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Modulation of relaxation to levcromakalim by S-nitroso-N-acetylpenicillamine (SNAP) and 8-bromo cyclic GMP in the rat isolated mesenteric artery

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1 Levcromakalim caused concentration-dependent relaxations of methoxamine-induced tone in both endothelium-denuded and intact vessels. Its potency was reduced by the nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP; 0.1 μM or 1 μM) in both denuded and intact vessels. The maximal relaxation (R_{max}) was reduced only in denuded vessels.

2 SNAP was more potent in endothelium-denuded than intact vessels but there were no differences in R_{max}. Glibenclamide (10 μM) did not affect relaxation to SNAP in endothelium-denuded or intact vessels.

3 The soluble guanylyl cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM) increased the potency and R_{max} of levcromakalim in endothelium-intact vessels. ODQ had no effect in denuded vessels.

4 ODQ (10 μM) reduced the vasorelaxant potency of SNAP in both intact and endothelium-denuded vessels by 190-fold and 620-fold, respectively.

5 8-bromo cyclic GMP (10 or 30 μM) reduced both the potency and R_{max} of levcromakalim in de-endothelialized vessels, but had no effect in intact vessels although it reduced both the potency and R_{max} of levcromakalim in intact vessels incubated with ODQ (10 μM).

6 In the presence of ODQ (10 μM), SNAP (0.1 μM or 1 μM) reduced the potency of levcromakalim in intact vessels, without altering R_{max}, but had no effect in denuded vessels. SNAP (50 μM) reduced both the potency and R_{max} of levcromakalim in intact and endothelium-denuded vessels.

7 Therefore, although SNAP causes relaxation principally through generation of cyclic GMP, it can modulate the actions of levcromakalim through mechanisms both dependent on, and independent of, cyclic GMP; the former predominate in endothelium-denuded vessels and the latter in intact vessels.

Keywords: Levcromakalim; rat mesenteric artery; nitric oxide; endothelium; cyclic GMP; 8-bromo cyclic GMP; SNAP (S-nitroso-N-acetylpenicillamine); ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one)

Introduction

ATP-sensitive potassium channels (K_{ATP}) are found in many types of vascular smooth muscle, and their activation by potassium channel activating agents (KCAs), such as levcromakalim, causes vasorelaxation. Hyperpolarization of the membrane, caused by efflux of K^+, may cause vasorelaxation by inhibiting the opening of voltage-operated calcium channels (VOCC; Cook, 1988). However, there is also evidence that hyperpolarization may inhibit inositol (1,4,5)trisphosphate synthesis (Ito *et al*., 1991), and promote refilling of intracellular Ca^{2+} stores (Criddle *et al*., 1995) which would also contribute to relaxation of agonist-induced tone. Recent findings suggest that activation of endothelial K_{ATP} channels may also release endothelium-derived nitric oxide (EDNO; Feleder & Adler-Graschinsky, 1997) or endothelium-derived hyperpolarizing factor (EDHF; White & Hiley, 1997b), contributing to the relaxant actions of KCAs.

There is growing evidence that K_{ATP} channel activity may be modulated by nitric oxide. Kubo *et al*. (1993) showed that nitric oxide donors cause activation of K_{ATP} channels in rat aorta by means of a cyclic GMP-dependent mechanism, possibly cyclic GMP-dependent protein kinase. Furthermore, Murphy & Brayden (1995) showed that nitric oxide caused smooth muscle hyperpolarization by a similar mechanism in the rabbit mesenteric artery. However it is now clear that nitric oxide may also have cyclic GMP-independent actions on K^+ channels (Bolotina *et al*., 1994). Indeed, Weidelt *et al*. (1997) showed that nitric oxide could activate K_{ATP} channels through a cyclic GMP-independent mechanism in the rat mesenteric artery. On the other hand, earlier work by Garland & McPherson (1992) on the rat mesenteric artery showed that, although nitric oxide could hyperpolarize smooth muscle cells via activation of K_{ATP} channels, this did not contribute to the relaxant actions of nitric oxide.

Inhibition of the relaxant activity of KCAs by nitric oxide has been demonstrated in the rabbit ear (Randall & Griffith, 1993) and the rat perfused mesenteric bed (McCulloch & Randall, 1996). However nitric oxide potentiated the actions of KCAs in guinea-pig ventricular myocytes (Shinbo & Iijima, 1997), an effect that could not be mimicked by the membrane-permeable cyclic GMP analogue, 8-bromo cyclic GMP. The aim of the present study was to investigate the interactions of nitric oxide with the K_{ATP} channel in the rat isolated mesenteric artery, and in particular to examine the contribution of cyclic GMP-dependent and cyclic GMP-independent mechanisms to these effects. In this respect, we have used the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) to release nitric oxide, 8-bromo cyclic GMP to determine the actions of cyclic GMP, and the guanylyl cyclase inhibitor ODQ (Garthwaite *et al*., 1995) to identify cyclic GMP-independent effects.

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Methods

Male Wistar rats (250–350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p., Sagatal, Rhone Merieux, Harlow, Essex, U.K.). The mesentery was removed and placed in ice-cold, gassed (95% O_2/5% CO_2), Krebs-Henseleit solution of the following composition (mM): NaCl, 118; KCl 4.7; $MgSO_4$, 1.2; KH_2PO_4 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 10. Segments (2 mm in length) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany-Halpern myograph (Model 500A, JP Trading, Aarhus, Denmark) as described in White & Hiley (1997a). Vessels were maintained at 37°C in Krebs-Henseleit solution, containing indomethacin (10 μM) and bubbled with 95% O_2/5% CO_2, and were allowed to equilibrate under zero tension for 60 min. After equilibration, vessels were normalized to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany & Halpern, 1977). The mean vessel diameter under these conditions was 365 ± 5 μm ($n = 72$). The vessels were left for another 30 min before experiments commenced. In experiments for which the endothelium was not required, it was removed by rubbing the intima with a human forearm hair.

Experimental protocol

After the equilibration period, the integrity of the endothelium was assessed by precontracting the vessels with methoxamine (10 μM) and then adding carbachol (10 μM). The mean tension generated by vessels in response to methoxamine was 13.1 ± 0.4 mN ($n = 72$). Tissues which relaxed to carbachol by greater than 90% were designated as endothelium-intact, and those in which carbachol caused less than 10% relaxation were designated as endothelium-denuded.

