATP}CO binding site are sensitive to L-arginine analogues is found when the concentration of L-NAME is increased to 300 μM. When the L-NAME concentration was increased, the pinacidil concentration response curve failed to be further displaced from the pinacidil concentration response curve that was produced in the presence of L-NAME (100 μM). If pinacidil was entirely sensitive to L-NAME, then it would be expected that an increased displacement of the concentration response curve to pinacidil would have occurred. It therefore follows that there is an insensitive component involved in the pinacidil induced vasorelaxations. This can be explained by the yellow part of the diagram that shows the commonality of pinacidil and cromakalim in binding to the K_{ATP}CO binding site on SUR2B. The model proposed suggests that an interaction between pinacidil and the L-arginine analogues is occurring at only a small part of the K_{ATP}CO binding site (blue region) hence explaining why there is an insensitive component to the pinacidil induced vasorelaxations.

In contrast to L-NAME, the pinacidil induced vasorelaxant and Rb efflux responses are insensitive to the L-ornithine analogue, L-NIO. Kontos and Wei, (1996) suggested that the L-arginine analogues interact with SUR2B, but no evidence can be found to suggest that L-NIO interacts with SUR2B. It is therefore proposed that L-NIO is not interacting in any way with the K_{ATP}CO binding site on SUR2B hence explaining why the vasorelaxant and Rb efflux responses are insensitive to L-NIO.

There are two K_{ATP}COs that are hard to fit into the two distinct groups characterised by pinacidil and cromakalim. These two K_{ATP}COs are DY-9708 and bimakalim. Both of these K_{ATP}COs are similar in structure to the benzopyran K_{ATP}CO, cromakalim, but these two K_{ATP}COs are sensitive to L-arginine analogues. DY-9708 and bimakalim therefore appear to bind to the K_{ATP}CO binding site in a different manner to cromakalim. A possible explanation for this sensitivity to the L-arginine analogues can be that when these K_{ATP}COs interact with the K_{ATP}CO binding site on SUR2B, some part of their structure interacts with the region on the K_{ATP}CO binding site that is sensitive to both pinacidil and L-arginine analogues. This would then explain why a larger concentration of these K_{ATP}COs is required to produce the same amount of relaxation in the presence of L-arginine analogues. In contrast, KB-R10101 is insensitive to L-arginine analogues suggesting that when KB-R10101 interacts with the K_{ATP}CO binding site, no part of KB-R10101 interacts with the part of the K_{ATP}CO binding site that is sensitive to L-arginine analogues. To conclude, the K_{ATP}COs used in this study show a large amount of commonality in their interactions with the K_{ATP}CO binding site on SUR2B. Their sensitivity to the L-arginine analogues derives from a section of their structure interacting with the part of the K_{ATP}CO binding site that is sensitive to the L-arginine analogues.

The L-arginine analogues, L-NNA and L-MMA have been shown to inhibit the cerebral arteriolar dilation from pinacidil in cats (Kontos and Wei, 1996). This effect was shown to be independent of NOS activity. The inhibitory effect of L-NNA and L-MMA was reversed by L-arginine suggesting that the effect of the L-arginine analogues on K_{ATP} channels may involve competition with L-arginine. It is therefore suggested that the requirement for L-arginine may be due to the presence of an arginine binding site on the SUR, which may need to be occupied to allow the K_{ATP} channel to open (Kontos and Wei, 1996, 1998). These studies support the proposed hypothesis that the binding of an L-arginine analogue to a site on SUR2B can influence how pinacidil interacts with the K_{ATP}CO binding site in rat isolated aorta devoid of endothelium.

The proposed model suggests how the K_{ATP}COs interact with the K_{ATP}CO binding site on the K_{ATP} channel. The model though cannot predict whether any additional responses are taking place following K_{ATP}CO application. Do the K_{ATP}COs bind to another part of the cell as well as the K_{ATP}CO binding site? Is an additional component linked to nitric oxide action? These additional questions are beyond the scope of this thesis, but provide thought provoking ideas for future research.

