ELSEVIER

European Journal of Pharmacology 357 (1998) 41–51

ejp

Effects of K^+ channel openers on relaxations to nitric oxide and endothelium-derived hyperpolarizing factor in rat mesenteric artery

Richard White, C. Robin Hiley *

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, UK

Received 4 May 1998; revised 10 July 1998; accepted 17 July 1998

Abstract

Relaxation of methoxamine-precontracted, endothelium-intact, rat mesenteric artery in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) and indomethacin (10 μM) is attributed to endothelium-derived hyperpolarizing factor (EDHF). The potency of carbachol in the presence (but not the absence) of L-NAME was reduced by levcromakalim and pinacidil, activators of ATP-sensitive K^+ channels (K_{ATP}). EDHF-mediated relaxation to Ca^{2+} ionophore A23187 was unaffected by these compounds but was inhibited by verapamil at the level of the smooth muscle. Levcromakalim and pinacidil had the same effects at both reduced and standard levels of tone. Glibenclamide (10 μM), a K_{ATP} blocker, alone did not affect carbachol relaxations but abolished both relaxation to levcromakalim and pinacidil and their inhibitory action on EDHF released by carbachol. Levcromakalim inhibited the endothelium-dependent hyperpolarization of mesenteric arteries to carbachol but not to A23187. Thus, levcromakalim or pinacidil inhibit EDHF, but not nitric oxide, release by carbachol through an action on the endothelium.

Keywords: EDHF (endothelium-derived hyperpolarizing factor); Mesenteric artery, rat; Endothelium; Levcromakalim; Nitric oxide (NO); Ca^{2+} ionophore A23187; Carbachol

1. Introduction

The K^+ channel activating agents are a structurally diverse group of compounds (Edwards and Weston, 1990) that open ATP-sensitive K^+ channels (K_{ATP}). K_{ATP} are present in many types of vascular smooth muscle, and their activation causes vasorelaxation that is thought to be a consequence of membrane hyperpolarization due to outward K^+ currents (Kühberger et al., 1993). Vasorelaxation may be due to the membrane hyperpolarization inhibiting the opening of voltage-operated Ca^{2+} channels (Cook, 1988), although there is also evidence that hyperpolarization may inhibit synthesis of inositol 1,4,5-trisphosphate (IP_3; Ito et al., 1991), which would also contribute to relaxation of agonist-induced tone.

K_{ATP} have also been found on endothelial cells (Janigro et al., 1993; Katnik and Adams, 1997) and activation of these channels may hyperpolarize the endothelium. This will increase the gradient for Ca^{2+} influx (Lückhoff and Busse, 1990) and therefore may promote the release of endothelium-derived factors. Indeed, Hutcheson and Griffith (1994) showed that activation of endothelial K_{ATP} may contribute to shear stress-induced release of endothelium-derived nitric oxide in the rabbit aorta. Furthermore, Kuo and Chancellor (1995) showed that adenosine dilated porcine coronary arteries by activating endothelial K_{ATP} and hence, stimulating release of nitric oxide by the endothelium. There is evidence that the vasorelaxation elicited in some tissues by K^+ channel activating agents may be dependent on the release of endothelium-derived factors; examples include the dog epicardial coronary artery (Drieu La Rochelle et al., 1992) and the rat perfused mesenteric bed (Feleder and Adler-Graschinsky, 1997). Also, we have recently demonstrated that K^+ channel activating agents release an endothelium-derived factor with properties similar to those of endothelium-derived hyperpolarizing factor (EDHF) in the rat isolated mesenteric artery (White and Hiley, 1997b).

McCulloch and Randall (1997) reported in an abstract that levcromakalim inhibited EDHF-mediated relaxation to

* Corresponding author. Tel.: +44-1223-334028; Fax: +44-1223-334040; E-mail: crh1@cam.ac.uk

0014-2999/98/$19.00
PII: S0014-2999(98)00538-X

carbachol in the rat perfused mesenteric bed. However, they did not examine whether the effect of levcromakalim was mediated by activation of K_{ATP} or was an effect specific to levcromakalim alone. Furthermore, although they suggested that inhibition of EDHF by K^+ channel activating agents occurred at the level of the smooth muscle, this may not necessarily be the case. In view of our recent findings that K^+ channel activating agents may have endothelium-dependent actions (White and Hiley, 1997b), and that inhibition of EDHF activity by K^+ channel modulators may occur at both the endothelium and smooth muscle (White and Hiley, 1997a), we have examined in more detail the nature of the inhibition of EDHF by K^+ channel activating agents in the rat isolated mesenteric artery.

In this study, we have used both levcromakalim and pinacidil to investigate whether or not inhibition of EDHF is mediated by structurally distinct K^+ channel activating agents. We have also used the K_{ATP} channel inhibitor, glibenclamide, to identify the possible role of these channels in inhibition of EDHF-mediated relaxation. As K_{ATP} are present on the endothelium and smooth muscle, the inhibitory effect of the K^+ channel activating agents on EDHF could be due to actions at either or both sites. In order to distinguish these possibilities, EDHF was released by the use of both carbachol and the Ca^{2+} ionophore A23187 in the presence of N^G-nitro-L-arginine methyl ester (L-NAME). A23187 elevates endothelial Ca^{2+} levels by exchange of one Ca^{2+} for two H^+ ions (Reed and Lardy, 1972), and its actions should therefore be largely independent of changes in membrane potential. Hence, inhibition of those actions of A23187 which are mediated by EDHF should reflect actions at the level of the smooth muscle, which we have verified using the L-type Ca^{2+} channel inhibitor verapamil. We have also examined, with respect to the degree of precontraction used, the possible influence of the protocol employed in studies investigating the interactions between vasorelaxant agents.

2. Materials and methods

2.1. Rat isolated mesenteric artery preparation

Male Wistar rats (250–350 g; Tucks, Rayleigh, Essex, UK) were killed with an overdose of sodium pentobarbitone (120 mg kg^{-1}, i.p., Sagatal, Rhone Merieux, Harlow, UK). 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, J.P. Trading, Aarhus, Denmark) as described in White and 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 normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany and Halpern, 1977). The mean vessel diameter under these conditions was 357 ± 4 μm ($n = 152$). The vessels were left for another 30 min before experiments commenced.

2.2. Experimental protocol

After the equilibration period, the integrity of the endothelium was assessed by pre-contracting the vessels with methoxamine (10 μM) and then adding carbachol (10 μM). The mean tension generated by vessels in response to 10 μM methoxamine was 12.7 ± 0.3 mN ($n = 152$); the size of this initial response to 10 μM methoxamine was used to standardise precontraction tone for subsequent relaxation experiments. Tissues which relaxed to carbachol by greater than 90% were designated as endothelium-intact.