The potency of levcromakalim was found to vary between vessels from different animals, but preliminary experiments revealed that three successive concentration-response curves to levcromakalim, separated by 30 min wash-out periods, could be obtained from a single preparation without significant changes in the parameters describing the responses (White & Hiley, 1997b). Hence matched control and experimental data for experiments involving levcromakalim were obtained from the same vessel.

When examining the effect of addition of a vasorelaxant such as 8-bromo cyclic GMP or SNAP, on subsequent responses to levcromakalim, it is essential to account for the reduction in tone that will be caused by the first drug added. Therefore, vessels were precontracted to a level of tone equal to that obtained in the test for endothelial integrity by addition of methoxamine (10 μM). 8-bromo cyclic GMP or SNAP was then added at a concentration producing approximately 25–35% relaxation of tone (near EC_{30} dose) or a higher concentration causing 45–55% relaxation (near EC_{50} dose) depending on the particular experiment. After vessel tone stabilized at the reduced level, methoxamine was added to give higher concentrations (30–100 μM) which returned tone to the level recorded before addition of vasorelaxant. After stabilization of tone at this level, a cumulative concentration-response curve to levcromakalim was constructed. These data were compared with control data for levcromakalim obtained using the standard level of tone (equal to that obtained in the test for endothelial integrity). In experiments where soluble guanylyl cyclase activity was inhibited using ODQ (10 μM), the inhibitor was added for 30 min prior to construction of the concentration-response curve and was then present throughout the experiment.

Since both SNAP and 8-bromo cyclic GMP were found to be more potent relaxants in endothelium-denuded than in intact vessels, the concentrations of each added for a given effect were lower in denuded vessels. Similarly, as ODQ potentiated relaxations to 8-bromo cyclic GMP in intact vessels (data not shown), the doses of 8-bromo cyclic GMP added were lowered in order to give the same level of relaxation as in experiments carried out in the absence of ODQ. However ODQ significantly inhibited the relaxant effects of SNAP; the EC_{50} dose of SNAP in the absence of ODQ caused $<10\%$ relaxation of tone in the presence of ODQ, therefore the second addition of methoxamine was reduced (10–15 μM) to produce the standard level of tone used in all experiments. SNAP was also added at a much higher dose, which caused 45–55% relaxation of tone in the presence of ODQ.

The K_{ATP} channel inhibitor, glibenclamide, was used to examine the role of these channels in relaxations to SNAP. Glibenclamide (10 μM) was added for 30 min prior to construction of the concentration-response curve and was then present throughout the experiment.

Data and statistical analysis

All relaxation responses are expressed as the percentage relaxation of the tone induced by methoxamine. As detailed above, all concentration-response curves were determined after the vessels had been contracted to a standard level of tone equal to that obtained on first exposure to 10 μM methoxamine during the initial test for endothelial integrity. Data are given as the mean $\pm$ s.e.mean. EC_{50} values for vasorelaxant responses were obtained from individual concentration-response curves by fitting the data to the logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{EC_{50} \cdot A^{n_H} + A^{n_H}}$$

where R is reduction in tone, A the concentration of the agonist, R_{max} the maximum reduction of established tone, n_H the slope function and EC_{50} the concentration of relaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph software (Synergy Software, Reading, PA, U.S.A) running on a Macintosh computer. Statistical analysis of the variables was carried out by two way analysis of variance and an *F*-test. All data are compared with controls carried out using vessels obtained from the same animal. *P* values less than 0.05 were considered to be statistically significant.

Drugs

All solutions were prepared on the day of the experiment. Methoxamine, carbachol and 8-bromo cyclic GMP (Sigma Chemical Company, Poole, Dorset) were dissolved in distilled water. S-nitroso-N-acetylpenicillamine (Calbiochem, Nottingham, U.K.) was dissolved in 100% ethanol. 1H-[1,2,4]oxadiazolo]4,3-a[quinoxaline-1-one (Tocris Cookson, Bristol) and glibenclamide (Aldrich) were dissolved in 100% DMSO. Levcromakalim (SmithKline Beecham, Betchworth, Surrey, U.K.) was dissolved in 70% (v/v) ethanol. Indomethacin (Sigma) was dissolved in 5% (w/v) $NaHCO_3$ solution. Dilutions were made in distilled water.

Results

Effect of SNAP on relaxation to levcromakalim

In endothelium-denuded vessels, levcromakalim caused concentration-dependent relaxation ($EC_{50}=0.27\pm0.02$ μM, $R_{max}=85.4\pm2.6\%$; $n=6$). Prior addition of a near EC_{30} concentration of SNAP (30 nM, causing $25.4\pm5.0\%$ relaxation of methoxamine-induced tone; $n=6$) had no significant effect on either the potency ($EC_{50}=0.23\pm0.01$ μM; $n=6$) or the maximum relaxation ($R_{max}=88.2\pm3.3\%$; $n=6$) to levcromakalim.

Addition of a higher, near EC_{50} concentration, of SNAP to endothelium-denuded vessels (0.1 μM, causing $45.4\pm8.6\%$ relaxation; $n=6$) significantly reduced the potency (control, $EC_{50}=0.40\pm0.02$ μM; with SNAP, $EC_{50}=0.94\pm0.07$ μM; $n=6$; $P<0.001$) and the maximal response to levcromakalim (control, $R_{max}=81.6\pm1.5\%$; with SNAP, $R_{max}=70.3\pm1.5\%$; $n=6$; $P<0.001$; Figure 1a).

In intact vessels, levcromakalim also caused concentration-dependent relaxations ($EC_{50}=0.15\pm0.02$ μM, $R_{max}=85.3\pm0.8\%$; $n=4$; Figure 1b). In view of the results obtained in endothelium-denuded vessels, only the near EC_{50} concentration of SNAP (1 μM, causing $62.6\pm10.0\%$ relaxation of methoxamine-induced tone; $n=4$) was used. This significantly reduced the potency of levcromakalim ($EC_{50}=0.47\pm0.08$ μM; $n=4$; $P<0.01$), but had no significant effect on the maximal relaxation ($R_{max}=89.2\pm2.5\%$; $n=4$; Figure 1b).