In contrast to this study that was performed in rat aorta, studies performed in the mesenteric arterial bed of Wistar rats showed that the concentration response curves to the K_{ATP}COs, levcromakalim and pinacidil were displaced to the left of the control curve in the presence of L-NAME. This suggests that basal nitric oxide has a modulatory effect on the interaction between K_{ATP}COs and K_{ATP} channels in this vascular bed (McCulloch & Randall, 1996). It is therefore possible that the L-arginine analogues have different effects on the vasorelaxant responses to K_{ATP}COs depending on where in the body the K_{ATP}COs are acting.

7.2 K_{ATP}CO Binding Site

Several studies have elucidated the regions of the SUR, which are involved in the activation of K_{ATP} channels by K_{ATP}COs such as pinacidil and cromakalim (Babenko *et al.*, 2000; Moreau *et al.*, 2000; Uhde *et al.*, 1999 and Schwanstecher *et al.*, 1998). Although the studies have determined the regions of the SUR, which confer the majority of the sensitivity to these K_{ATP}COs, they have commented that to obtain a complete K_{ATP}CO induced activation of the K_{ATP} channel, additional regions of the SUR are required. This study has shown that the pinacidil induced vasorelaxant and Rb efflux responses are sensitive to L-NAME, whereas the cromakalim induced vasorelaxant and Rb efflux responses are insensitive to L-NAME. If additional SUR regions are required to produce a complete pinacidil or cromakalim induced activation of the K_{ATP} channel, then this suggests that the some aspects of the binding sites for pinacidil and cromakalim may be different and

supports the previously discussed model explaining how pinacidil and cromakalim interact with the K_{ATP}CO binding site on SUR2B.

7.3 Common K_{ATP}CO Pharmacophore

Common features between cyanoguanidine and benzopyran K_{ATP}COs have been described and have led to the suggestion that there is a requirement for K_{ATP}COs to have a hydrophobic group, an electron deficient aromatic ring and a hydrogen bonding site (Atwal, 1992). Other common pharmacophore models have described the need for the K_{ATP}CO to have four common regions. Two of these areas represent areas of lipophilic interaction and the other two are hydrogen bonding regions (Koga *et al.*, 1993). If cromakalim and pinacidil have common binding sites, then one would believe that the vasorelaxant and Rb efflux responses to these K_{ATP}COs would be identical, in other words, they would both be either sensitive or insensitive to L-arginine analogues. The findings of the study show that there are similarities between pinacidil and cromakalim as cromakalim is insensitive to L-NAME and pinacidil has an L-NAME insensitive component, but the findings also show that pinacidil and cromakalim have a differential sensitivity to L-NAME, suggesting that elements of their respective binding sites are different in nature. The hypothesis suggesting differences in the cyanoguanidine and benzopyran K_{ATP}CO binding sites is supported in a study examining the cyanoguanidine K_{ATP}CO, P1075 and the benzopyran K_{ATP}CO, PKF217-744 (Manley *et al.*, 1993: 2001). The study shows that substituents of the benzopyran ring of PKF217-744 make contact with SUR2B. Therefore, some parts of the binding site of PKF217-744 are not utilised by P1075, suggesting that the binding sites of the two K_{ATP}COs are not identical.

7.4 Sites of Action

The K_{ATP} channel antagonist, glibenclamide, was shown to reduce the Rb efflux induced by pinacidil and cromakalim. This supports the action of pinacidil and cromakalim involving K_{ATP} channels in rat isolated aorta devoid of endothelium. The Na^+/K^+-ATPase pump inhibitor ouabain, failed to modify the Rb efflux produced by pinacidil or cromakalim. The failure to see a

modification of the pinacidil or cromakalim induced Rb efflux, suggests that the mechanisms of action induced by pinacidil and cromakalim involve the K_{ATP} channel and do not involve the Na^+/K^+ATPase pump in rat isolated aorta.

