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Modulation of vasorelaxant responses to potassium channel openers by basal nitric oxide in the rat isolated superior mesenteric arterial bed

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1 We have used the isolated buffer-perfused mesenteric arterial bed of the rat to assess the modulation of vasorelaxation to potassium channel openers (KCOs) by basal nitric oxide.

2 The dose-response curves to the KCOs, levcromakalim and pinacidil, in preconstricted preparations were significantly shifted to the left in the presence of the nitric oxide synthase inhibitor (100 μM) N^G-nitro-L-arginine methyl ester (levcromakalim, $ED_{50} = 4.47 \pm 0.70$ nmol vs. 1.73 ± 0.26 nmol, $P < 0.001$; pinacidil, $ED_{50} = 16.1 \pm 4.8$ nmol vs. 5.43 ± 1.10 nmol, $P < 0.001$). The vasorelaxant responses to papaverine, a vasodilator which acts independently of potassium channels was unaffected by N^G-nitro-L-arginine methyl ester (L-NAME).

3 Removal of the endothelium, by perfusion with the detergent CHAPS (0.3%), significantly ($P < 0.001$) increased the potency of levcromakalim as a vasodilator (ED_{50} 4.47 ± 0.70 nmol vs. 2.59 ± 0.31 nmol). The subsequent administration of L-NAME following perfusion with CHAPS did not lead to any additional enhancement of responses to levcromakalim.

4 The presence of the non-selective adenosine antagonist, 8-phenyltheophylline (8-PT, 10 μM) significantly ($P < 0.001$) shifted the dose-response curve to levcromakalim to the left (ED_{50} 4.47 ± 0.70 nmol vs. 1.11 ± 0.32 nmol). In the presence of both L-NAME and 8-PT, the dose-response curve to levcromakalim was also significantly ($P < 0.01$) shifted to the left compared with control (ED_{50} in the presence of both L-NAME and 8-PT was 0.42 ± 0.08 nmol).

5 The presence of 8-bromo cyclic GMP (10 μM) reversed the increase potency of levcromakalim, observed following inhibition of nitric oxide synthase (ED_{50} in the presence of L-NAME was 0.59 ± 0.01 nmol and in the presence of 8-bromo cyclic GMP plus L-NAME the ED_{50} was 3.17 ± 0.80 nmol). However in the absence of L-NAME, the cell permeable analogue of cyclic GMP, 8-bromo cyclic GMP, did not affect the dose-response curve to levcromakalim compared with control (control ED_{50} value was 4.16 ± 0.52 nmol vs. 3.85 ± 1.13 nmol in the presence of 8-bromo cyclic GMP).

6 The present investigation demonstrates that both basal nitric oxide and adenosine modulate vasorelaxation to the KCOs levcromakalim and pinacidil. The modulatory effect of nitric oxide may be mediated via cyclic GMP.

Keywords: Potassium channel openers (KCOs); ATP-sensitive potassium (K_{ATP}) channels; levcromakalim; pinacidil; nitric oxide; cyclic GMP; mesenteric arterial bed; N^G-nitro-L-arginine methyl ester (L-NAME); adenosine; endothelium

Introduction

ATP-sensitive potassium (K_{ATP}) channels were first identified by Noma (1983) in cardiac muscle and have been shown to exist in various tissues, including pancreatic β cells (Cook & Hales, 1984), skeletal muscle (Spruce *et al.*, 1985), neurones (Ashford *et al.*, 1988), and vascular smooth muscle (Standen *et al.*, 1989, for review, see Ashcroft & Ashcroft, 1990). These channels are regulated by a variety of endogenous factors, e.g. the ratio of intracellular ATP to ADP i.e. micromolar intracellular ATP results in channel closure while micromolar ADP opposes this inhibition (Nichols & Lederer, 1991, review) and other nucleotide diphosphates such as GDP may also modulate the activity of K_{ATP} channels, (Pilsudski *et al.*, 1989). Intracellular magnesium (Lederer & Nichols, 1989) and intracellular pH (Fan *et al.*, 1994) may also have a role in the modulation of K_{ATP} channel activity. Furthermore, it has recently been shown that intracellular messengers may also modulate the activity of K_{ATP} channels e.g. adenosine 3':5'-cyclic monophosphate (cyclic AMP) enhances the effect of the K^+ channel opener, P1075 (Linde & Quast, 1995), and guanosine 3':5'-cyclic monophosphate (cyclic GMP) activates K_{ATP} channels in rabbit mesenteric arteries (Murphy & Brayden, 1995). Various endogenous ligands have been reported to be involved in K_{ATP} channel activation and these include such diverse agents as adenosine (Kirsch *et al.*, 1990; von Beckerath *et al.*, 1991; Dart & Standen, 1993; Akatsuka *et al.*, 1994; Randall, 1995), somatostatin (Fosset *et al.*, 1988), calcitonin gene-related peptide (Nelson *et al.*, 1990), and vasoactive intestinal polypeptide (Standen *et al.*, 1989). Recently, K_{ATP} channels have been shown to be involved in vasorelaxation to β-adrenoceptor stimulation (Cook *et al.*, 1993; Randall & McCulloch, 1995).

ATP-sensitive potassium channels are activated by a variety of appropriately named potassium channel opening (KCO) agents. Members of this group of drugs are structurally diverse, ranging from the benzopyran, levcromakalim, to the pyridine, pinacidil and the benzothiadiazine, diazoxide (for reviews, see Edwards & Weston, 1990; 1993). Their actions are all inhibited by the sulphonylurea, glibenclamide, which is known to inhibit K_{ATP} channels (Sturgess *et al.*, 1985), and this led Quast & Cook, (1989) to propose that potassium channel openers act on the K_{ATP} channels to produce their electrical and vasorelaxant effects.

Recent evidence has shown that inhibition of basal nitric oxide release results in augmented vasorelaxant responses to

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the potassium channel opener, levcromakalim, in the rabbit ear (Randall & Griffith, 1993; Randall *et al.*, 1994) and in the porcine cardiovascular system (Herity *et al.*, 1994).