Effect of glibenclamide on relaxation to SNAP

Glibenclamide (10 μM) had no significant effect on relaxation to SNAP either in the presence of endothelium (control, $EC_{50}=72\pm6$ nM, $R_{max}=78.2\pm2.0\%$; $n=4$; with glibenclamide, $EC_{50}=61\pm9$ nM, $R_{max}=78.7\pm3.6\%$; $n=4$), or in denuded vessels (control, $EC_{50}=41\pm4$ nM, $R_{max}=76.1\pm2.4\%$; $n=4$; with glibenclamide, $EC_{50}=26\pm8$ nM, $R_{max}=72.6\pm1.6\%$; $n=4$).

Effect of ODQ on relaxation to SNAP

The nitric oxide donor SNAP caused relaxations of endothelium-intact vessels ($EC_{50}=0.17\pm0.03$ μM, $R_{max}=80.2\pm4.1\%$; $n=4$), but was found to be significantly ($P<0.05$) more potent at relaxing endothelium-denuded vessels, although there was no significant difference in maximum response ($EC_{50}=66\pm6$ nM, $R_{max}=85.4\pm2.5\%$; $n=4$; Figure 2).

Incubation of vessels with the soluble guanylyl cyclase inhibitor ODQ (10 μM) significantly reduced the vasorelaxant potency of SNAP in both intact and endothelium-denuded vessels (Figure 2). ODQ caused a 190-fold parallel rightward shift in the concentration-response curve to SNAP in endothelium-intact vessels (to an EC_{50} of 32.2 ± 2.9 μM; $n=6$; $P<0.001$), and a parallel rightward shift of 620-fold in endothelium-denuded vessels (to an EC_{50} of 40.4 ± 3.2 μM; $n=4$; $P<0.001$). Solubility limitations prevented definition of a true maximum response, however the relaxations obtained at the highest concentration that could be used (100 μM) were $67.9\pm11.3\%$ in the presence of endothelium ($n=6$) and $60.1\pm14.0\%$ in its absence ($n=4$).

Effect of ODQ on relaxation to levcromakalim

Incubation of vessels with ODQ (10 μM) significantly ($P<0.001$) increased both the vasorelaxant potency of levcromakalim in endothelium-intact vessels (control, $EC_{50}=0.17\pm0.03$ μM; $n=4$; in the presence of ODQ, $EC_{50}=95\pm5$ nM; $n=8$) and the maximum response (control in the absence of ODQ, $R_{max}=86.3\pm1.7\%$; $n=4$; in the presence of ODQ, $R_{max}=94.3\pm1.6\%$; $n=8$; Figure 3a).

However in endothelium-denuded vessels, ODQ (10 μM) had no significant effect on either the potency of levcromakalim (control, $EC_{50}=0.27\pm0.03$ μM; $n=6$; with ODQ, $EC_{50}=0.26\pm0.02$ μM; $n=8$) or the maximum response (control in the absence of ODQ, $R_{max}=87.1\pm2.4\%$; $n=6$; in the presence of ODQ, $R_{max}=93.4\pm2.4\%$; $n=8$; Figure 3b).

Effect of 8-bromo cyclic GMP on relaxation to levcromakalim

Levcromakalim relaxation of endothelium-denuded vessels ($EC_{50}=0.34\pm0.01$ μM, $R_{max}=83.4\pm4.2\%$; $n=10$) was not

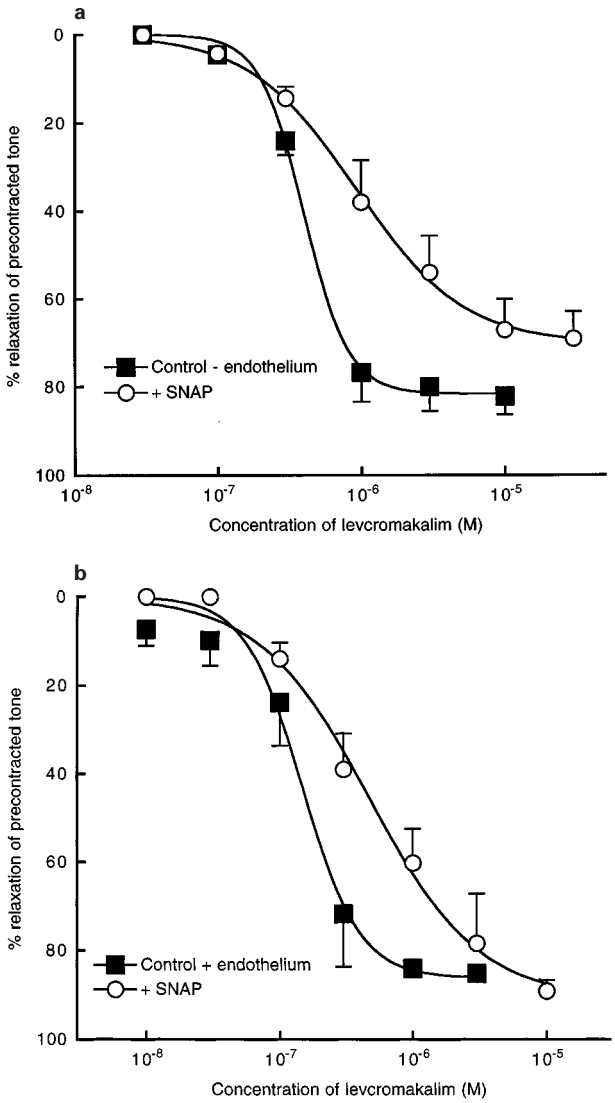

Figure 1 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by levcromakalim. Relaxations were determined in the presence or absence of SNAP either (a) in the absence (0.1 μM SNAP) or (b) in the presence (1 μM SNAP) of a functional endothelium. Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) $n=6$ for both curves, and in (b) $n=4$ for both curves.