The concentration response curves produced to K_{ATP}COs in the presence of L-arginine analogues, have been displaced to the right of the control curves without modifying the maximal response. This process is called surmountable antagonism. If all the K_{ATP}COs interacted with the K_{ATP}CO binding site in rat isolated aorta in the same manner, then it would be expected that the Hill co-efficients would be the same. As the K_{ATP}COs have been shown to interact with the K_{ATP}CO binding site in different ways, then a range of Hill co-efficient values have been obtained.

7.5 Cyanoguanidine K_{ATP}COs

The new, thienylcyanoguanidine K_{ATP}COs, which are similar in structure to pinacidil, have played an important role in establishing the particular moieties of the K_{ATP}CO structure, which have an affinity to the K_{ATP}CO receptor in rat isolated aorta (Yoshiizumi *et al.*, 1997). The findings of the study have shown that the vasorelaxant responses to pinacidil, KB-R6844, KB-R6907 and KB-R10757 are sensitive to L-MMA, whereas the vasorelaxant responses to KB-R10101 and KB-R10758 are insensitive to L-MMA (Table 7). These observations suggest that pinacidil, KB-R6844, KB-R6907 and KB-R10757 activate a relaxant mechanism in rat aorta, which is independent of the mechanism(s) operated by KB-R10101 and KB-R10758. The findings of the study add further support to the hypothesis that some aspects of the K_{ATP}COs binding sites on SUR2B are different. If all of the K_{ATP}COs interact with the same binding site in the same way on SUR2B, then it would be expected that the K_{ATP}COs would either all be sensitive or insensitive to L-arginine analogues. As the K_{ATP}COs have a differential sensitivity to the L-arginine analogues, then this suggests that element(s) of their binding sites are different.

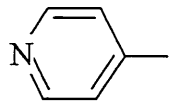
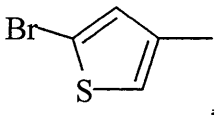
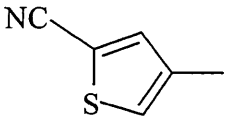
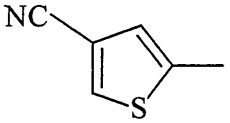
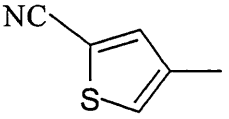
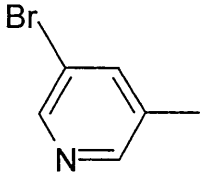
Compound	R_1	R_2	R_3	Effect of L-MMA on Activity
Pinacidil		$-C(CH_3)$	-H	Reduced
KB-R10101		$-CH_2.CH_3$	$-CH_3$	No Change
KB-R6844		$-CH_2.CH_3$	$-CH_3$	Reduced
KB-R10757		$-CH_2.CH_3$	$-CH_3$	Reduced
KB-R6907		$-C(CH_3)_3$	-H	Reduced
KB-R10758		$-CH_2.CH_3$	$-CH_3$	No Change

Table 7. A comparison of a series of cyanoguanidines K_{ATP}COs. The effect of L-MMA on the K_{ATP}COs activity is shown.

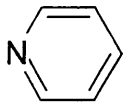
The structural differences between the cyanoguanidine K_{ATP}COs that are sensitive and those that are insensitive to L-arginine analogues have been compared. The vasorelaxant responses to KB-R6844 are sensitive to L-MMA whereas the vasorelaxant responses to KB-R10101 are insensitive to L-MMA. The only structural difference between these two compounds, is the substitution of a –Br group on KB-R10101 for a –CN group on KB-R6844 at position R_1 (Table 7). This substitution may therefore for account for the sensitivity of KB-R6844 to L-MMA in rat isolated aorta devoid of endothelium. The substitution may also result in KB-R6844 interacting with the part of the K_{ATP}CO binding site on SUR2B that is sensitive to L-arginine analogues.

The vasorelaxant responses to both KB-R10757 and KB-R6844 are sensitive to L-MMA in rat isolated aorta devoid of endothelium. The only structural difference between KB-R10757 and KB-R6844 is the positioning of the -S group on the thiophene ring at position R_1. The R_2 and R_3 groups on KB-R10757 are identical to those in KB-R6844. Therefore, the structural modification at position R_1 appears not to affect the sensitivity of KB-R10757 and KB-R6844 to L-MMA. KB-R6907 has the same R_1 substitution as KB-R6844, and the same R_2 and R_3 modifications as pinacidil. Despite these structural differences, the vasorelaxant responses to KB-R6907 are sensitive to L-MMA. This suggests that the structural modifications in KB-R6907 do not affect the sensitivity of KB-R6907 to L-MMA.