The actions of EDHF were examined by assessing the vasorelaxation to carbachol and A23187 in the presence of L-NAME which was added 30 min before, and was then present throughout, construction of the agonist concentration–response curves. Although preincubation of tissues with L-NAME (100 μM, 30 min) had no effect on resting tension, the vasoconstrictor effect of methoxamine was greatly augmented. In view of this, the concentration of methoxamine used to precontract vessels was reduced (to 1–3 μM) such that an equivalent level of tone was induced to that obtained in response to 10 μM methoxamine in the same tissue in the absence of L-NAME.

When examining the effect of addition of a vasorelaxant agent (drug A) on subsequent responses to a different vasodilator (drug B), it is important to account for the reduction in tone caused by the presence of the first drug added (drug A). We have used two protocols for this (Fig. 1). In the first, control responses were assessed at a 'reduced' level of precontracted tone, obtained by reducing the concentration of methoxamine added. This was the approach taken by, for example, McCulloch et al. (1997). Alternatively, control responses to drug B can be assessed at 'standard' levels of tone. For the assessment of the responses to drug B in the presence of drug A, the concentration of methoxamine was increased so that the reduction in tone caused by addition of drug A was reversed. This approach was used by Plane and Garland (1996).

2.2.1. Reduced tone protocol

Vessels were first precontracted to a level of tone equal to that obtained in the test for endothelial integrity by addition of 10 μM methoxamine (Fig. 1a). Drug A

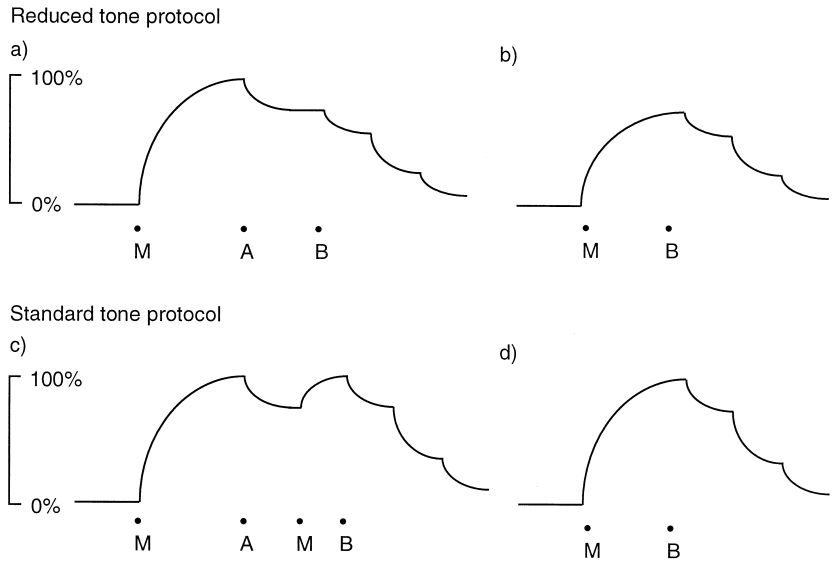

Fig. 1. Schematic representations of the protocols used to assess interactions between vasodilator drugs. (a) and (b) were used to compare responses at a reduced level of tone as compared to that induced by 10 μM methoxamine in the initial test for endothelial integrity. (c) and (d) show the protocols for comparison of responses at the same level of tone as was induced by 10 μM methoxamine in the test for endothelial function. M represents addition of methoxamine, A addition of the interacting vasodilator (which was used at a fixed concentration) and B the start of determination of the concentration/effect curve for the vasodilator under test.

(levcromakalim, pinacidil or verapamil) was then added at a concentration that gave 25–40% relaxation of the precontraction. After vessel tone stabilised at this reduced level, a cumulative concentration–response curve to drug B (carbachol, A23187 or verapamil) was constructed. These data were compared with control data for drug B obtained after contracting vessels to a lower tone level (60–75% of that obtained in the test for endothelial integrity; Fig. 1b) by precontraction with a lower concentration of methoxamine (1–3 μM in the absence of L-NAME, 0.3–1 μM in its presence). Preliminary experiments showed that higher concentrations of the K^+ channel activating agents (causing 45–65% relaxation of methoxamine-precontracted tone) did not produce a stable level of tone and therefore could not be used under this protocol.

2.2.2. Standard tone protocol

As shown in Fig. 1c, vessels were first precontracted, by addition of methoxamine (10 μM in the absence of L-NAME, 1–3 μM in its presence), to a level of tone equal to that which had been obtained in the test for endothelial integrity. Drug A (levcromakalim, pinacidil or verapamil) was then added at a concentration producing approximately 25–35% relaxation of tone. After the tone of the vessel had stabilised at this reduced level, the methoxamine concentration was increased (to 30–50 μM in the absence of L-NAME or 5–20 μM in its presence) such that tone returned to the level obtained before addition of drug A. After stabilisation of tone at this standard level (that is, equal to that obtained in the test for endothelial integrity), a cumulative concentration–response curve to drug B was constructed. These data were compared with control data for drug B alone using the standard level of tone (Fig. 1d).

2.3. Electrophysiological studies

Mesenteric arteries were removed from rats as described above and were then cut open along the long axis before being pinned, intimal surface upwards, to the silicone rubber base of an organ chamber (volume 0.5 ml). When required, endothelium was removed by rubbing the intimal surface with a moistened cotton bud. The chamber was perfused with Krebs–Henseleit solution, containing 100 μM L-NAME and 10 μM indomethacin, at 3 ml min^{-1} and 37°C. Transmembrane potential was recorded with glass microelectrodes filled with 1 M KCl (tip resistance 50–80 MΩ) which were connected to the headstage of a recording amplifier with capacitance neutralization (Axoclamp 2B; Axon Instruments, Foster City, CA, USA). Output was recorded on a Gould chart recorder (Valley View, OH, USA). Cells were impaled (as determined by the sudden development of a negative potential held for at least 10 min) from the intimal side and were held under current-clamp conditions. Drugs were applied by changing the perfusing solution to Krebs–Henseleit containing the required concentrations of the agents under investigation.

Levcromakalim (0.1 μM) was applied, where appropriate, 10 min before addition of carbachol or A23187. When used, glibenclamide (10 μM) was added 10 min before the levcromakalim.

2.4. Drugs

All solutions were prepared on the day of the experiment. Methoxamine, carbachol and L-NAME (all from Sigma) were dissolved in distilled water. Ca^{2+} ionophore A23187 and verapamil (Sigma) were dissolved in 100% ethanol as 10 mM stock solutions. Pinacidil (RBI, Natick, MA, USA) and levcromakalim (SmithKline Beecham, Betchworth, UK) were dissolved in 70% (v/v) ethanol as 10 mM stock solutions. Dilutions were made in distilled water. The maximum volume of solvent added to the 10 ml myograph bath was 10 μl.

2.5. Statistical analysis

All relaxation responses are expressed as the percentage relaxation of the tone induced by methoxamine. Data are given as the mean ± S.E.M. 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 (Synergy Software, Reading, PA, USA) running on a Macintosh computer. Except for the electrophysiology, statistical analysis of the variables was carried out by two-way analysis of variance and an F-test. Comparison of electrophysiological data was by Student's unpaired t-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.