The present investigation was designed to investigate whether basal nitric oxide modulates the interaction between KCOs and K_{ATP} channels, and/or the ensuing hyperpolarization, in the rat superior mesenteric arterial bed. Specifically, the vasorelaxant responses to the KCOs, levcromakalim and pinacidil, were examined in the absence and presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, Moore *et al.*, 1990) and a comparison was made with vasorelaxant responses to papaverine, which acts independently of K_{ATP} channels.

In the guinea-pig heart, nitric oxide synthase inhibition results in a significant compensatory increase in adenosine release (Kostic & Schrader, 1992). It has also been shown that adenosine receptors are coupled to K_{ATP} channels in a variety of tissues (Kirsch *et al.*, 1990; von Beckerath *et al.*, 1991; Dart & Standen, 1993; Randall, 1995) and that adenosine receptor activation enhances the potency of KCOs in the rabbit ear (Randall & Griffith, 1993; Randall *et al.*, 1994). In view of this we have investigated the effect of the nonselective adenosine antagonist, 8-phenyltheophylline (8-PT) on vasorelaxation to KCOs in the absence and presence of L-NAME.

A preliminary account of this work has been communicated to the British Pharmacological Society (McCulloch & Randall, 1995).

Methods

Preparation of the isolated buffer-perfused superior mesenteric arterial bed

Male Wistar rats (200–390 g; Bantin & Kingman, Hull, Humberside) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p., Sagatal, Rhône Mérieux, Harlow, Essex). A midline incision was made, a cannula (polythene tubing, i.d. = 0.5 mm, o.d. = 1.0 mm) was inserted into the superior mesenteric artery and the vascular bed was flushed with Krebs-Henseleit solution. The arterial vasculature was dissected away from the guts, transferred to a jacketed organ bath (37°C) as described by Randall & Hiley (1988), and perfused at 5 ml min^{-1} with gassed (95% O_2/5% CO_2) Krebs-Henseleit solution at 37°C (composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10), by means of a peristaltic pump (Watson-Marlow 504S).

The perfusion pressure in the superior mesenteric arterial bed was continuously monitored by means of a pressure transducer coupled to a Maclab 4e recording system (AD Instruments, New South Wales, Australia). Flow was maintained constant (5 ml min^{-1}) and therefore changes in perfusion pressure represented alterations in vascular resistance. At the end of each experiment, the cannula pressure was measured and subtracted from the recorded basal perfusion pressure in order to determine the pressure drop across the bed.

Experimental protocol

Following a 30 min equilibration period, perfusion pressure was raised by addition of methoxamine (10–30 μM) to the perfusion buffer to achieve a submaximal increase in perfusion pressure of 80–90 mmHg. The vasodilator effects of the ATP-sensitive potassium channel opening (KCO) drugs, levcromakalim and pinacidil, and papaverine, a vasodilator which acts independently of K_{ATP} channels were assessed in the absence and presence of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM). In view of the augmented vasoconstrictor responses in the presence of L-NAME, the concentration of methoxamine used in the experiments was reduced (1–3 μM) to induce an equivalent level of tone. Vasodilators were administered close-arterially as bolus doses in random order. Each preparation was used to investigate the effects of up to 2 of the vasodilators under control and experimental conditions.

The ability of an exogenous nitric oxide donor and cyclic GMP to reverse the effects of L-NAME were assessed independently using the nitric oxide donor sodium nitroprusside (SNP, 3 nM) and the cell permeable analogue of cyclic GMP, 8-bromo guanosine monophosphate (8-bromo cyclic GMP, 10 μM) respectively. The concentrations of SNP and 8-bromo cyclic GMP used were determined as the EC_{50} values from cumulative concentration-response curves established in preliminary experiments. In view of the fact that addition of SNP and 8-bromo cyclic GMP reduced vascular tone by approximately 50%, appropriate low tone controls were used. This was achieved by addition of a lower concentration of methoxamine (1–3 μM in the absence of L-NAME, 0.5–0.75 μM in the presence of L-NAME) to the buffer, so as to produce an equivalent level of tone as that observed in the presence of SNP or 8-bromo cyclic GMP.

The effects of L-NAME were compared with endothelial removal with the use of the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 0.3% (w/v) in distilled water, which was infused close arterially (4 ml over 50 s). In some preparations the subsequent effects of L-NAME were investigated by its addition to the perfusion fluid after treatment with CHAPS. In all cases verapamil (1.22 nmol) and carbachol (1.64 nmol) were introduced to confirm the vasorelaxant viability of the preparation and the functional presence and absence of the endothelium, respectively.

The possibility of increased endogenous adenosine release following inhibition of nitric oxide synthase influencing vasorelaxant responses to levcromakalim was investigated by use of the adenosine antagonist, 8-phenyltheophylline (8-PT, 10 μM).

Data and statistical analysis

All data are presented as mean ± s.e.mean. Perfusion pressures were compared by either paired or unpaired Student's *t* test, as appropriate. ED_{50} values for vasorelaxant responses were obtained from individual dose-response curves as the dose at which the half-maximal relaxant response occurred. These variables were determined by fitting the data to the logistic equation:

$$R = \frac{R_{max} \times A^{n_H}}{ED_{50}{}^{n_H} + A^{n_H}}$$

where R is the reduction in tone, A the dose of the vasorelaxant, R_{max} the maximum reduction of established tone, n_H the slope function and ED_{50} the dose of vasorelaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph Software (Synergy, Reading, PA, U.S.A.) running on a Macintosh LCIII computer. Statistical analysis of the variables was carried out by a two way analysis of variance and *F*-test (Prism, GraphPad, CA, U.S.A.).

Drugs

All drugs were prepared on the day of the experiment. Levcromakalim (a generous gift from SmithKline Beecham, Surrey), pinacidil (a generous gift from Leo, Bucks.) and verapamil (Sigma Chemical Company, Poole, Dorset) were dissolved in absolute ethanol and diluted in 0.9% saline. Methoxamine, L-NAME, papaverine, sodium nitroprusside and carbachol (all from Sigma Chemical Company) were all dissolved at the relevant concentrations in Krebs-Henseleit solution. 8-bromo cyclic GMP and 8-phenyltheophylline (also from Sigma Chemical Company) were dissolved in 0.1 M NaOH at the appropriate concentrations. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS, Sigma Chemical Company) was dissolved in distilled water.