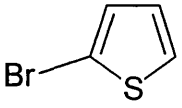
The findings of the study suggest that the -CN group on the thiophene ring at position R_1, is required for the thienylcyanoguanidine K_{ATP}COs to show sensitivity to L-MMA, as the substitution of the –CN group for a –Br group results in a loss of sensitivity to L-MMA.

An examination of the R_1 substitutions shows that the nitrogen on pinacidil possesses a lone pair of electrons (i). The nitrogen is sp^2 hybridized. The bromine on KB-R10101 also possesses a lone pair of electrons, but is more electronegative than the nitrogen, hence it is less willing to share the lone pair (ii). The bromine is sp^3 hybridized. However, in KB-R6844 and KB-R6907 (iii),

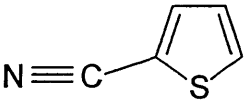
the nitrogen once more possesses a lone pair of electrons, but is sp hybridised, which means that the electrons are held closer to the nucleus than in the cases of sp^2 and sp^3 hybridization.



(i) Pinacidil



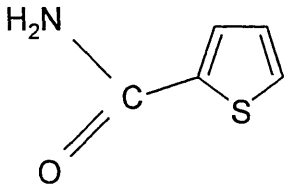
(ii) KB-R10101



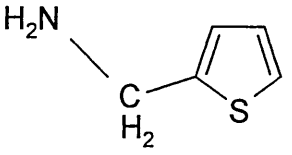
(iii) KB-R6844 & KB-R6907

In conclusion, there appears to be a requirement for a lone pair donor to be present at an appropriate site on the $K_{ATP}CO$, to enable binding to the $K_{ATP}CO$ receptor to occur. In addition, the atom containing the lone pair of electrons, has to be willing to give the lone pair up, as seen in example (i) with pinacidil. Despite the fact that the bromine on KB-R10101 contains a lone pair of electrons, it is less willing to donate the lone pair of electrons, as the bromine on KB-R10101 is more electronegative than the nitrogen on pinacidil.

This suggests that there is a balance between the willingness of the atom to donate a lone pair and the electronegativity characteristics of the group in question. A net effect of these two features appears to have a role in determining the sensitivity of the vasorelaxant responses of these $K_{ATP}COs$ to L-MMA. To investigate these effects further, it would be useful to examine additional $K_{ATP}COs$ with similar properties. To serve this purpose, an amide (iv) or an amine group (v) could be substituted at position R_1. Both the amide and amine possess a nitrogen group, which is sp^3 hybridized and is less electronegative than the –Br on KB-R10101. The electrons in sp^3 hybridised atoms are held less closely to the nucleus than the electrons in sp and sp^2 hybridised atoms, hence the sp^3 hybridized nitrogen group in the amide and amine compounds should be more willing to donate their lone pair of electrons than in the cases of pinacidil, KB-R6844 and KB-R6907.



(iv) Amide



(v) Amine

The effect of L-MMA on the vasorelaxant responses to K_{ATP}COs possessing the amide and amine groups could then be compared to the current study and provide additional information about the relationship between the L-arginine analogues, the K_{ATP}COs, the K_{ATP}CO binding site on SUR2B and the role that the lone pair of electrons play in this interaction.