3. Results

3.1. Effect of K^+ channel activating agents on relaxation to carbachol and verapamil

In the presence of L-NAME, and at a reduced level of methoxamine-induced tone, carbachol caused concentration-dependent relaxation (Fig. 2, Table 1). Prior addition of either of the K^+ channel activating agents levcromakalim (150 nM) or pinacidil (1 μM) significantly ($P < 0.001$) reduced the potency of carbachol 3-fold and 2.5-fold, respectively, but had no effect on the maximum relaxation (Fig. 2, Table 1).

In the absence of L-NAME, carbachol also caused concentration-dependent relaxations at a reduced level of tone with an $EC_{50} = 74 \pm 2$ nM and an $R_{max} = 86.0 \pm 0.7\%$ ($n = 6$). In this case, however, prior addition of levcromakalim (200 nM) had no effect on either the potency or maximum relaxation to carbachol ($EC_{50} = 80 \pm 7$ nM, $R_{max} = 79.6 \pm 0.2\%$, $n = 8$, Fig. 3a).

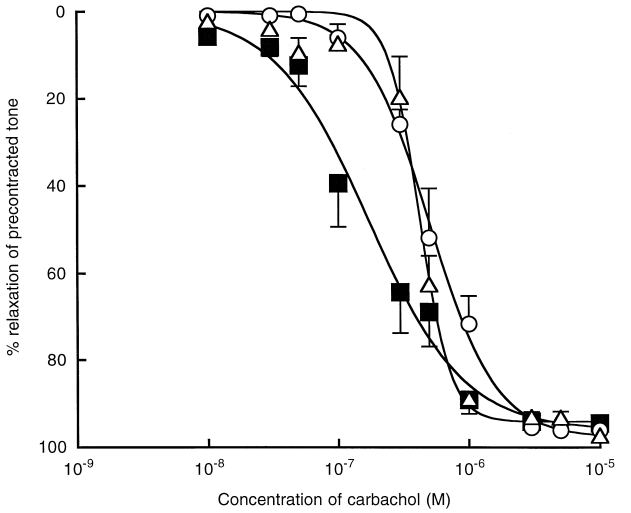

Fig. 2. Concentration–response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery in the presence of 100 μM L-NAME. Relaxation was induced by carbachol in the absence (filled squares; $n = 10$) and presence of the K^+ channel activators, levcromakalim (150 nM; open circles; $n = 4$) and pinacidil (1 μM; open triangles; $n = 4$). Reduced tone protocols were used for the control experiments such that relaxation was from the same methoxamine-induced tone in the presence and absence of the K^+ channel activators (Table 1). Values are shown as mean and vertical lines indicate S.E.M. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in Table 1.

In the presence of L-NAME, and at a reduced level of methoxamine-induced tone, verapamil caused concentration-dependent relaxations ($EC_{50} = 0.15 \pm 0.04$ μM, $R_{max} = 88.8 \pm 0.6\%$, $n = 8$). Prior addition of levcromakalim (200 nM) had no significant effect on either the potency or maximum relaxation to verapamil ($EC_{50} = 0.15 \pm 0.01$ μM, $R_{max} = 96.0 \pm 1.2\%$, $n = 8$, Fig. 3b).

3.2. Effect of K^+ channel activating agents on relaxation to A23187 in the presence of L-NAME

In the presence of L-NAME, and at a reduced level of methoxamine-induced tone, A23187 caused concentration-dependent relaxation (data not shown). Prior addition of either of the K^+ channel activating agents levcromakalim (100 nM) or pinacidil (500 nM) had no significant effect on either the potency or maximum relaxation to A23187 (Table 1).

3.3. Effect of glibenclamide on relaxation to carbachol

Glibenclamide (10 μM) had no significant effect on basal tone or that induced by methoxamine (data not

Table 1
Effects of levcromakalim and pinacidil on relaxation to carbachol and A23187 in the presence of L-NAME in the rat isolated mesenteric artery with intact endothelium

	Initial tone (%)	EC_{50} [b]	R_{max} (%)	*n*
Carbachol				
Reduced tone				
Control	76.4 ± 1.7	0.17 ± 0.02	96.1 ± 3.9	10
+ Levcromakalim	75.7 ± 3.4	0.51 ± 0.02[a]	97.8 ± 1.2	4
+ Pinacidil	72.5 ± 2.4	0.42 ± 0.02[a]	94.2 ± 2.7	4
Standard tone				
Control	98.3 ± 3.6	0.32 ± 0.02	90.7 ± 2.2	6
+ Levcromakalim	103.7 ± 2.8	0.84 ± 0.09[a]	83.3 ± 3.1	6
A23187				
Reduced tone				
Control	72.6 ± 6.4	45.1 ± 9.4	96.5 ± 4.8	9
+ Levcromakalim	65.3 ± 10.8	47.2 ± 2.9	93.7 ± 1.6	8
+ Pinacidil	64.0 ± 6.0	49.0 ± 10.7	98.8 ± 6.7	8
Standard tone				
Control	98.0 ± 11.4	137 ± 30	96.5 ± 10.3	4
+ Pinacidil	92.5 ± 4.4	140 ± 41	95.9 ± 9.1	4

In reduced tone experiments, control responses were obtained with 0.3–1 μM methoxamine in order to start relaxation from the same tone as in the presence of the K^+ channel activating agents. Relaxation in the presence of levcromakalim and pinacidil was recorded in the presence of 1–3 μM methoxamine.
In standard tone experiments, control relaxations were obtained in the presence of 1–3 μM methoxamine. In the presence of levcromakalim and pinacidil, a higher concentration of methoxamine (5–20 μM) was added to give an equivalent level of tone to control vessels.
Data are expressed as mean ± S.E.M. % Initial tone shows the tone that was present at the start of a relaxation curve as a percentage of the tone elicited by 10 μM methoxamine in the initial test for endothelial integrity. EC_{50} and R_{max} values were obtained from the curve fitting procedure described in Section 2.
n-Values indicate the number of animals used.
[a] $P < 0.001$ indicates significant differences from control values.
[b] EC_{50} values for carbachol are in μM, for A23187 in nM.

shown). Glibenclamide (10 μM) also had no significant effect on the relaxation by carbachol of the standard level of methoxamine-induced tone either in the absence of the nitric oxide synthase inhibitor, L-NAME (control, $EC_{50} = 0.40 \pm 0.02$ μM, $R_{max} = 87.9 \pm 1.3\%$, $n = 4$; glibenclamide, $EC_{50} = 0.48 \pm 0.07$ μM, $R_{max} = 91.5 \pm 4.1\%$, $n = 4$, Fig. 4a) or in its presence (control, $EC_{50} = 0.64 \pm 0.01$ μM, $R_{max} = 92.8 \pm 0.6\%$, $n = 9$; glibenclamide, $EC_{50} = 0.69 \pm 0.08$ μM, $R_{max} = 94.0 \pm 3.1\%$, $n = 7$, Fig. 4b).