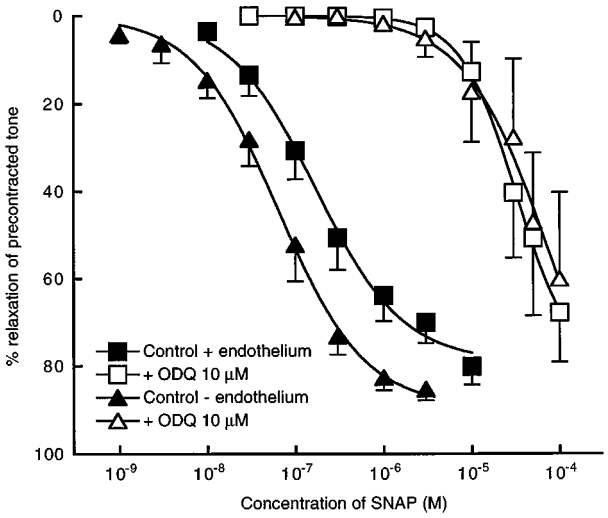

Figure 2 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by SNAP. Relaxations were determined in the absence or presence of ODQ (10 μM) in either intact or endothelium-denuded vessels. Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. $n=4$ for all except for relaxations of intact vessels in the presence of ODQ (10 μM; $n=6$).

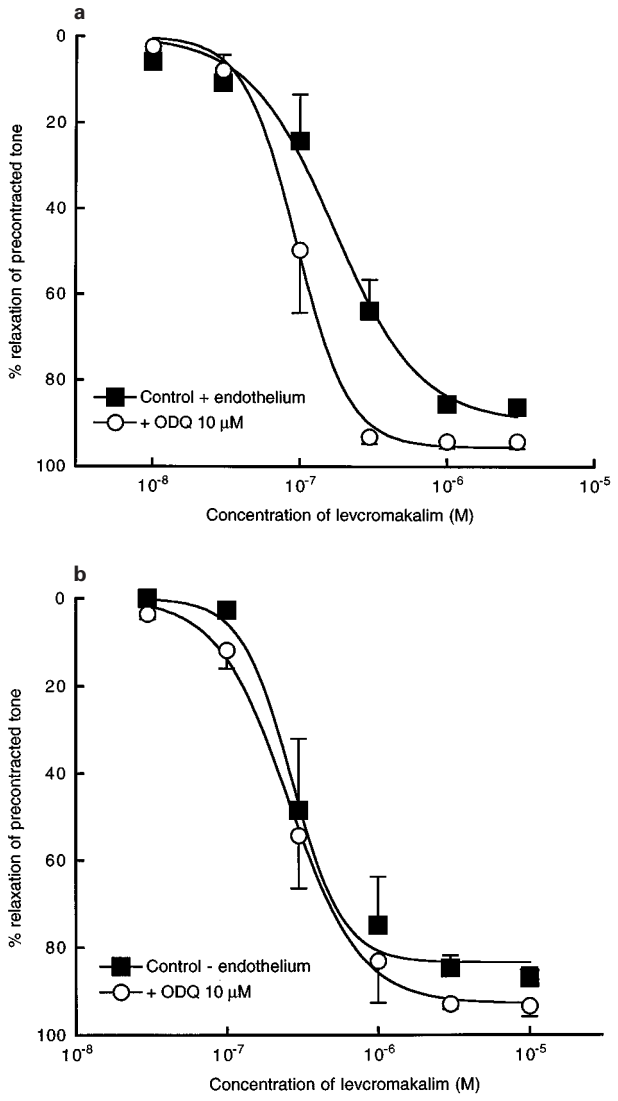

Figure 3 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by levcromakalim. Relaxations were determined in the presence or absence of ODQ (10 μM) either (a) in the presence of a functional endothelium or (b) in its absence. Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) control, $n=4$; with ODQ, $n=8$; for (b) control, $n=6$; with ODQ, $n=8$.

affected by prior addition of a near EC_{30} concentration of 8-bromo cyclic GMP (5 μM, causing $26.9\pm2.0\%$ relaxation; $n=10$). In the presence of 8-bromo cyclic GMP, the concentration-response curve for relaxations to levcromakalim had an EC_{50} of 0.31 ± 0.01 μM and an R_{max} of $87.3\pm4.5\%$ ($n=10$).

A higher, near EC_{50}, concentration of 8-bromo cyclic GMP (10 μM, causing $56.4\pm4.3\%$ relaxation; $n=4$) significantly reduced both the potency (control, $EC_{50}=0.48\pm0.02$ μM; with 8-bromo cyclic GMP, $EC_{50}=0.78\pm0.09$ μM; $n=4$; $P<0.05$) and the maximal relaxation to levcromakalim (control, $R_{max}=91.0\pm0.7\%$; with 8-bromo cyclic GMP, $R_{max}=71.1\pm4.9$; $n=4$; $P<0.01$; Figure 4a).

Figure 4b shows that relaxation of endothelium-intact vessels by levcromakalim ($EC_{50}=0.18\pm0.01$ μM, $R_{max}=82.0\pm1.4\%$; $n=6$) was unaffected by a near EC_{50} concentration of 8-bromo cyclic GMP (30 μM, causing $45.2\pm5.3\%$ relaxation; $n=6$). In the presence of 8-bromo cyclic GMP, relaxations to levcromakalim had an EC_{50} of 0.24 ± 0.02 μM and an R_{max} of $81.1\pm2.7\%$ ($n=6$; Figure 4b).

However in the presence of ODQ (10 μM), levcromakalim relaxation of endothelium-intact vessels ($EC_{50}=0.19\pm0.01$ μM, $R_{max}=90.9\pm2.1\%$; $n=6$) was attenuated by a near EC_{50} concentration of 8-bromo cyclic GMP (10 μM, causing $48.7\pm4.1\%$ relaxation; $n=6$). The cyclic GMP analogue significantly reduced the potency ($EC_{50}=0.43\pm0.04$ μM; $n=6$; $P<0.001$) and the maximum response to levcromakalim ($R_{max}=68.5\pm7.9\%$; $n=6$; $P<0.001$; Figure 4c).