7.6 Endothelium

In the presence of a functional endothelium, the vasorelaxant responses to pinacidil are sensitive to L-NAME, L-MMA and L-NNA and the vasorelaxant responses to KB-R6907 and KB-R6844 are sensitive to L-MMA. In addition, the vasorelaxant responses to KB-R6844 are also sensitive to the NOS inhibitor L-NIO and the guanylate cyclase inhibitor methylene blue. In the absence of a functional endothelium, the vasorelaxant responses to KB-R6844 are insensitive to L-NIO and methylene blue. Therefore, the removal of a functional endothelium appears to also remove the sensitivity of the vasorelaxant responses to KB-R6844 to L-NIO and methylene blue in rat isolated aorta. In contrast, the vasorelaxant responses to pinacidil, KB-R6844 and KB-R6907 show sensitivity to L-arginine analogues in the presence or absence of a functional endothelium. NOS inhibitors have previously been shown to modify the vasorelaxant responses to K_{ATP}COs, but their effects have been associated with endothelium-derived NO and cGMP-dependent pathways (Deka *et al.*, 1998; Maczewski and Beresewicz, 1997; Lawson *et al.*, 1993).

The enzyme NOS is central to the control of NO biosynthesis. There are three forms of NOS: an inducible form (iNOS, expressed in macrophages and

Kupffer cells, neutrophils, fibroblasts, vascular smooth muscle and endothelial cells in response to pathological invasion) and two constitutive forms that are present under physiological conditions in endothelium (eNOS) and in neurons (nNOS) (Anggard, 1994).

The activity of constitutive forms of NOS is controlled by intracellular calcium-calmodulin. The most important stimuli controlling NO synthesis in vessels under physiological conditions are pulsatile flow and shear stress (Anggard, 1994). In addition, endothelial cells possess receptors for several dilators including acetylcholine and substance P, occupation of which increases intracellular calcium and stimulates endothelial NO biosynthesis. Once NOS has been activated, it converts L-arginine into L-citrulline, and in the process produces NO. The NO then diffuses from the endothelium into the vascular smooth muscle where it activates the enzyme guanylate cyclase, which in turn converts GTP into cGMP and causes relaxation (Anggard, 1994; Nathan, 1992).

If the sensitivity of the vasorelaxant responses of pinacidil, KB-R6844 and KB-R6907 to the L-arginine and L-ornithine analogues were entirely due to endothelial NO production, then in the absence of a functional endothelium, it would be expected that this sensitivity would not occur. It is possible that pinacidil but not cromakalim could activate NOS and in turn, the sensitivity of pinacidil to L-NAME could be attributed to pinacidil directly activating NOS. However the insensitivity of pinacidil to L-NIO supports the hypothesis that NOS action is not involved in the mechanisms that underpin the response of the rat isolated aorta devoid of endothelium to K_{ATP}COs. The insensitivity of pinacidil to L-NIO in the absence of a functional endothelium also shows that any NOS activity in smooth muscle is minimal. Therefore, the sensitivity of the K_{ATP}COs to L-arginine analogues in rat isolated aorta devoid of endothelium, suggests that the action of the L-arginine analogues is independent of the NO:L-arginine pathway.

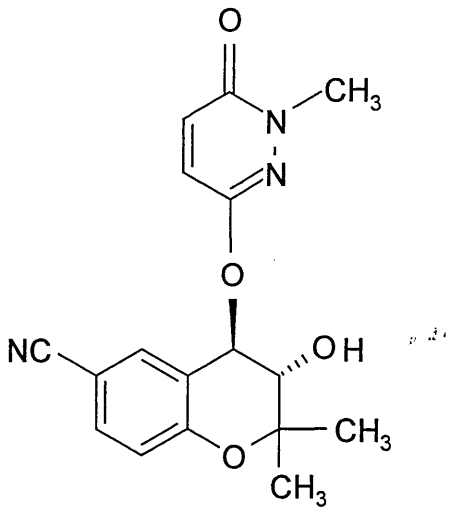
7.7 Future Biological Work

The findings of this study have shown that the vasorelaxant responses to several K_{ATP}COs are sensitive to L-arginine analogues. Binding studies could be used to examine whether the L-arginine analogues are having a direct effect on the K_{ATP}CO receptor in rat vascular smooth muscle. An inhibition of [^{3}H] P1075 binding by the L-arginine analogues would suggest that the L-arginine analogues are having a direct effect at the K_{ATP}CO receptor.