In the presence of glibenclamide (10 μM), the relaxant effect of levcromakalim (300 nM) was abolished ($n = 4$). Furthermore, levcromakalim no longer significantly affected relaxation to carbachol in the presence of L-NAME and glibenclamide ($EC_{50} = 0.89 \pm 0.10$ μM, $R_{max} = 89.6 \pm 3.5\%$, $n = 4$, Fig. 4c). Glibenclamide also abolished the ability of pinacidil both to cause relaxation and inhibit EDHF-mediated relaxations to carbachol (data not shown).

3.4. Effect of verapamil on relaxation to carbachol and A23187 in the presence of L-NAME

Fig. 5a shows that, in the presence of L-NAME and at a reduced level of methoxamine-induced tone, carbachol caused concentration-dependent relaxations. Prior addition of the L-type Ca^{2+} channel blocker verapamil (150 nM), which has effects only at the level of the smooth muscle, significantly ($P < 0.001$) reduced the potency of carbachol 3.4-fold but had no significant effect on the maximum response (Fig. 5a).

With a similar lower degree of tone, relaxations to A23187 in the presence of L-NAME had an EC_{50} of 38 ± 7 nM and an R_{max} of 95.0 ± 4.4% ($n = 8$). Prior addition of verapamil (300 nM; Fig. 5b) also significantly

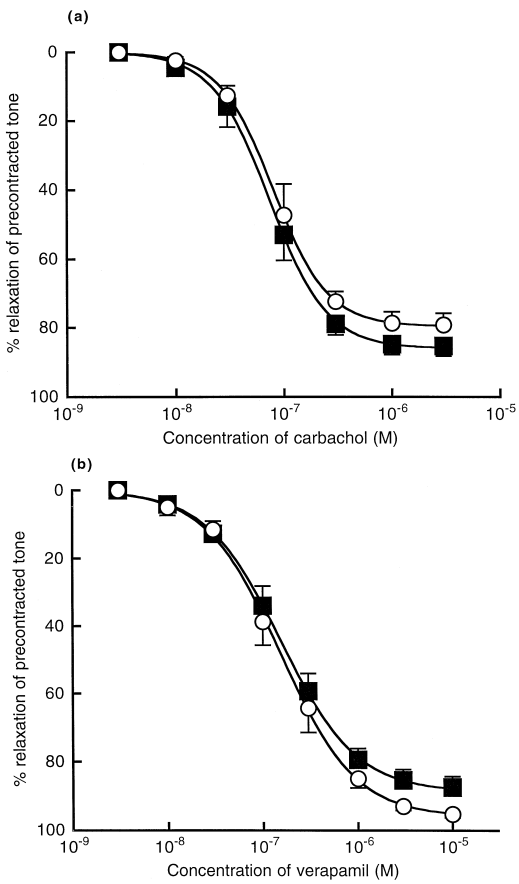

Fig. 3. Concentration–response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery. Relaxation was induced by (a) carbachol in the absence of L-NAME or (b) verapamil in the presence of L-NAME, and was determined in the absence (filled squares) and presence (open circles) of levcromakalim (200 nM). Values are shown as mean and vertical lines indicate S.E.M. Reduced tone protocols were used such that relaxation was from the same tone in the presence and absence of levcromakalim (initial tone as a percentage of the first response to 10 μM methoxamine. (a) Control, 63.4 ± 9.8%; levcromakalim, 58.8 ± 9.2%. (b) Control, 71.5 ± 5.1%; levcromakalim, 74.8 ± 1.7%). The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. $n = 8$ for each curve except the control for carbachol in which $n = 6$.

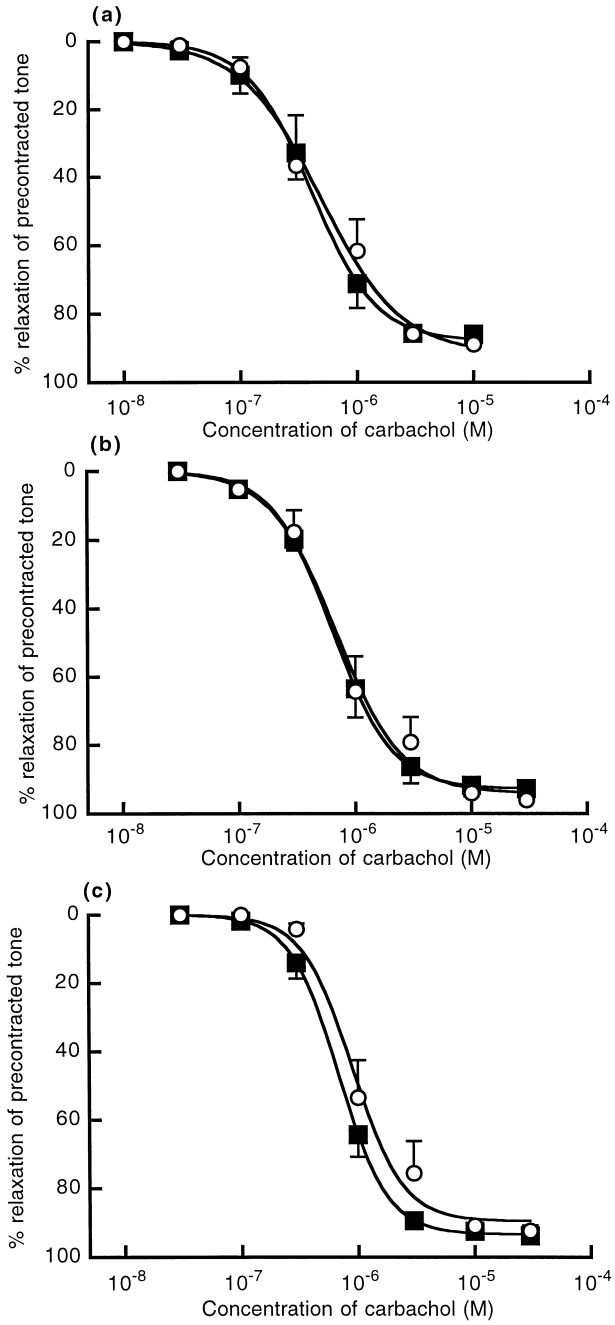

Fig. 4. Concentration–response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery. Relaxation was induced by carbachol and was determined in the absence (filled squares) or presence (open circles) of 10 μM glibenclamide (a) in the absence, or (b) the presence of 100 μM L-NAME, or (c) the presence of L-NAME and 300 nM levcromakalim. Values are shown as mean and vertical lines indicate S.E.M. Standard tone protocols were used (i.e., relaxation was from the tone induced by 10 μM methoxamine alone). The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text. For (a) and (c), $n = 4$ for both curves. For (b) control, $n = 9$; in the presence of glibenclamide, $n = 7$.