Results

Basal perfusion pressures and established tone

In the 101 preparations used, basal perfusion pressure was 20.8±0.9 mmHg, and addition of methoxamine (10–30 μM) increased tone by 85.5±2.8 mmHg ($n=51$). The presence of L-NAME (100 μM) in the perfusion fluid did not significantly influence basal perfusion pressure, while the addition of methoxamine (1–3 μM) increased basal perfusion pressure by 115±6 mmHg ($n=33$). In the case of the low tone experiments, in the absence of L-NAME, methoxamine (1–3 μM) increased perfusion pressure by 56.6±6.0 mmHg ($n=5$) and in the presence of L-NAME, methoxamine (0.5–0.75 μM) raised perfusion pressure by 53.0±4.9 mmHg ($n=8$).

After perfusion with CHAPS, basal perfusion pressure was raised by 10.8±2.2 mmHg ($n=7$) and the increase in perfusion pressure in response to methoxamine (10–30 μM) was 66.6±8.9 mmHg ($n=7$). The adenosine antagonist, 8-PT (10 μM) reduced established tone by 32.6±4.7% ($n=22$), and the further addition of methoxamine (30–60 μM) re-established tone at 66.3±7.4 mmHg ($n=13$).

Sodium nitroprusside (SNP, 3 nM) reduced pre-established tone by 55.3±1.1% ($n=6$), which resulted in a low tone of 58.2±3.6 mmHg over basal ($n=6$). The cyclic GMP analogue, 8-bromo cyclic GMP (10 μM) reduced pre-established tone by 43.5±4.5% ($n=7$), resulting in a perfusion pressure of 64.6±5.2 mmHg over basal ($n=7$).

Vasorelaxant responses to levcromakalim, pinacidil and papaverine

In the presence of elevated tone, levcromakalim (35 pmol–105 nmol), pinacidil (0.1 nmol–3.8 μmol) and papaverine (84 pmol–2.8 μmol) all produced dose-related relaxations of established tone as shown in Figures 1a, b and c and the variables for the dose-response curves are given in Table 1.

Effects of L-NAME on vasorelaxant responses to levcromakalim, pinacidil and papaverine

Dose-response curves for levcromakalim, pinacidil and papaverine in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) are shown in Figures 1a, b and c respectively and the variables for the dose-response curves are given in Table 1. In the presence of L-NAME the dose response-curve for levcromakalim was significantly ($P<0.001$, $n=6$) shifted to the left, with no significant change in maximal response. In the case of pinacidil, the ED_{50} was also significantly ($P<0.001$) reduced with the dose-response curve being shifted to the left, while there was a significant ($P<0.01$, $n=6$) increase in maximal response. There was no change in either the ED_{50} or R_{max} for papaverine in the presence of L-NAME ($n=4$).

Effects of endothelium removal on vasorelaxant responses to levcromakalim

To confirm functionally that the endothelium had been destroyed after treatment with CHAPS and that the tissue was still viable, 1.64 nmol carbachol and 1.22 nmol verapamil were independently injected in bolus doses. The vasorelaxation to carbachol was reduced from 83.9±3.0% before CHAPS to 8.41±2.67% after treatment ($n=7$). In the case of verapamil vasorelaxation to the test dose was only reduced from 42.0±1.6% to 31.9±1.9% after CHAPS treatment ($n=3$). The dose-response curve to levcromakalim was significantly ($P<0.001$) shifted to the left after treatment with CHAPS compared with control (ED_{50} 4.47±0.70 nmol vs. 2.59±0.31 nmol), with maximal relaxation unchanged (R_{max} 104±5% vs. 107±2%, see Figure 2). This was not significantly different from the dose-response curve obtained in the other preparations treated with L-NAME. In a further 3 preparations the addition of L-NAME after perfusion with

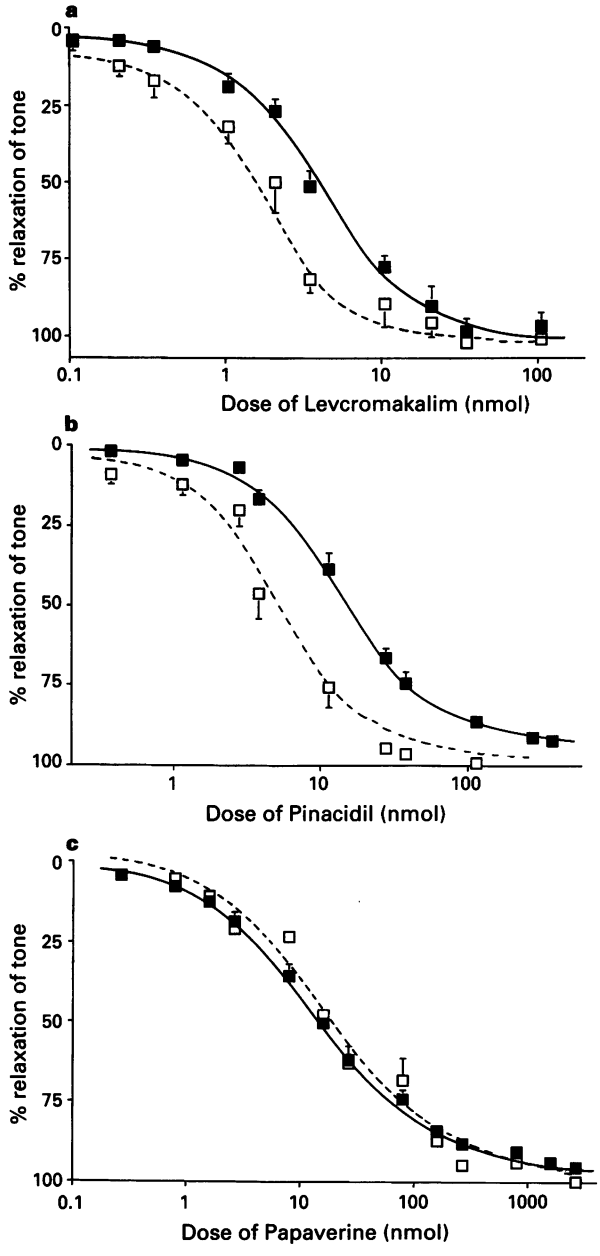

Figure 1 Dose-response curves for the relaxation of methoxamine-induced tone in the rat isolated perfused superior mesenteric arterial bed by (a) levcromakalim ($n=10$) (b) pinacidil ($n=6$) and (c) papaverine ($n=4$) in the absence (■) and presence (□) of 100 μM L-NAME. Values are shown as mean±s.e.mean.