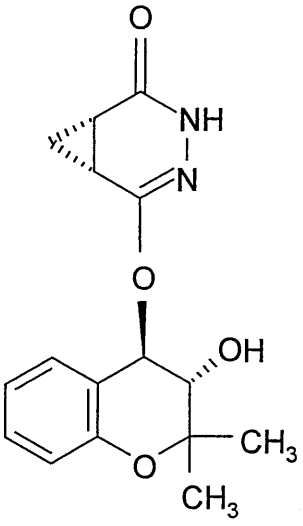
A demonstration that NOS was not involved in the sensitivity of K_{ATP}COs to L-arginine analogues would be useful to eliminate any effects of NO on the mechanism of action of K_{ATP}COs. Cloned K_{ATP}CO receptors could be used to elucidate why certain K_{ATP}COs are sensitive to L-arginine analogues but are not sensitive to L-NIO and where the L-arginine analogues are having their effects.

The membrane currents produced by stable smooth muscle cell lines expressing SUR2B and Kir6.2 could be examined using the whole cell patch clamp technique. The current produced as a consequence of K_{ATP}CO activation could be measured in the presence of L-arginine analogues. In addition, the possible effects of protein kinase A and protein kinase C activators and inhibitors on this K_{ATP}CO induced current could be examined.

7.8 Future Work – Alternative K_{ATP}COs

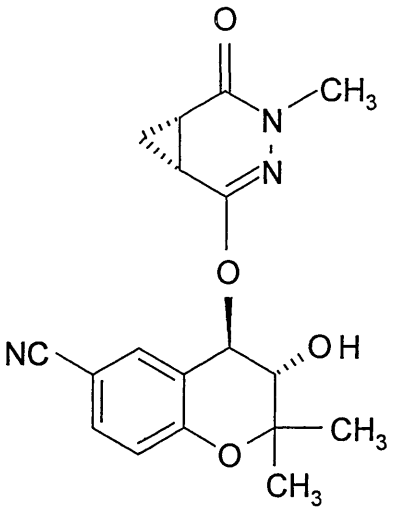


Symakalim

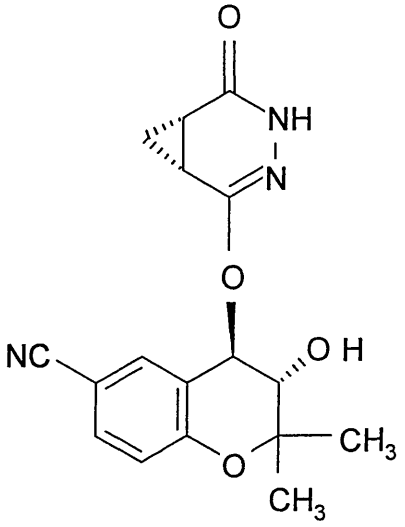


DY-9708

The vasorelaxant responses to DY-9708 but not those to symakalim are sensitive to L-MMA. This suggests that DY-9708 and symakalim interact with the K_{ATP}CO binding site in a different manner. At present, it is not possible to suggest the particular moieties of DY-9708 and symakalim, which allow this difference in L-MMA sensitivity to occur. To examine the difference in L-MMA sensitivity, it would be beneficial to synthesize some K_{ATP}COs that are similar in structure to both symakalim and DY-9708. Some suggestions of suitable compounds can be seen below.

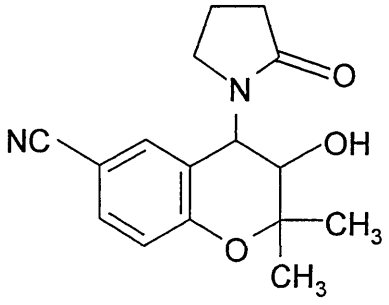


Compound (vi)

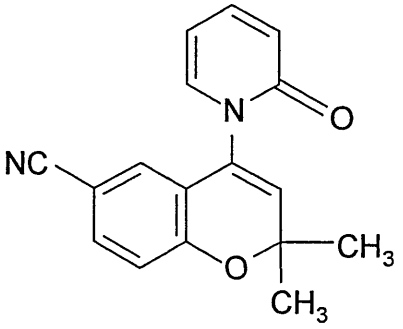


Compound (vii)

These two compounds fill the structural gaps between symakalim and DY-9708 and should provide some explanation as to the ability of L-MMA to modify the vasorelaxant responses to DY-9708, but not to symakalim. The compounds will show the parts of the $K_{ATP}CO$ that are crucial in binding to the $K_{ATP}CO$ receptor in SUR2B.