($P < 0.01$) reduced the potency of A23187 by 3.5-fold ($EC_{50} = 132 \pm 24$ nM, $n = 9$) but had no effect on the maximum response ($R_{max} = 95.6 \pm 7.8\%$).

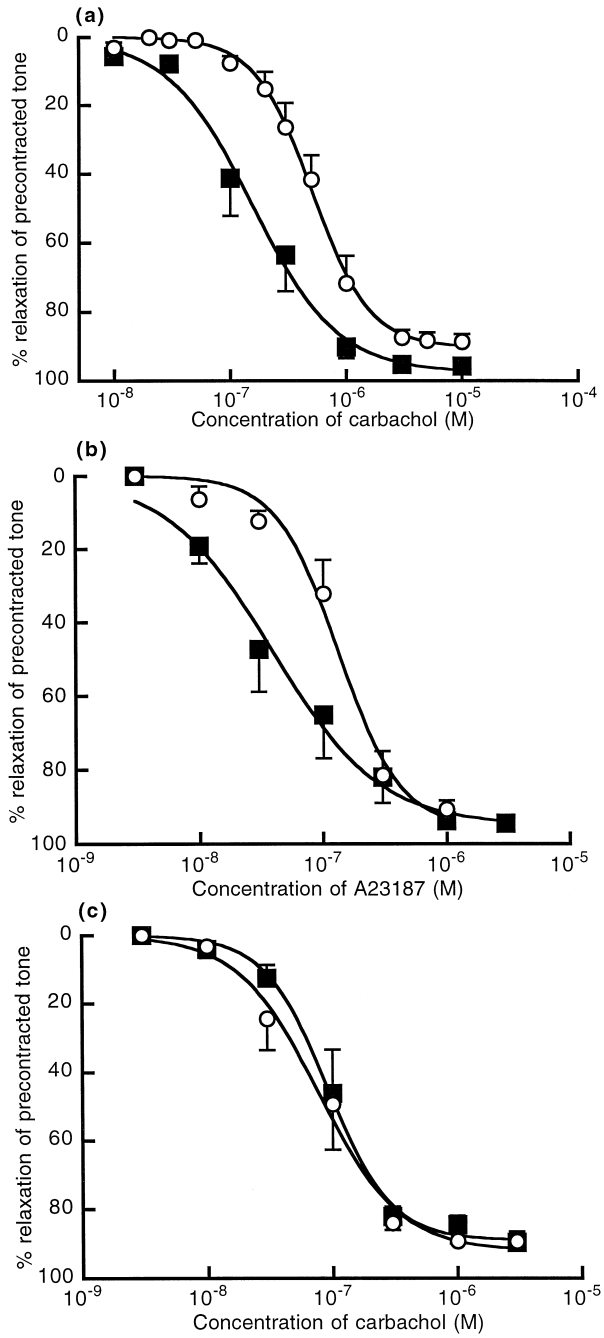

Fig. 5. Concentration–response curves for relaxation of methoxamine-induced tone in the rat isolated mesenteric artery. Relaxations were induced in the absence (filled squares) or presence (open circles) of verapamil by (a) carbachol or (b) A23187 in the presence of 100 μM L-NAME or (c) carbachol in the absence of L-NAME. Reduced tone protocols were used; the concentrations of verapamil used (a, 150 nM; b, 300 nM; c, 200 nM) gave the same relaxation of the tone initially induced by 10 μM methoxamine. In (a) Control, $n = 9$, initial tone = 76.0 ± 1.4% of tone induced by the test administration of 10 μM methoxamine; in the presence of verapamil, $n = 8$, initial tone = 71.2 ± 4.3%. In (b) Control, $n = 8$, and initial tone = 70.6 ± 6.9%; verapamil, $n = 9$, and initial tone = 70.9 ± 3.6%. In (c) Control, $n = 5$, initial tone = 66.5 ± 4.7%; verapamil, $n = 4$, initial tone = 60.6 ± 5.3%. Values are shown as mean and vertical lines indicate S.E.M. The curves drawn are those obtained from the curve-fitting procedure and the parameters describing the curves are given in the text.

3.5. Effect of verapamil on relaxation to carbachol in the absence of L-NAME

The concentration–response curve for relaxation of a reduced level of methoxamine-induced tone by carbachol in the absence of L-NAME was described by an EC_{50} 91 ± 7 nM and an R_{max} $89.0 \pm 2.1\%$ ($n = 5$). Prior addition of verapamil (200 nM, causing $39.4 \pm 5.3\%$ relaxation of methoxamine-induced tone, $n = 4$) had no effect on either the potency or maximum relaxation to carbachol (EC_{50} 77 ± 10 nM, R_{max} $92.1 \pm 3.5\%$, $n = 4$, Fig. 5c).

3.6. Comparison of standard and lowered tone protocols

Table 1 shows that, in the presence of L-NAME and at the standard level of methoxamine-induced tone (identical to the test contraction to 10 μM methoxamine in the absence of L-NAME), prior addition of levcromakalim (300 nM) followed by further addition of methoxamine to restore tone to the previous level, significantly ($P < 0.001$) reduced the potency of carbachol, although there was no change in the maximum response. The magnitude of the rightward shift in the carbachol concentration–response curve (2.6 fold) was similar to that found using lower tone controls (3 fold).

Similarly, prior addition of pinacidil (500 nM) and further addition of methoxamine to restore tone to the previous level, caused no significant change in either the potency or maximum response to A23187 in the presence of L-NAME (Table 1). This is also consistent with the results from the experiments employing the lower tone controls.

In the absence of L-NAME, carbachol caused concentration-dependent relaxation of the standard level of methoxamine-induced tone with EC_{50} 0.23 ± 0.07 μM and R_{max} $85.5 \pm 0.8\%$ ($n = 6$). With prior addition of levcromakalim (200 nM) followed by further addition of methoxamine to restore tone to the previous level, there was no change in either the potency or maximum response to carbachol (EC_{50} 0.24 ± 0.05 μM, R_{max} $79.1 \pm 0.5\%$, $n = 6$; data not shown), consistent with findings from experiments using the lower tone controls.

3.7. Effect of lower tone on vasorelaxation

Table 2 shows that reducing the level of tone developed by the vessels significantly increased the potency of each of the vasorelaxant agents studied ($P < 0.001$ for all). The leftward shifts in the concentration–response curves were similar in each case, for carbachol in the absence of L-NAME (2.6 fold), carbachol in the presence of L-NAME (4.3 fold), A23187 in the presence of L-NAME (3.7 fold), levcromakalim (2.1 fold), pinacidil (2.7 fold) and verapamil (2.8 fold).