Effect of SNAP on relaxation to levcromakalim in the presence of ODQ

Although the presence of ODQ (10 μM) in intact vessels greatly reduced the relaxant effect of SNAP (the near EC_{50} dose in the absence of ODQ, 1 μM, causing only $5.9\pm2.4\%$ relaxation in the presence of the inhibitor; $n=8$), the nitric oxide donor still caused a 1.9-fold reduction in the potency of levcromakalim (control in the presence of ODQ, $EC_{50}=0.17\pm0.01$ μM; $n=6$; with SNAP, $EC_{50}=0.32\pm0.02$ μM; $n=8$; $P<0.001$), although there was no significant decrease in the maximum response (control in the presence of ODQ, $R_{max}=94.0\pm6.8\%$; $n=6$; with SNAP, $R_{max}=88.1\pm1.9\%$; $n=8$; Figure 5a).

The relaxant effect of SNAP (0.1 μM, the near EC_{50} dose in the absence of ODQ) was also virtually abolished by ODQ in denuded vessels (relaxation $0.8\pm0.5\%$; $n=4$). In contrast to the results obtained in the absence of ODQ, SNAP now had no significant effect on either the potency of levcromakalim (control in the presence of ODQ, $EC_{50}=0.32\pm0.05$ μM; $n=4$; with ODQ and SNAP, $EC_{50}=0.44\pm0.02$ μM; $n=4$) or the maximum response (control in the presence of ODQ, $R_{max}=93.4\pm2.4\%$; $n=4$; with ODQ and SNAP, $R_{max}=92.2\pm3.5\%$; $n=4$; Figure 5b).

A higher concentration of SNAP (50 μM, causing $62.7\pm11.4\%$ relaxation; $n=6$) caused a 2.2-fold reduction in the potency of levcromakalim in intact vessels (control in the presence of ODQ, $EC_{50}=95\pm5$ nM; $n=8$; with ODQ and

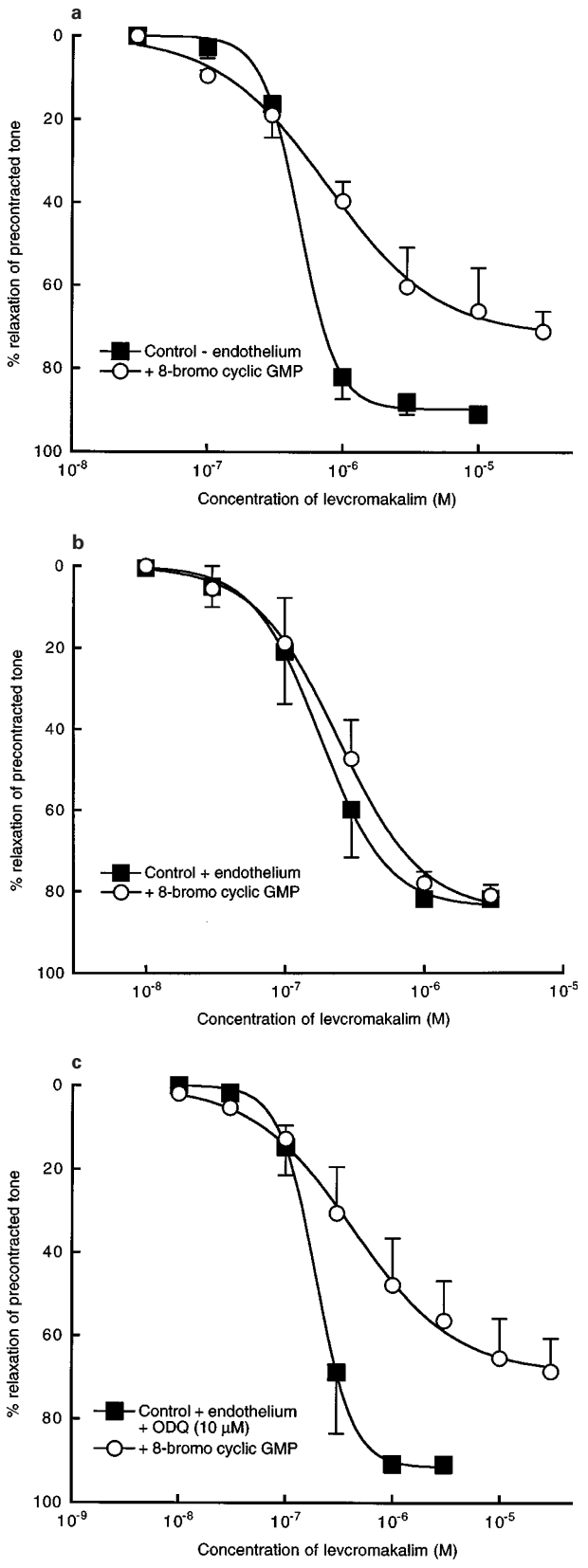

Figure 4 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by levcromakalim. Relaxations were determined in the presence or absence of 8-bromo cyclic GMP either (a) in the absence of endothelium but with 8-bromo cyclic GMP (10 μM) or (b) in the presence of endothelium and 8-bromo cyclic GMP (30 μM), or (c) in the presence of endothelium, 8-bromo cyclic GMP (10 μM) and ODQ (10 μM). Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) $n=4$ for both curves, and for (b) and (c) $n=6$ for both curves.

SNAP, $EC_{50}=0.21\pm0.01$ μM; $n=6$; $P<0.001$), but now also reduced the maximal response (control in the presence of ODQ, $R_{max}=95.6\pm1.9\%$; $n=8$; with ODQ and SNAP, $R_{max}=83.4\pm1.5\%$; $n=6$; $P<0.001$; Figure 6a).

In endothelium-denuded vessels, the higher concentration of SNAP (50 μM, causing $44.8\pm13.5\%$ relaxation; $n=4$) significantly reduced the potency of levcromakalim (control in the presence of ODQ, $EC_{50}=0.22\pm0.01$ μM; with SNAP, $EC_{50}=0.55\pm0.06$ μM; $n=4$; $P<0.01$) and the maximal relaxation (control in the presence of ODQ, $R_{max}=92.6\pm1.0\%$; with SNAP, $R_{max}=82.1\pm1.3\%$; $n=4$; $P<0.001$; Figure 6b).