CHAPS did not have any additional influence on the responses to levcromakalim; in this respect the variables for the control were 7.07±0.45 nmol (ED_{50}) and 92.6±2.6% (R_{max}), following perfusion with CHAPS the respective values were 4.76±0.10 nmol and 113±4% and after the subsequent addition of L-NAME, $ED_{50}=3.27\pm0.87$ nmol and $R_{max}=98.9\pm0.6\%$.

Effects of 8-PT and L-NAME on vasorelaxation to levcromakalim, pinacidil and papaverine

Addition of 8-PT (10 μM) significantly shifted the dose-response curves for levcromakalim ($P<0.001$) and pinacidil ($P<0.01$) to the left compared with control (Figure 3, Table 1). In the presence of both 8-PT and L-NAME there was a significant ($P<0.01$) leftward shift in the dose-response curve for levcromakalim ($n=5$) compared with control and a significant

($P < 0.01$) leftward shift compared with L-NAME alone ($n = 6$). There was no significant difference between the ED_{50} values obtained in the presence of L-NAME and 8-PT with those of 8-PT alone (see Figure 3a and Table 1).

The dose-response curve to pinacidil was also significantly ($P < 0.01$, $n = 5$) shifted to the left in the presence of both L-NAME and 8-PT compared with control ($n = 6$, Figure 3b, Table 1) and this was also shifted significantly ($P < 0.01$, $n = 7$) to the left compared with 8-PT alone and L-NAME alone ($P < 0.05$, $n = 6$).

The dose-response curve to papaverine was not significantly altered by the presence of both L-NAME and 8-PT compared with control or compared with that in the presence of L-NAME alone, but was significantly ($P < 0.01$) shifted to the right in the presence of 8-PT alone (variables are given in Table 1).

Effects of sodium nitroprusside in the presence of L-NAME on vasorelaxation to levcromakalim

The dose-response curve for levcromakalim in the presence of L-NAME and SNP is shown in Figure 4 (variables are given in Table 2). In the presence of both L-NAME and SNP there was no significant change in either the ED_{50} or R_{max} for levcromakalim compared with the values obtained in the presence of L-NAME alone.

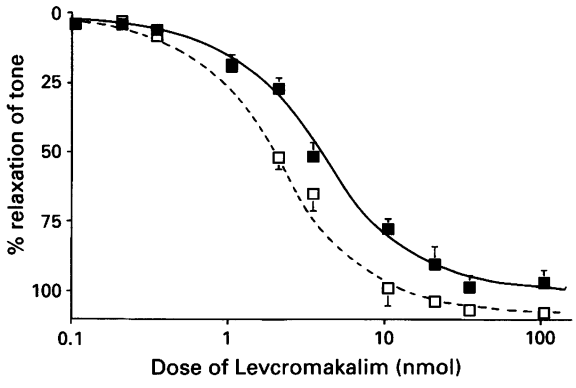

Figure 2 Dose-response curves for the relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed to levcromakalim (control data taken from Figure 1a) before (■, $n = 10$) and after (□, $n = 7$) treatment with CHAPS.

Effects of 8-bromo cyclic GMP on vasorelaxation to levcromakalim in the presence of L-NAME

In the presence of both L-NAME and 8-bromo cyclic GMP, the dose-response curve to levcromakalim was significantly ($P < 0.001$) shifted to the right compared with L-NAME alone (ED_{50} in the presence of L-NAME alone was 0.59 ± 0.01 nmol, $n = 6$, ED_{50} for L-NAME plus 8-bromo cyclic GMP was

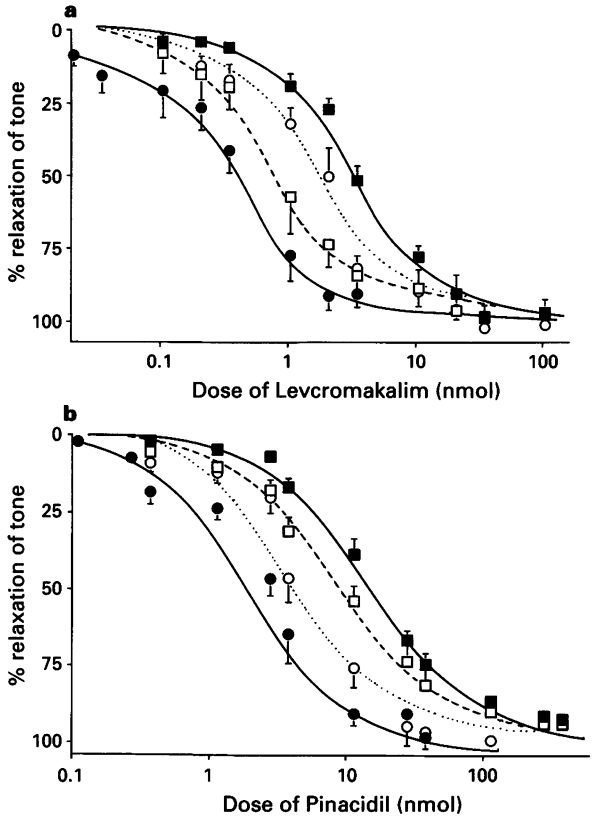

Figure 3 Dose-response curve for the relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed to (a) levcromakalim (b) pinacidil in the absence (■) and presence of L-NAME (○), 8-PT (□) and both L-NAME and 8-PT (●). The control data for levcromakalim and pinacidil are taken from Figure 1a and 1b respectively and have been included for comparison. Values are shown as mean ± s.e.mean.