3.8. Electrophysiological studies

The resting membrane potential of mesenteric arteries with intact endothelium in the presence of 10 μM indomethacin and 100 μM L-NAME was -55.2 ± 1.5 mV ($n = 25$). Both carbachol (Fig. 6a) and A23187 (Fig. 6b) caused concentration-dependent hyperpolarization, which can be attributed to EDHF, at concentrations similar to those causing vasorelaxation. Removal of the endothelium abolished the hyperpolarizations to carbachol and A23187 (data not shown). Addition of levcromakalim (0.1 μM) caused a hyperpolarization of 12.4 ± 1.3 mV ($n = 9$) and significantly reduced the change in membrane potential induced by subsequent addition of a submaximal concentration of carbachol (1 μM; Fig. 6a). In contrast the presence of levcromakalim did not affect the change in membrane potential caused by subsequent addition of A23187, again at a submaximal concentration (1 μM; Fig. 6b). The maximum recorded responses to levcromakalim occurred at 1 μM and were 25.2 ± 2.0 mV ($n = 5$). Incubation of vessels with glibenclamide (10 μM) significantly

Table 2
Effect of lowering precontracted tone on the actions of vasorelaxant agents in the rat isolated mesenteric artery with intact endothelium

	Normal tone			Reduced tone		
	EC_{50} (μM)	R_{max} (%)	n	EC_{50} (μM)	R_{max} (%)	n
A23187 + L-NAME	0.17 ± 0.03	95.8 ± 6.3	5	0.05 ± 0.01^{b}	96.4 ± 4.8	9
Carbachol	0.25 ± 0.01	86.3 ± 1.5	7	0.10 ± 0.01^{b}	86.2 ± 1.1	4
Carbachol + L-NAME	0.65 ± 0.02	92.8 ± 0.6	8	0.15 ± 0.02^{b}	96.1 ± 2.5	10
Levcromakalim	0.11 ± 0.01	89.0 ± 1.7	10	0.04 ± 0.01^{b}	97.8 ± 0.5^{b}	9
Pinacidil	1.44 ± 0.14	90.7 ± 4.0	4	0.54 ± 0.05^{b}	88.1 ± 2.8	6
Verapamil	0.45 ± 0.03	94.4 ± 1.6	4	0.16 ± 0.03^{b}	88.8 ± 0.5^{a}	8

Data are expressed as mean ± S.E.M. EC_{50} and R_{max} values were obtained from the curve fitting procedure described in Section 2.
n-Values indicate the number of animals used.
[a] $P < 0.01$, [b] $P < 0.001$ indicate significant differences from control values.

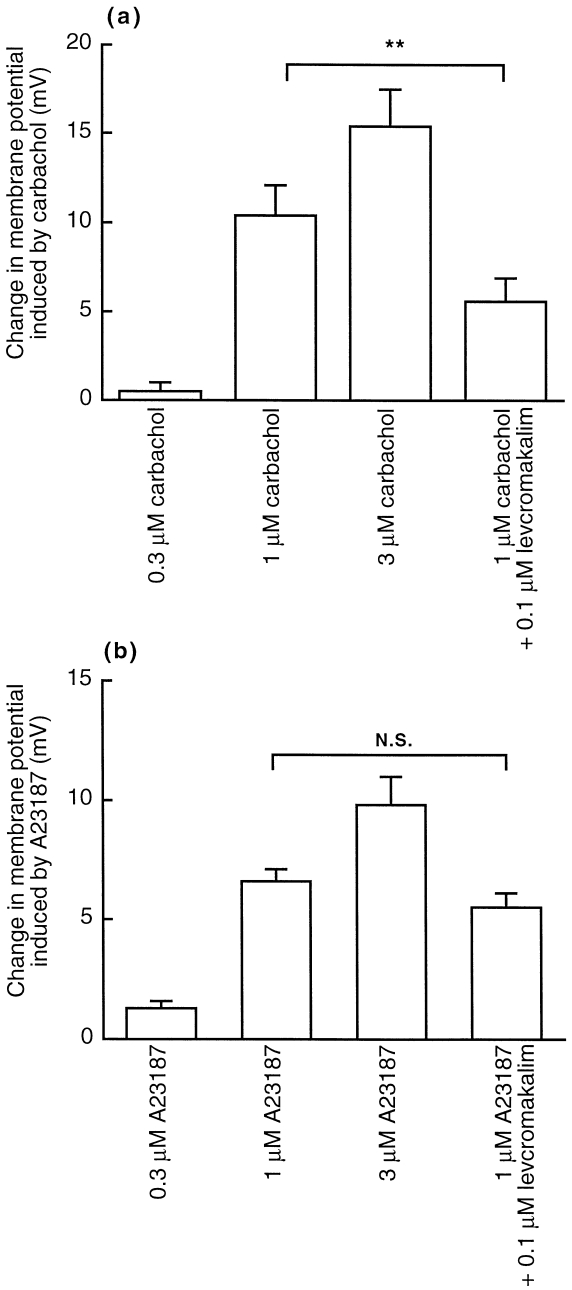

Fig. 6. Hyperpolarizations induced by (a) carbachol and (b) calcium ionophore A23187 in rat isolated, endothelium-intact, mesenteric arteries in the presence and absence of levcromakalim. L-NAME (100 μM) and indomethacin (10 μM) were present throughout. Values are shown as mean and vertical lines indicate S.E.M. $n = 4–6$ for all experiments. Comparison between 1 μM carbachol and 1 μM A23187 in the presence and absence of 0.1 μM levcromakalim was made by Student's *t*-test; N.S. denotes not significant ($P > 0.05$) and ** statistically different with $P < 0.01$.

($P < 0.001$) reduced the hyperpolarization induced by 0.1 μM levcromakalim to 1.7 ± 0.9 mV ($n = 3$).

4. Discussion

The major finding of the present study is that the K^+ channel activating agents, levcromakalim and pinacidil, inhibit relaxation to carbachol mediated by EDHF but not that mediated by nitric oxide. This effect is sensitive to glibenclamide and none of the effects observed can be mediated by cyclo-oxygenase products since 10 μM indomethacin was present in all experiments. In contrast, the K^+ channel activating agents do not inhibit EDHF-mediated relaxation to A23187. These results suggest that K^+ channel activating agents cause inhibition of EDHF release by carbachol by activating K_{ATP} channels at the level of the endothelium, but not the smooth muscle. We have also shown that, although lowering the level of precontracted tone increases the potency of a range of vasodilator agents, this apparently does not alter the interactions between these agents that we have examined.

Levcromakalim and pinacidil both inhibited relaxations to carbachol obtained in the presence of L-NAME, and which are attributed to EDHF. However, levcromakalim did not inhibit relaxations to carbachol in the absence of L-NAME, nor did it inhibit relaxations to verapamil (a blocker of L-type voltage-operated Ca^{2+} channels), ruling out non-specific inhibition of vasorelaxation. These results are similar to those published in the abstract of McCulloch and Randall (1997), however they postulated that inhibition by levcromakalim of EDHF-mediated relaxation in the rat perfused mesenteric bed was due to the effect of levcromakalim on smooth muscle.