Discussion

The results of the present study show for the first time that, although the nitric oxide donor SNAP causes relaxation

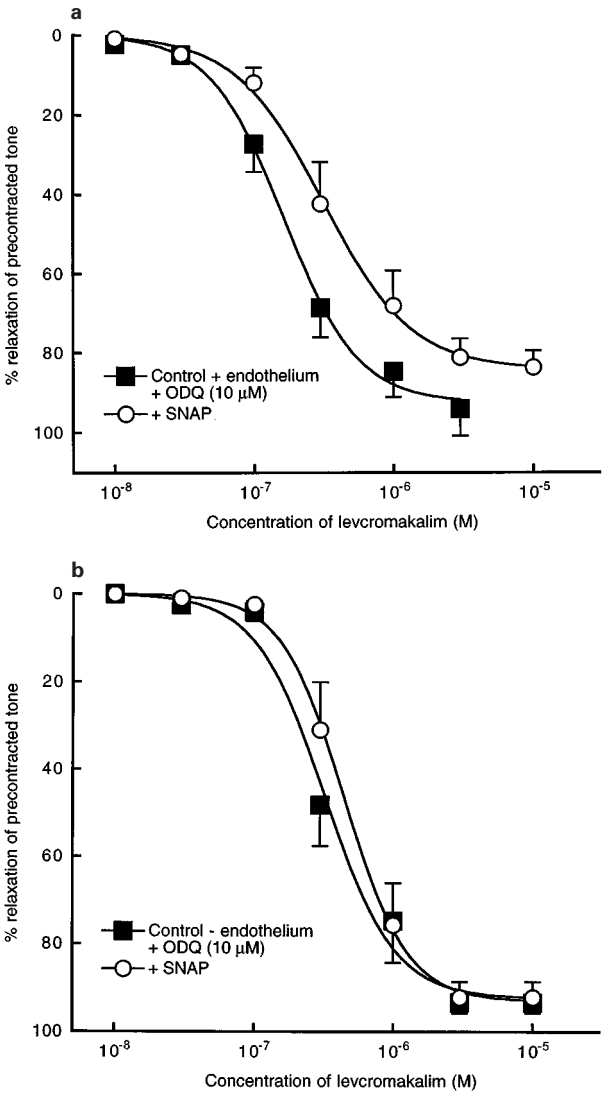

Figure 5 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by levcromakalim in the presence of ODQ (10 μM). Relaxations were determined in the presence or absence of SNAP either (a) in the presence of endothelium (1 μM SNAP) or (b) in its absence (0.1 μM SNAP). Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) control, $n=6$; with SNAP, $n=8$; for (b) $n=4$ for both curves.

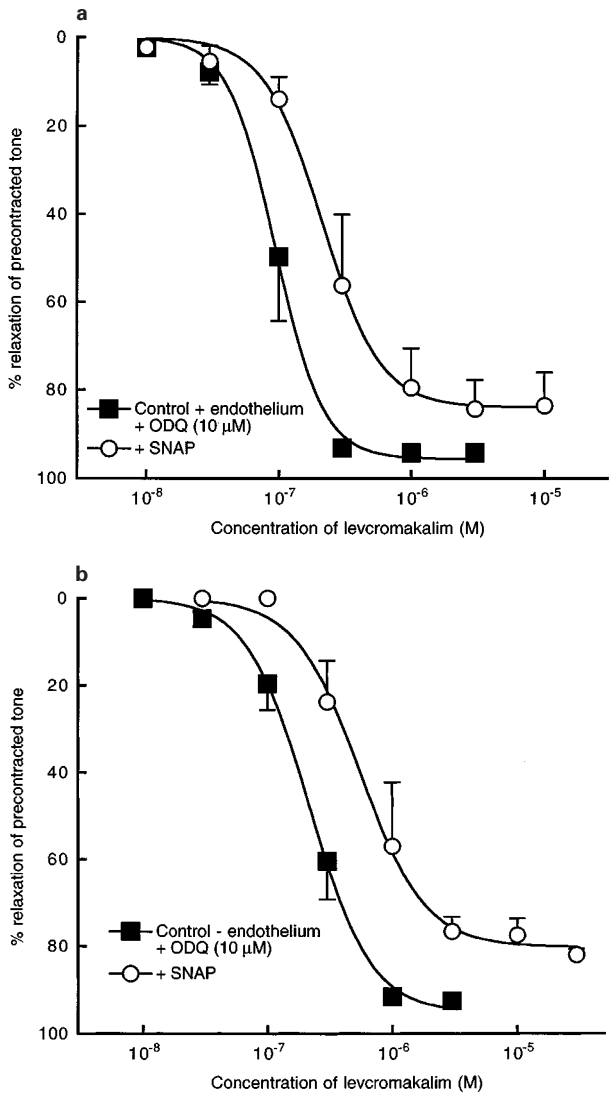

Figure 6 Concentration-response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery by levcromakalim in the presence of ODQ (10 μM). Relaxations were determined in the presence or absence of SNAP (50 μM) either (a) in the presence or (b) in the absence of a functional endothelium. Values are shown as mean and vertical lines indicate s.e.mean. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) control, $n=8$; with SNAP, $n=6$; for (b) $n=4$ for both curves.

principally via cyclic GMP-dependent (and K_{ATP}-independent) processes, it can modulate the K_{ATP} channel through mechanisms which are both dependent and independent of the actions of cyclic GMP. The former predominate in endothelium-denuded vessels and the latter in intact preparations. Modulation of K_{ATP} by either mechanism does not contribute to the relaxation produced by SNAP.

SNAP was significantly more potent at relaxing endothelium-denuded than intact vessels, a common feature of nitric oxide donors (Busse *et al.*, 1990; Douglas & Hiley, 1991). The selective, reversible guanylyl cyclase inhibitor, ODQ (Garthwaite *et al.*, 1995) greatly reduced the vasorelaxant activity of SNAP in both intact and endothelium-denuded vessels. It should also be noted that there was no significant difference in the potency of SNAP between endothelium-intact and -denuded vessels in the presence of ODQ. Hence it is possible that the greater relaxant potency of SNAP in denuded vessels in the absence of ODQ may reflect greater ability to activate cyclic GMP-dependent processes in these than in intact vessels. The large inhibitory effect of ODQ on relaxation in response to SNAP shows that the nitric oxide donor causes vasorelaxation primarily via stimulation of guanylyl cyclase in this preparation. The relaxations caused by very high concentrations of SNAP in the presence of ODQ may, however, reflect a cyclic GMP-independent mechanism of relaxation, although it may also be that such high concentrations may overcome the inhibitory effects of ODQ. Some of these results contrast markedly with those obtained by Plane *et al.* (1996), who found that the relaxant potency of the nitric oxide and superoxide donor, SIN-1, was not affected either by the presence of endothelium or ODQ. The reasons for this discrepancy are not clear, however they are unlikely to be due to generation of superoxide by SIN-1, as superoxide dismutase did not alter these results (Plane *et al.*, 1996).