Table 1 Variables for the dose-response curves for levcromakalim, pinacidil and papaverine under control conditions, in the presence of 100 μM L-NAME, in the presence of 10 μM 8-PT and in the presence of L-NAME (100 μM) plus 8-PT (10 μM)

	n	ED_{50} (nmol)	R_{max} (%)
Levcromakalim	10	4.47 ± 0.70	104 ± 5
Levcromakalim + L-NAME	6	1.73 ± 0.26***	101 ± 4
Levcromakalim + 8-PT	5	1.11 ± 0.32***	98.2 ± 3.4
Levcromakalim + L-NAME/8-PT	5	0.42 ± 0.08***	93.2 ± 2.0
Pinacidil	6	16.1 ± 4.8	93.2 ± 2.1
Pinacidil + L-NAME	6	5.43 ± 1.10***	103 ± 3**
Pinacidil + 8-PT	7	7.78 ± 1.25**	96.5 ± 2.6
Pinacidil + L-NAME/8-PT	5	2.46 ± 0.30**	98.2 ± 6.4
Papaverine	4	14.7 ± 2.1	95.3 ± 1.7
Papaverine + L-NAME	4	21.4 ± 3.7	101 ± 2
Papaverine + 8-PT	5	24.5 ± 6.9**	92.5 ± 3.3
Papaverine + L-NAME/8-PT	5	23.4 ± 8.7	104 ± 4

Values are given as mean ± s.e.mean. (** represents $P < 0.01$ and *** represents $P < 0.001$ statistically significant differences compared with the variables from the appropriate control dose-response curve).

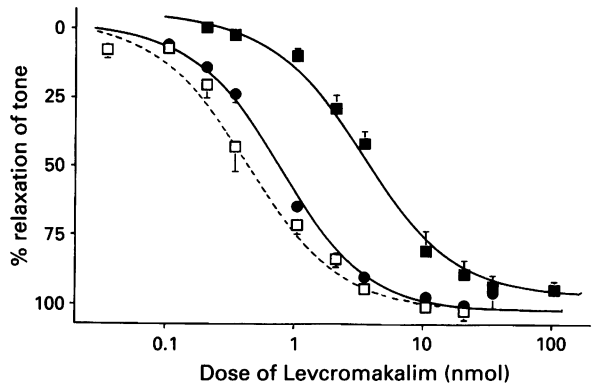

Figure 4 Dose-response curve for the relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed to levcromakalim (low tone) in the absence (■, $n=5$) and presence of L-NAME (□, $n=6$), and L-NAME plus SNP (●, $n=6$). Values are shown as mean ± s.e.mean.

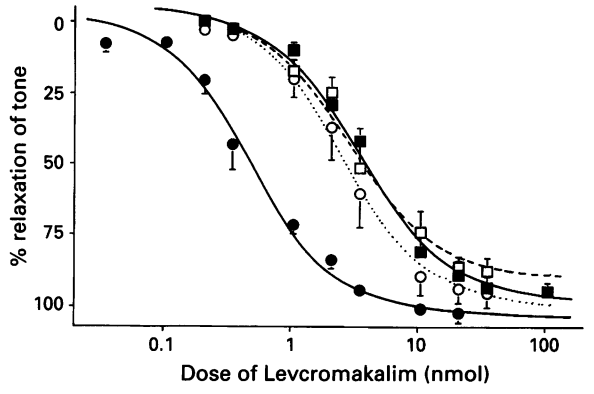

Figure 5 Dose-response curve for the relaxation of methoxamine-induced tone in the rat isolated superior mesenteric arterial bed to levcromakalim (low tone) in the absence (■, $n=5$; taken from Figure 4) and presence of L-NAME (●, $n=6$), 8-bromo cyclic GMP (□, $n=5$) and L-NAME plus 8-bromo cyclic GMP (○, $n=5$). Values are shown as mean ± s.e.mean.

Table 2 Variables for the dose-response curves to levcromakalim in the absence and presence of L-NAME, SNP and 8-bromo cyclic GMP

	n	ED_{50} (nmol)	R_{max} (%)
Levcromakalim low tone	5	4.16 ± 0.52	96.9 ± 3.1
Levcromakalim low tone + L-NAME	6	0.59 ± 0.01***	102 ± 3.0
Levcromakalim + L-NAME/SNP	6	0.72 ± 0.06***	99.5 ± 2.0
Levcromakalim + LNAME/8-bromo cyclic GMP	5	3.17 ± 0.80	98.2 ± 2.6
Levcromakalim + 8-bromo cyclic GMP	3	3.85 ± 1.13	94.5 ± 4.8

The control data and the experiments in the presence of L-NAME alone were performed under low tone conditions, where the concentration of methoxamine was reduced to match tone to the levels found in the presence of SNP or 8-bromo cyclic GMP. Values are stated as mean ± s.e.mean. (***represents $P<0.001$, compared with the low tone control).

3.17 ± 0.80 nmol, $n=5$, Figure 5 and Table 2). There was no significant difference between the low tone control values and those values obtained in the presence of 8-bromo cyclic GMP alone (Figure 5, Table 2). The values obtained in the presence of L-NAME and 8-bromo cyclic GMP did not differ significantly from those in the presence of 8-bromo cyclic GMP alone or from those of low tone control.

Discussion

The results of the present investigation clearly indicate a modulatory effect of basal nitric oxide on vasorelaxation to potassium channel openers (KCOs) in the rat mesenteric arterial bed. In this respect, removal of basal nitric oxide significantly augmented the responses to both levcromakalim and pinacidil. The augmentation following inhibition of nitric oxide synthase was reversed by 8-bromo cyclic GMP, suggesting that the modulatory effect of nitric oxide is mediated by cyclic GMP.

The vasorelaxant responses to the ATP-sensitive potassium (K_{ATP}) channel openers, levcromakalim and pinacidil, were augmented following the removal of basal nitric oxide by L-NAME or destruction of the endothelium. This observation suggests that basal nitric oxide has a modulatory effect on the interaction between KCOs and K_{ATP} channels in this vascular bed and this accords with previous observations in the rabbit ear (Randall & Griffith, 1993; Randall *et al.*, 1994) and the porcine cardiovascular system (Herity *et al.*, 1994). That vasorelaxation to papaverine was unaffected by L-NAME suggests that nitric oxide has a selective modulatory effect on the responses to KCOs, rather than on the vasorelaxation in general.