However, levcromakalim and pinacidil had no significant effect on EDHF-mediated relaxations to A23187 (i.e., those obtained in the presence of L-NAME). As A23187 causes a direct increase in endothelial Ca^{2+} levels, it stimulates release of EDHF which is known to be Ca^{2+}-dependent (Nagao et al., 1992). Therefore it seems unlikely that levcromakalim and pinacidil exert their inhibitory action on EDHF at the level of the smooth muscle, as if this were the case they would inhibit EDHF-mediated relaxation to A23187.

Selective inhibition by levcromakalim of the endothelium-dependent hyperpolarization to carbachol, but not of that to A23187, was also observed. Levcromakalim produced a glibenclamide-sensitive hyperpolarization of mesenteric artery smooth muscle cells, confirming that K_{ATP} were being activated by the concentration of levcromakalim used. Although levcromakalim (0.1 μM) alone gave an approximate 12 mV hyperpolarization, our observations showed that further hyperpolarization could still occur due to release of EDHF by A23187. Further evidence that the vascular smooth muscle can hyperpolarize considerably beyond the extent seen with 0.1 μM levcromakalim is shown by the maximal response to the K^+ channel activator being 25 mV. Therefore, the decreased change in membrane potential seen with carbachol in the presence of levcromakalim cannot be due simply to the hyperpolarizing effect of levcromakalim on the smooth muscle.

In order to verify the sensitivity of EDHF-mediated relaxations to agents acting at the level of the smooth

muscle, we examined the effects of verapamil on the responses to A23187 in the presence of L-NAME. As the actions of EDHF involve smooth muscle membrane hyperpolarization, which then leads to the closure of voltage-operated L-type Ca^{2+} channels, inhibitors of the L-type Ca^{2+} channel should attenuate EDHF-mediated relaxation. This has been demonstrated in the rat isolated mesenteric artery using nifedipine (Plane and Garland, 1996). However as there are no L-type Ca^{2+} channels on endothelial cells (Nilius et al., 1997), verapamil should have no effect on the endothelium. Here, verapamil (used at a concentration established in preliminary experiments as being its EC_{50} for relaxation of tone induced by depolarising K^+ solution) was found to inhibit EDHF-mediated relaxations to both carbachol and A23187, but had no effect on relaxation to carbachol in the absence of L-NAME which is predominantly mediated by nitric oxide. This shows that agents acting at the level of the smooth muscle cause inhibition of relaxation mediated by EDHF. The observation that verapamil did not inhibit nitric oxide-mediated relaxation to carbachol is also consistent with the results obtained by Plane and Garland (1996) using nifedipine.

Taken together, these results suggest that the inhibitory effect of the K^+ channel activating agents on EDHF-mediated relaxation to carbachol occurs at the level of the endothelium and not the smooth muscle. As A23187 directly elevates Ca^{2+} levels in endothelial cells, the K^+ channel activating agents cannot act to inhibit a process subsequent to Ca^{2+} mobilisation, as they would then cause inhibition of relaxation to A23187. It therefore seems likely that K^+ channel activating agents inhibit the ability of carbachol to cause release of EDHF. This effect would not necessarily inhibit release of nitric oxide, as Bauersachs et al. (1996) showed that nitric oxide attenuated bradykinin-stimulated release of EDHF by reducing the ability of bradykinin to elevate endothelial Ca^{2+} levels, but that this did not inhibit nitric oxide release by bradykinin. Therefore, it is possible that nitric oxide synthesis is stimulated by smaller increases in Ca^{2+} levels than EDHF synthesis, as is the case for prostacyclin (Parsaee et al., 1992).

The mechanism by which the K^+ channel activating agents inhibit release of EDHF by carbachol is not clear. Glibenclamide, a blocker of K_{ATP}, abolished both the relaxant and hyperpolarizing effects of levcromakalim, and its inhibitory action on EDHF, suggesting that all involve activation of K_{ATP}. However glibenclamide had no effect on carbachol relaxation in the presence or absence of L-NAME, showing that activation of K_{ATP} does not normally contribute to agonist-stimulated release of endothelium-derived factors. This confirms the findings of Hutcheson and Griffith (1994) in the rabbit aorta. Our observation that glibenclamide does not potentiate EDHF-mediated relaxation to carbachol indicates that, under the conditions of our experiments, K_{ATP} are not normally active in the absence of K^+ channel activators.

Carbachol is thought to act on M_3 muscarinic receptors in the rat mesentery (Adeagbo and Triggle, 1993). This receptor is coupled to $G_{q/11}$, and activation thus releases intracellular Ca^{2+} stores by an IP_3-dependent mechanism. Sustained release of endothelium-derived factors requires influx of extracellular Ca^{2+}, which is thought to occur via non-selective cation channels (Fukao et al., 1997). The stimulus for mobilisation of extracellular Ca^{2+} has not been identified, but probably involves activation of K^+ channels, as blockers of these channels inhibit hyperpolarization of the endothelium by agonists (Chen and Cheung, 1992). Activation of K^+ channels, which may involve an intermediate 'Ca^{2+} influx factor' (Hoebel et al., 1997), increases the driving force for Ca^{2+} entry.

On the basis of these arguments, K^+ channel activators should also cause an increase in endothelial cytoplasmic Ca^{2+} levels and this has been confirmed experimentally (Lückhoff and Busse, 1990; Langheinrich et al., 1998). This will lead to release of endothelium-derived factors (Feleder and Adler-Graschinsky, 1997; White and Hiley, 1997b) and might therefore be expected to potentiate the actions of carbachol. However, when a vessel is developing tone, relaxation can only occur when there is a change in the balance of factors giving rise to that tone. In the experiments reported here, carbachol must increase the amount of EDHF released if a relaxation is to occur and this must presumably require an increase in the level of endothelial Ca^{2+} beyond that generated by the levcromakalim or pinacidil alone. Therefore, it is possible that the K^+ channel activators inhibit the relaxant actions of carbachol by reducing its ability to increase endothelial cytoplasmic Ca^{2+} concentrations above those that they themselves induce. One way this could happen is if the hyperpolarization caused by K^+ channel activators reduced the driving force for K^+ efflux through the channels activated by carbachol. Alternatively, prior hyperpolarization by K^+ channel activators may inhibit the opening of the K^+ channel normally activated by carbachol. K^+ channel activating agents have also been shown to inhibit IP_3 synthesis (Ito et al., 1991), and may inhibit intracellular Ca^{2+} store refilling (Bray et al., 1991) in smooth muscle cells; if they have similar effects in endothelial cells, this could also explain their subsequent inhibition of the actions of carbachol.