In the present study, SNAP inhibited relaxations to levcromakalim both in the presence and absence of endothelium. However the maximal relaxation to levcromakalim was only reduced in endothelium-denuded vessels. Both SNAP and the membrane-permeant cyclic GMP analogue, 8-bromo cyclic GMP, were only effective at inhibiting relaxations to levcromakalim at concentrations near their respective EC_{50}, but not EC_{30}, values. This may indicate that the modulatory actions of these agents on levcromakalim, are concentration-related, but distinct from their relaxant effects.

In an extensive study on the effects of different protocols on interactions between vasorelaxant agents we found that, although the absolute level of precontracted tone does influence the potency of vasodilators, it does not affect the interactions between them (White & Hiley, unpublished results). The most important factor was that control and test relaxations should be evaluated from the same relative level of tone, as was done in the present study.

Several observations suggest that these effects of SNAP on the actions of levcromakalim are mediated by cyclic GMP-independent mechanisms, as well as those involving cyclic GMP synthesis. Firstly, the membrane-permeable cyclic GMP analogue, 8-bromo cyclic GMP, inhibited relaxations to levcromakalim in endothelium-denuded vessels, but had no effect in intact vessels. Similar results were obtained by McCulloch & Randall (1996) in the rat perfused mesenteric bed. However, ODQ significantly potentiated relaxations to levcromakalim in the presence, but not in the absence, of endothelium, providing evidence that basal cyclic GMP synthesis, stimulated by EDNO, leads to inhibition of KCA activity, as was previously suggested by McCulloch & Randall (1996). It is interesting to note that ODQ only very slightly increased the maximal relaxation to levcromakalim in intact vessels, although this did reach statistical significance. Hence, although the actions of basal production of endogenous cyclic GMP may be similar to those found for 8-bromo cyclic GMP in the present study (both reduce the maximum response to levcromakalim), this cannot be firmly concluded from our results.

The ability of 8-bromo cyclic GMP to mimic the inhibitory effect of SNAP in the absence, but not in the presence, of endothelium suggests that, although nitric oxide may inhibit KCA activity by a cyclic GMP-dependent mechanism in endothelium-denuded vessels (leading to a decrease in the maximum response to levcromakalim), a cyclic GMP-independent mechanism is likely to predominate in endothelium-intact vessels which is not associated with depression of the R_{max}. Similarly, Plane *et al.* (1996) showed that SIN-1, caused relaxation not through a cyclic GMP-dependent mechanism in endothelium-denuded vessels, but through a

cyclic GMP-independent process in intact vessels. Since 8-bromo cyclic GMP retained its relaxant effects in intact vessels, it again seems likely that these may be dissociated from the process by which it modulates K_{ATP}. Hence the observation that the presence of endothelium abolishes the modulatory effect of 8-bromo cyclic GMP (at a near EC_{50} concentration), but does not affect the relaxant response to the cyclic GMP analogue, strongly suggests that this agent does not inhibit relaxations to levcromakalim as a result of a physiological antagonism based on its relaxant actions.

Secondly, although 8-bromo cyclic GMP did not inhibit relaxation to levcromakalim in endothelium-intact vessels, the addition of ODQ to intact vessels revealed an inhibitory effect for the cyclic GMP analogue similar to that observed in the absence of endothelium. These findings suggest that basal release of nitric oxide by the endothelium, stimulating cyclic GMP synthesis in intact vessels, inhibits the actions of exogenous cyclic GMP (Plane *et al*., 1996). Recent evidence suggests that this may involve upregulation of cyclic GMP-specific phosphodiesterase by cyclic GMP in this preparation (Smith *et al*., 1997), although it is also possible that the putative cyclic GMP-dependent mechanism is maximally stimulated by basal release of nitric oxide by the endothelium.

Although the concentration of SNAP established as the approximate EC_{50} for control vessels had only a very small relaxant effect in ODQ-treated vessels, whether endothelium-denuded or intact, this concentration of SNAP retained some inhibitory effect on relaxation to levcromakalim in intact, but not denuded vessels. This inhibition did not produce a reduction in the maximum response. These results indicate that SNAP may normally modulate the K_{ATP} channel via cyclic GMP-independent mechanisms in endothelium-intact vessels. This may again reflect functional inhibition of the actions of cyclic GMP in endothelium-intact vessels (Plane *et al*., 1996; Smith *et al*., 1997), or that the putative modulatory mechanism is already maximally activated by EDNO, as detailed above. Conversely, in denuded vessels, SNAP at the concentrations employed acts solely through cyclic GMP. The observation that inhibition of levcromakalim did not involve a reduction in the maximum response is consistent with the actions of SNAP in intact vessels in the absence of ODQ, whereas 8-bromo cyclic GMP was found to reduce the maximum response; this provides further evidence that SNAP is acting through a different mechanism to cyclic GMP. The precise nature of this mechanism is unclear, although it may involve a direct interaction of nitric oxide with specific residues on the K_{ATP} channel protein (Bolotina *et al*., 1994).

At the higher concentrations of SNAP (approximating to its EC_{50} in the presence of ODQ), inhibition of levcromakalim relaxation was seen in both endothelium-denuded and intact vessels, with the R_{max} for levcromakalim being significantly reduced in each case. These results suggest that very high concentrations of SNAP can surmount the inhibitory effects of ODQ, and cause both relaxation and modulation of the K_{ATP} channel via the actions of cyclic GMP.