Since Furchgott & Zawadzki (1980) demonstrated the involvement of the endothelium in relaxation to acetylcholine, the relaxation to muscarinic agonists has been used as a functional indicator of the absence or presence of the endothelium in blood vessels. In the present investigation, CHAPS treatment reduced relaxation to carbachol by 90%, a functional demonstration of the selective loss of the integrity of the endothelium, while vasorelaxation to verapamil was still present. There was no difference in the vasorelaxant responses to levcromakalim in the presence of L-NAME, compared with after perfusion with CHAPS, indicating that removal of nitric oxide by inhibition of nitric oxide synthase or loss of the endothelium results in the enhanced vasorelaxation to KCOs. Furthermore, in some preparations the presence of L-NAME after treatment with CHAPs had no additional modulatory effect, which confirms that the treatments were functionally equivalent and that the nitric oxide was endothelial in origin.

There have been several investigations into the effects of basal nitric oxide release on vascular smooth muscle ion channels. In 1990, Tare *et al.* demonstrated that nitric oxide was capable of hyperpolarizing (by increasing potassium conductance), as well as relaxing, guinea-pig uterine artery, while Krippeit-Drews *et al.* (1992) showed that nitric oxide hyperpolarized rat aortic smooth muscle cells. There have subsequently been reports that nitric oxide can activate calcium-activated potassium (K_{Ca}) channel, directly (Bolotina *et al.*, 1994; Miyoshi & Nakaya, 1994) or indirectly via a cyclic GMP-dependent protein kinase (Robertson *et al.*, 1993) and can also activate K_{ATP} channels (Miyoshi *et al.*, 1994). These reports may provide an explanation of the potentiated responses to KCOs after nitric oxide synthase inhibition in the current investigation. If nitric oxide is activating K_{ATP} channels, the inhibition of nitric oxide synthase may result in reduced channel activation and therefore there will be more

channels available for KCOs to interact with, which may explain the increased potency observed. Murphy & Brayden (1995) have recently shown that nitric oxide, by activation of K_{ATP} channels, hyperpolarizes the vascular smooth muscle membrane of rabbit mesenteric arteries. However, we have found no effect of the K_{ATP} channel blocker, glibenclamide, on the vasorelaxation to carbachol, suggesting that nitric oxide has little effect on K_{ATP} channels in the rat mesentery (McCulloch & Randall, 1996). Alternatively, if nitric oxide also causes hyperpolarization via other potassium channels, there will be less scope for further hyperpolarization and thus impaired relaxation to KCOs. Thus, removal of this hyperpolarizing input will enable KCOs to have a greater impact.

Gardiner *et al.* (1991) reported that levcromakalim had a hypotensive effect in conscious rats, which, contrary to our experiments, was not influenced by the presence of L-NAME. This difference may relate to the fact that the cardiovascular reflexes are intact *in vivo*, and therefore the present findings cannot readily be compared with their study. Furthermore, in their study administration of L-NAME increased vascular resistance and therefore the vasorelaxant properties of levcromakalim were compared at different levels of tone. Herity *et al.* (1994), studying the cardiovascular effects of levcromakalim in the anaesthetized pig, found that L-NAME did, however, potentiate the vasodilatation produced by levcromakalim.

In the guinea-pig heart, inhibition of nitric oxide synthesis results in a 'compensatory' release of adenosine (Kostic & Schrader, 1992). Randall *et al.* (1994) reported that in the rabbit isolated ear the adenosine A_1 agonist, N^6-cyclohexyladenosine (CHA) enhanced the potency of levcromakalim. Furthermore, there are also several reports that adenosine A_1 receptors are to coupled K_{ATP} channels (Kirsch *et al.*, 1990; Dart & Standen, 1993). The coupling of adenosine A_2 receptors to K_{ATP} channels (von Beckerath *et al.*, 1991; Akatsuka *et al.*, 1994) in various vascular tissues, has also been demonstrated. In this respect adenosine stimulates cyclic AMP production via interaction with A_2 receptors (see Olah & Stiles, 1992 for review), while the sensitization of the K_{ATP} channels for KCOs by increased cyclic AMP levels has been recently demonstrated by Linde & Quast (1995). It may therefore be the case that in the rat mesentery the increased potency of KCOs following the inhibition of nitric oxide is due to an increase in adenosine release. However, the addition of the nonselective adenosine A_1/A_2 receptor antagonist, 8-phenyltheophylline (8-PT), did not reverse the effects of L-NAME on vasorelaxation to levcromakalim and pinacidil. In fact, the presence of 8-PT further enhanced the vasorelaxation to levcromakalim and pinacidil, suggesting that basal adenosine too may modulate KCO-induced vasorelaxation in the rat mesentery. Neither L-NAME alone nor L-NAME in the presence of 8-PT had an effect on the vasorelaxation to papaverine, indicating that neither L-NAME nor 8-PT were having non-specific effects on the vascular smooth muscle. If it is the case in the rat mesentery that there is a compensatory adenosine release following nitric oxide inhibition and that adenosine is capable of stimulating K_{ATP} channels, then it follows that on removal of the adenosine, the potency of KCOs will be increased as there will be a greater proportion of inactivated K_{ATP} channels with which KCOs can interact.

The possibility that nitric oxide influences the ability of the KCOs to produce vasorelaxation was investigated with the use of the spontaneous nitric oxide donor, sodium nitroprusside (SNP). SNP had no effect on the augmented KCO responses observed in the presence of L-NAME. Since exogenous nitric oxide did not reverse the effects of L-NAME, it may suggest that basal nitric oxide does not modulate the KCO/K_{ATP} interaction. However, there may be differences in the distribution of endogenous and SNP-derived nitric oxide throughout the tissue. Specifically, nitric oxide has differing activities throughout a vascular bed (Griffith *et al.*, 1987), a role which co-ordinates the blood flow of the vascular network (i.e. perfusion-demand matching). In contrast, SNP-derived nitric oxide would have an even distribution throughout the vascular bed and would therefore not be acting in the same manner as endogenous nitric oxide. This may perhaps result in the inability of this exogenous nitric oxide to reverse the effect of L-NAME on vasorelaxation to KCOs.