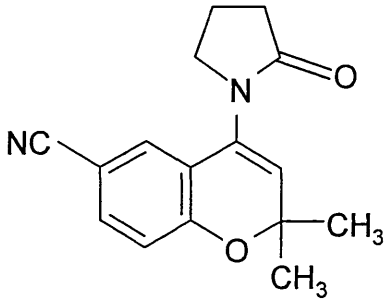
Cromakalim



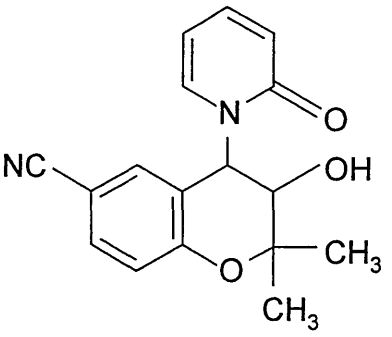
Bimakalim

The vasorelaxant responses to bimakalim, but not those to cromakalim are sensitive to L-arginine analogues. This suggests that cromakalim and bimakalim interact with the $K_{ATP}CO$ binding site in a different manner. At present, it is not possible to suggest the particular moieties of cromakalim and bimakalim that allow this difference in L-arginine analogue sensitivity to occur.

Two compounds have been suggested below, which may help in the elucidation of the L-MMA sensitivity.



Compound (viii)



Compound (ix)

These two compounds may help to explain the differences in L-arginine analogue sensitivity seen with cromakalim and bimakalim. The compounds should also show which parts of the $K_{ATP}CO$ are crucial in binding to the $K_{ATP}CO$ receptor.

8 Conclusion

The K_{ATP}COs used in this study appear to fall into two categories. The K_{ATP}COs typified by pinacidil are sensitive to L-arginine analogues whereas the K_{ATP}COs typified by cromakalim are insensitive to L-arginine analogues. This sensitivity appears to be independent of NOS action as the vasorelaxant and Rb efflux responses to pinacidil are insensitive to the NOS inhibitor L-NIO. The presence of a –CN group rather than a –Br group in thienylcyanoguanidine K_{ATP}COs appears to be important in relation to the sensitivity of these K_{ATP}COs to L-MMA as substituting the –CN group for a –Br group removes the sensitivity for L-MMA.

A model explaining the differential vasorelaxant and Rb efflux responses to the K_{ATP}COs has been suggested. Pinacidil and cromakalim are believed to have a degree of commonality in how they interact with the K_{ATP}CO binding site on SUR2B. Some parts of pinacidil though are believed to interact with the area of the K_{ATP}CO binding site that recognises L-arginine analogues explaining why an increased amount of pinacidil is required to produce the same degree of relaxation in the presence of L-arginine analogues. In contrast, no part of cromakalim is believed to interact with this particular region of the K_{ATP}CO binding site, which explains the insensitivity of cromakalim to L-NAME. In contrast to L-NAME, L-NIO is not believed to interact with the K_{ATP}CO binding site on SUR2B explaining why it is unable to modify the vasorelaxant responses to pinacidil.

In future, binding studies could be used to examine whether the L-arginine analogues are having a direct effect on the K_{ATP}CO receptor in rat vascular smooth muscle. A demonstration that NOS is not involved in the sensitivity of K_{ATP}COs to L-arginine analogues would be useful to eliminate any effects of NO on the mechanism of action of K_{ATP}COs. Cloned K_{ATP}CO receptors could be used elucidate why certain K_{ATP}COs are sensitive to L-arginine analogues but are not sensitive to L-NIO.

Such information will help in the generation of agents that may play a valuable therapeutic role in the treatment of conditions caused by high levels of cell excitability.

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