It is therefore likely that SNAP may modulate the K_{ATP} channel via both cyclic GMP-dependent and -independent mechanisms. However relaxations to SNAP in the presence or absence of endothelium were unaffected by the K_{ATP} channel inhibitor glibenclamide, indicating that activation of such channels does not contribute to the relaxant effects of SNAP. This may indicate that although nitric oxide may modulate the activity of the K_{ATP} channel, it does not itself activate the channel, which has also been demonstrated by Shinbo & Iijima (1997) in guinea-pig ventricular cells.

Our results do not entirely rule out activation of the K_{ATP} channel by nitric oxide, however. Nitric oxide donor compounds have been shown to activate K_{ATP} channels via a cyclic GMP-dependent mechanism, presumably involving activation of cyclic GMP-dependent protein kinase, in rat aortic smooth muscle cells (Kubo *et al*., 1993) and rabbit mesenteric artery (Murphy & Brayden, 1995), and by a cyclic GMP-independent mechanism in the rat mesenteric artery (Weidelt *et al*., 1997). However Garland & McPherson (1992) showed that, although nitric oxide caused hyperpolarization of the rat mesenteric artery through activation of K_{ATP} channels and that this could be blocked by glibenclamide,this did not contribute to the vasorelaxant actions of nitric oxide. These findings are in accordance with those of the present study, which suggest that SNAP may modulate the K_{ATP} channel via a cyclic GMP-independent mechanism, but that this does not contribute to the relaxant effects of the nitric oxide donor.

Furthermore, there is evidence that the relationship between K_{ATP} channel activation and vasorelaxation may be more complex than was previously thought. In particular, Bray & Quast (1992) showed that although glibenclamide and tedisamil inhibited K_{ATP} channel activation by levcromakalim with similar potencies, they showed markedly different potencies at inhibiting relaxation to this KCA. Similarly, Quast *et al*. (1995) showed that Ba^{2+} was much more potent at inhibiting K_{ATP} channel activation by levcromakalim than at inhibiting its relaxant effects, and estimated that 97% of channels could be blocked before the relaxant effects were inhibited. Such dissociation between channel opening and vasorelaxation suggests that nitric oxide could activate K_{ATP} channels without causing vasorelaxation.

In summary, the present study has shown that relaxation to the KCA, levcromakalim, can be inhibited by the nitric oxide donor, SNAP, apparently by both cyclic GMP-dependent and cyclic GMP-independent mechanisms. Our results also show that the presence of a functional endothelium modulates the actions of vasodilator agents. Further studies, including electrophysiology, are required to elucidate the precise mechanism by which SNAP and 8-bromo cyclic GMP modulate K_{ATP} at the single channel level.

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References

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

BRAY, K. & QUAST, U. (1992). Differential inhibition by tedisamil (KC 8857) and glibenclamide of the responses to cromakalim and minoxidil sulphate in rat isolated aorta. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **345,** 244–250.

BÜSSE, R., POHL, U., MULSCH, A. & BASSENGE, E. (1990). Modulation of the vasodilator action of SIN-1 by the endothelium. *J. Cardiovasc. Pharmacol.,* **14 (suppl. 11),** S81–S85.

COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.,* **9,** 21–28.

CRIDDLE, D.N., JAZBIK, W. & SOARES DE MOURA, R. (1995). Differential vasorelaxant effects of levcromakalim and P1060 in the isolated KCl- and RbCl precontracted human saphenous vein: possible involvement of intracellular Ca^{2+} stores. *Eur. J. Pharmacol.,* **286,** 123–130.

DOUGLAS, S.A. & HILEY, C.R. (1991). Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16-21) and other endothelin-related peptides in the rat. *Br. J Pharmacol.,* **104,** 311–320.

FELEDER, E.C. & ADLER-GRASCHINSKY, E. (1997). Endothelium-mediated and N^{ω}-nitro-L-arginine methyl ester-sensitive responses to cromakalim and diazoxide in the rat mesenteric bed. *Eur. J. Pharmacol.,* **319,** 229–238.

GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.,* **105,** 429–435.

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSEN, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.,* **48,** 184–188.

ITO, S., KAJIKURI, J., ITOH, T. & KURIYAMA, H. (1991). Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. *Br. J. Pharmacol.,* **104,** 227–233.

KUBO, M., NAKAYA, Y., MATSUOKA, S., SAITO, K. & KURODA, Y. (1993). Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle. *Circ. Res.,* **74,** 471–476.

MCCULLOCH, A.I. & RANDALL, M.D. (1996). Modulation of vasorelaxant responses to potassium channel openers by basal nitric oxide in the rat isolated superior mesenteric arterial bed. *Br. J. Pharmacol.,* **117,** 859–866.

MULVANY, M.J. & HALPERN, W. (1977). Contractile responses of small resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19–26.

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

PLANE, F., HURRELL, A., JEREMY, J.Y. & GARLAND, C.J. (1996). Evidence that potassium channels make a major contribution to SIN-1 evoked relaxation of rat isolated mesenteric artery. *Br. J. Pharmacol.,* **119,** 1557–1562.

QUAST, U., BAUMLIN, Y. & LOFFLER, C. (1995). Ba^{2+} differentially inhibits the Rb^+ efflux promoting and the vasorelaxant effects of levcromakalim and minoxidil sulphate in rat isolated aorta. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **353,** 86–93.

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.

SHINBO, A. & IIJIMA, T. (1997). Potentiation by nitric oxide of the ATP-sensitive K^+ current induced by K^+ channel openers in guinea-pig ventricular cells. *Br. J. Pharmacol.,* **120,** 1568–1574.

SMITH, J.J., PLANE, F. & GARLAND, C.J. (1997). Modulation of cyclic GMP-mediated dilatation in the rat isolated mesenteric artery by basally released nitric oxide. *Br. J. Pharmacol.,* **122,** 46P.

WEIDELT, T., BOLDT, W. & MARKWARDT, F. (1997). Acetylcholine-induced K^+ currents in smooth muscle cells of intact rat small arteries. *J Physiol.,* **500,** 617–630.

WHITE, R. & HILEY, C.R. (1997a). A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. *Br. J. Pharmacol.,* **122,** 1573–1584.

WHITE, R. & HILEY, C.R. (1997b). Endothelium and cannabinoid receptor involvement in levcromakalim relaxation. *Eur. J. Pharmacol.,* **339,** 157–160.

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