To investigate the effects of cyclic GMP on the vasorelaxant responses to KCOs, the cell permeable analogue of cyclic GMP, 8-bromo guanosine cyclic monophosphate (8-bromo cyclic GMP) was used in the presence of L-NAME. Williams *et al.* (1988) had previously found in bovine aortic smooth muscle cells that cyclic GMP modulated the activity of K_{Ca} channels and suggested that it may therefore mediate the action of some vasodilators (e.g. SNP, adenosine, atrial natriuretic peptide). In subsequent experiments, Murphy & Brayden (1995) demonstrated that nitric oxide is capable of hyperpolarizing rabbit mesenteric arteries by the elevation of cyclic GMP and subsequent activation of K_{ATP} channels. In the present investigation, the augmented vasorelaxant responses to levcromakalim in the presence of L-NAME alone were reversed on addition of 8-bromo cyclic GMP, suggesting that it is this part of the nitric oxide-pathway which modulates the K_{ATP} channel response to KCOs. Inhibition of basal nitric oxide production would subsequently reduce the tonic stimulation of soluble guanylate cyclase in the vascular smooth muscle and intracellular cyclic GMP would fall (Rapoport & Murad, 1983; Miller *et al.*, 1984). The effect of cyclic GMP on the K_{ATP} channels would be reduced allowing a greater effect of levcromakalim on the channels. The mechanism of action of cyclic GMP on the K_{ATP} channels is at present unknown, but it may be that cyclic GMP causes phosphorylation of the channels via cyclic GMP-dependent protein kinases or even cyclic AMP-dependent protein kinases by cross-activation (Jiang *et al.*, 1992), and therefore channel gating is modulated, possibly by increasing the open state probability of channels. On removal of nitric oxide, control via this putative mechanism would be reduced and accordingly there would be more channels available in the closed state for levcromakalim to activate, increasing the potency of this agent. Alternatively, phosphorylation of the channels by a cyclic GMP-dependent protein kinase may reduce the affinity of the K_{ATP} channels for KCOs and therefore removal of this phosphorylation control, via the removal of nitric oxide, would result in enhanced affinity of K_{ATP} channels for KCOs and thus their dose-response curves are shifted to the left. It should be noted that the presence of 8-bromo cyclic GMP alone did not influence the vasorelaxation to levcromakalim, suggesting that under basal conditions (i.e. in the presence of basal nitric oxide), this putative control mechanism is maximally activated. In view of this proposed control mechanism it is of interest that Janigro *et al.* (1993) reported a regulatory role of K_{ATP} channels over the endothelium and this may suggest a reciprocal control relationship between the nitric oxide pathway and K_{ATP} channels.

In summary, the results of the present investigation demonstrate that both basal nitric oxide and adenosine modulate vasorelaxation to the K_{ATP} channel openers, levcromakalim and pinacidil, in the rat mesenteric arterial bed in that removal of both nitric oxide and blockade of adenosine receptors potentiates the effects of the KCOs. The results of these experiments suggest that the modulatory effects of nitric oxide may be mediated by cyclic GMP. These findings have important implications for the therapeutic use of KCOs in the treatment of disease states associated with endothelial dysfunction such as hypertension. (Lüscher & Vanhoutte, 1986) and hypercholesterolaemia (Verbeuren *et al.*, 1986).

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References

AKATSUKA, Y., EGASHIRA, K., KATSUDA, Y., NARISHIGE, T., UENO, H., SHIMOKAWA, H. & TAKESHITA, A. (1994). ATP sensitive potassium channels are involved in adenosine A_2 receptor mediated coronary vasodilatation in the dog. *Cardiovasc. Res.*, **28,** 906–911.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell. Signalling*, **2,** 197–214.

ASHFORD, M.L.J., STURGESS, N.C., TROUT, N.J., GARDNER, N.J. & HALES, C.N. (1988). Adenosine-5′-triphosphate-sensitive ion channels in neonatal rat cultured central neurones. *Pflügers Arch. Pharmacol.*, **412,** 297–304.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates Ca-activated potassium channels in vascular smooth muscle. *Nature*, **368,** 850–853.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic β-cells. *Nature*, **311,** 271–273.

COOK, S.J., SMALL, R.C., BERRY, J.L., CHIU, P., DOWNING, S.J. & FOSTER, R.W. (1993). β-Adrenoceptor subtypes and the opening of plasmalemmal K^+ channels in trachealis muscle: electrophysiological and mechanical studies in guinea-pig tissue. *Br. J. Pharmacol.*, **109,** 1140–1148.

DART, C. & STANDEN, N.B. (1993). Adenosine-activated potassium current in smooth muscle cells isolated from the pig coronary artery. *J. Physiol.*, **471,** 767–786.

EDWARDS, G. & WESTON, A.H. (1990). Potassium channel openers and vascular smooth muscle relaxation. *Pharmacol. Ther.*, **48,** 237–258.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33,** 597–637.

FAN, Z., TOKUYAMA, Y. & MAKIELSKI, J.C. (1994). Modulation of ATP-sensitive K^+ channels by internal acidification in insulin secreting cells. *Am. J. Physiol.*, **267,** C1036–C1044.

FOSSET, M., SCHMID-ANTOMARCHI, H., DE WEILLE, J.R. & LAZDUNSKI, M. (1988). Somatostatin activates glibenclamide-sensitive and ATP-regulated K^+ channels in insulinoma cells via a G-protein. *FEBS Lett.*, **242,** 94–96.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373–376.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats. *Br. J. Pharmacol.*, **104,** 731–737.

GRIFFITH, T.M., EDWARDS, D.H., DAVIES, R. Ll., HARRISON, T.J. & EVANS, K.T. (1987). EDRF coordinates the behaviour of vascular resistance vessels. *Nature*, **329,** 442–445.

HERITY, N.A., ALLEN, J.D., SILKE, B. & ADGEY, A.A.J. (1994). Assessment of the ability of levcromakalim and sodium nitroprusside to reverse the cardiovascular effects of nitric oxide synthase inhibition in the anaesthetised pig. *Cardiovasc. Res.*, **28,** 894–900.