It is clear that, when examining the effect of addition of one relaxant agent on the actions of another, the reduction in tone caused by the initial agent must be taken into consideration. We provide evidence that the two approaches that we have used, which involved either evaluating control data from reduced levels of tone similar to those in the presence of the relaxant, or alternatively adding higher concentrations of methoxamine to restore the tone of vessels treated with the relaxant, give similar results. In the presence of L-NAME, levcromakalim had similar effects on relaxation to carbachol, and pinacidil on relaxation to A23187, with either protocol. Furthermore, in

the absence of L-NAME, levcromakalim had no effect on relaxation to carbachol in either protocol. The only difference found was that all the relaxant agents studied were more potent (i.e., had a lower EC_{50}) when causing relaxation of the lower degree of precontraction, as has previously been found for endothelium-derived nitric oxide (Dainty et al., 1990). The increase in potency was similar for all agents studied, suggesting that lowering the level of precontraction has a general effect to potentiate vasorelaxation rather than an effect specific to any one relaxant mechanism. It is therefore essential that, in studies measuring relaxation of precontracted tone, control and experimental data are evaluated from the same relative level of precontracted tone. In the present study, the interactions between vasorelaxant agents were found to be similar at both the standard and lowered levels of precontraction. This indicates that the absolute level of precontracted tone may have little effect, providing that control and experimental data are evaluated at matched levels of tone.

In conclusion, we have shown that K^+ channel activating agents inhibit carbachol-mediated relaxation in the presence of L-NAME and indomethacin. This relaxation to carbachol is likely to be due to EDHF as shown by electrophysiology. Relaxations to carbachol mediated by nitric oxide were unaffected by levcromakalim. Since levcromakalim had no effect on relaxations to EDHF released by the Ca^{2+} ionophore A23187, or on A23187-induced hyperpolarization, it probably has its inhibitory effect by acting on the endothelium and not on smooth muscle. This finding strengthens the evidence that K^+ channel activating agents have important actions on vascular endothelium, in addition to their well-characterised effects on smooth muscle.

Acknowledgements

RW is an A.J. Clark student of the British Pharmacological Society. We wish to thank SmithKline Beecham for the gift of levcromakalim. We are also grateful to Dr. B.A. Callingham (University of Cambridge) and Drs. R.D. Latimer and A. Vuylsteke (Papworth Hospital) for the loan of a myograph. We are indebted to Drs. K. Lee and R.D. Pinnock of the Parke–Davis Neuroscience Research Centre for assistance with the electrophysiology.

References

Adeagbo, A.S.O., Triggle, C.R., 1993. Varying extracellular K^+: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. J. Cardiovasc. Pharmacol. 21, 423–429.

Bauersachs, J., Popp, R., Hecker, M., Sauer, E., Fleming, I., Busse, R., 1996. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. Circulation 94, 3341–3347.

Bray, K.M., Weston, A.H., Duty, S., Newgreen, D.T., Longmore, J., Edwards, G., Brown, T.J., 1991. Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta. Br. J. Pharmacol. 102, 337–344.

Chen, G., Cheung, D.W., 1992. Characterization of acetylcholine-induced membrane hyperpolarization in endothelial cells. Circ. Res. 70, 257–263.

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

Dainty, I.A., McGrath, J.C., Spedding, M., Templeton, A.G.B., 1990. The influence of the initial stretch and the agonist-induced tone on the effect of basal and stimulated release of EDRF. Br. J. Pharmacol. 100, 767–773.

Drieu La Rochelle, C., Richard, V., Dubois-Rande, J.L., Roupie, E., Guidicelli, J.F., Hittinger, L., Berdeaux, A., 1992. Potassium channel openers dilate large epicardial coronary arteries in conscious dogs by an indirect, endothelium-dependent mechanism. J. Pharmacol. Exp. Ther. 263, 1091–1096.

Edwards, G., Weston, A.H., 1990. Structure-activity relationships of K^+-channel openers. Trends Pharmacol. Sci. 11, 417–422.

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.

Fukao, M., Hattori, Y., Kanno, M., Sakuma, I., Kitabatake, A., 1997. Sources of Ca^{2+} in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. Br. J. Pharmacol. 120, 1328–1334.

Hoebel, B.G., Kostner, G.M., Graier, W.F., 1997. Activation of microsomal cytochrome *P*450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells. Br. J. Pharmacol. 121, 1579–1588.

Hutcheson, I., Griffith, T.M., 1994. Heterogeneous populations of K^+ channels mediate EDRF release to flow but not agonists in rabbit aorta. Am. J. Physiol. 266, H590–H596.

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.

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.

Katnik, C., Adams, D.J., 1997. Characterization of ATP-sensitive potassium channels in freshly dissociated rabbit aortic endothelial cells. Am. J. Physiol. 272, H2507–H2511.

Kühberger, E., Kukovetz, W.R., Groschner, K., 1993. Cromakalim inhibits multiple mechanisms of smooth muscle activation with similar stereoselectivity. J. Cardiovasc. Pharmacol. 21, 947–954.

Kuo, L., Chancellor, J.D., 1995. Adenosine potentiates flow-induced dilation of coronary arterioles by activating K_{ATP} in endothelium. Am. J. Physiol. 269, H541–H549.

Langheinrich, U., Schnitzler, M.M.Y., Daut, J., 1998. Ca^{2+}-transients induced by K^+ channel openers in isolated coronary capillaries. Pflugers Archiv. Eur. J. Physiol. 435, 435–438.

Lückhoff, A., Busse, R., 1990. Activators of potassium channels enhance calcium influx into endothelial cells as a consequence of potassium currents. Naunyn Schmiedeberg's Arch. Pharmacol. 342, 94–99.

McCulloch, A.I., Randall, M.D., 1997. The effects of levcromakalim on nitric-oxide- and EDHF-mediated relaxations in the rat isolated mesenteric arterial bed. Br. J. Pharmacol. 120, 155P.

McCulloch, A.I., Bottrill, F.E., Randall, M.D., Hiley, C.R., 1997. Characterisation and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric bed. Br. J. Pharmacol. 120, 1431–1438.

Mulvany, M.J., Halpern, W., 1977. Contractile responses of small resistance vessels in spontaneously hypertensive and normotensive rats. Circ. Res. 41, 19–26.

Nagao, T., Illiano, S., Vanhoutte, P.M., 1992. Calmodulin antagonists inhibit endothelium-dependent hyperpolarization in the canine coronary artery. Br. J. Pharmacol. 107, 382–386.

Nilius, B., Viana, F., Droogmans, G., 1997. Ion channels in vascular endothelium. Annu. Rev. Physiol. 59, 145–170.

Parsaee, H., McEwan, J.R., Joseph, S., MacDermot, J., 1992. Differential sensitivities of the prostacyclin and nitric oxide biosynthetic pathway to cytosolic calcium in bovine aortic endothelial cells. Br. J. Pharmacol. 107, 1013–1019.

Plane, F., Garland, C.J., 1996. Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. Br. J. Pharmacol. 119, 191–193.

Reed, P.W., Lardy, H.A., 1972. A23187: a divalent cation ionophore. J. Biol. Chem. 247, 6970–6977.

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.