JANIGRO, D., WEST, G.A., GORDON, E.L. & WINN, H.R. (1993). ATP-sensitive K^+ channels in rat aorta and brain microvascular endothelial cells. *Am. J. Physiol.*, **265,** C812–C821.

JIANG, H., SHABB, J.B. & CORBIN, J.D. (1992). Cross-activation: overiding cAMP/cGMP selectivities of protein kinases in tissues. *Biochem. Cell Biol.*, **70,** 1283–1289.

KIRSCH, G.E., CODINA, J., BIRNBAUMER, L. & BROWN, A.M. (1990). Coupling of ATP-sensitive K^+ channels to A_1 receptors by G proteins in rat ventricular myocytes. *Am. J. Physiol.*, **259,** H820–H826.

KOSTIC, M.M. & SCHRADER, J. (1992). Role of nitric oxide in reactive hyperaemia of guinea pig heart. *Circ. Res.*, **70,** 208–212.

KRIPPEIT-DREWS, P., MOREL, N. & GODFRAIND, T. (1992). Effect of nitric oxide on membrane potential and contraction of rat aorta *J. Cardiovasc. Pharmacol.*, **20** (Suppl. 12), S72–S75.

LEDERER, W.J. & NICHOLS, C.G. (1989). Nucleotide modulation of the activity of rat heart ATP-sensitive K^+ channels in isolated membrane patches. *J. Physiol.*, **419,** 193–211.

LINDE, C. & QUAST, U. (1995). Potentiation of P1075-induced K^+ channel opening by stimulation of adenylate cyclase in rat isolated aorta. *Br. J. Pharmacol.*, **115,** 515–521.

LÜSCHER, T.F. & VANHOUTTE, P.M. (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*, **8,** 344–348.

MCCULLOCH, A.I. & RANDALL, M.D. (1995). Modulation of vasorelaxation to potassium channels openers by basal nitric oxide in isolated perfused rat superior mesenteric arterial bed. *Br. J. Pharmacol.*, **116,** 174P.

MCCULLOCH, A.I. & RANDALL, M.D. (1996). Characterisation of endothelium-dependent relaxations in the isolated perfused rat superior mesenteric arterial bed: the involvement of EDHF. *Br. J. Pharmacol.*, (Proc. Suppl. in press).

MILLER, R.C., MONY, M., SCHINI, V., SCHOEFFTER, P. & STOCLET, J.C. (1984). Endothelial mediated inhibition of contraction and increase in cyclic GMP levels evoked by the α-adrenoceptor agonist B-HT 920 in rat isolated aorta. *Br. J. Pharmacol.*, **83,** 903–908.

MIYOSHI, H. & NAKAYA, Y. (1994). Endotoxin-induced non-endothelial nitric oxide activates the Ca^+-activated K^+ channel in cultured vascular smooth muscle cells. *J. Mol. Cell. Cardiol.*, **26,** 1487–1495.

MIYOSHI, H., NAKAYA, Y. & MORITOKI, H. (1994). Nonendothelial-derived nitric oxide activates the ATP-sensitive K^+ channels of vascular smooth muscle cells. *FEBS Lett.*, **345,** 47–49.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine (L-NOARG), a novel, (L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99,** 408–412.

MURPHY, M.E. & BRAYDEN, J.E. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J. Physiol.*, **486,** 47–58.

NELSON, M.T., HUANG, Y., BRAYDEN, J.E., HESCHELER, J. & STANDEN, N.B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature*, **344,** 770–773.

NICHOLS, C.G. & LEDERER, W.J. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.*, **261,** H1675–H1686.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature*, **305,** 147–148.

OLAH, M.E. & STILES, G.L. (1992). Adenosine receptors. *Annu. Rev. Physiol.*, **54,** 211–225.

PILSUDSKI, R., ROUGIER, O. & TOURNEUR, Y. (1989). Activation of an ATP-sensitive K^+ current is promoted by internal GDP in frog atrial myocytes. *Pflügers Arch. Pharmacol.*, **414** (Suppl. 1), S177.

QUAST, U. & COOK, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10,** 431–435.

RANDALL, M.D. (1995). The involvement of ATP-sensitive potassium channels and adenosine in the regulation of coronary flow in the isolated perfused rat heart. *Br. J. Pharmacol.*, **116,** 3068–3074.

RANDALL, M.D. & GRIFFITH, T.M. (1993). Modulation of vasodilatation to levcromakalim by hypoxia and EDRF in the rabbit isolated ear: a comparison with pinacidil, sodium nitroprusside and verapamil. *Br. J. Pharmacol.*, **109,** 386–393.

RANDALL, M.D. & HILEY, C.R. (1988). Effect of phenobarbitone pretreatment upon endothelium-dependent relaxation to acetylcholine in rat superior mesenteric arterial bed. *Br. J. Pharmacol.*, **94,** 977–983.

RANDALL, M.D. & MCCULLOCH, A.I. (1995). The involvement of ATP-sensitive potassium channels in β-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. *Br. J. Pharmacol.*, **115,** 607–612.

RANDALL, M.D., UJIIE, H. & GRIFFITH, T.M. (1994). Modulation of vasodilatation by adenosine analogues in the rabbit ear: an explanation for hypoxic augmentation. *Br. J. Pharmacol.*, **112,** 49–54.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ. Res.*, **52,** 352–357.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K^+ channels in cerebral artery smooth muscle cells. *Am. J. Physiol.*, **265,** C299–C303.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316,** 736–738.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarising vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **24,** 177–180.

STURGESS, N.C., ASHFORD, M.L.J., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **ii,** 474–475.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarisation and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **346,** 69–71.

VERBEUREN, T.J., JORDAENS, F.H., ZONNEKEYN, L.L., VAN HOVE, C.E., COENE, M-C. & HERMAN, A.G. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ. Res.*, **58,** 552–564.

VON BEKERATH, N., CYRYS S., DISCHNER, A. & DAUT, J. (1991). Hypoxic vasodilatation in isolated, perfused guinea-pig heart: an analysis of the underlying mechanisms. *J. Physiol.*, **442,** 297–319.

WILLIAMS, Jr, D.L. KATZ, G.M., ROY-CONTANCIN, L. & REUBEN, J.P. (1988). Guanosine 5′-monophosphate modulates gating of high conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 9360–9364.

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