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Interaction of cyclic AMP modulating agents with levcromakalim in the relaxation of rat isolated mesenteric artery

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Abstract

The effect of cyclic AMP modulating agents on levcromakalim-induced relaxation was investigated in myograph-mounted rat mesenteric arteries. Forskolin (adenylyl cyclase activator), dibutyryl cyclic AMP (protein kinase A activator) and 5′-*N*-ethylcarboxamidoadenosine (NECA; adenosine receptor agonist) all potentiated the vasorelaxant effects of levcromakalim. The modulatory and relaxant effects of dibutyryl cyclic AMP, NECA and forskolin were sensitive to the protein kinase A inhibitor, Rp-cAMPS. However, relaxation to these three agents was unaffected by the K_{ATP} inhibitor, glibenclamide. Dibutyryl cyclic AMP and NECA also caused levcromakalim to induce relaxation in the sub-nanomolar concentration range, however, this effect was Rp-cAMPS- and glibenclamide-insensitive. These results suggest that cyclic AMP modulating agents modulate K_{ATP}, even though this channel does not contribute to their relaxant effects.

Keywords: Mesenteric artery, rat; cAMP; Levcromakalim; Forskolin; Protein kinase A; K_{ATP} channels

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}) were first identified in cardiac muscle by Noma (1983), and it has since been shown that activation of these channels by endogenous factors such as vasoactive intestinal peptide (Standen et al., 1989) and adenosine (Kleppisch and Nelson, 1995) causes hyperpolarisation and relaxation of vascular smooth muscle. K_{ATP} can also be activated by K^+ channel activating agents such as levcromakalim (the active enantiomer of cromakalim) and pinacidil, and it has recently been shown that the relaxant effects of levcromakalim in rat mesenteric arteries are inhibited by endogenous and exogenous nitric oxide and cyclic GMP (McCulloch and Randall, 1996; White and Hiley, 1997b, 1998a). Interestingly, although nitric oxide activates K_{ATP} in mesenteric arteries from the rat (Garland and McPherson, 1992) and rabbit (Murphy and Brayden, 1995), activation of K_{ATP} apparently does not contribute to the relaxant effects of either endogenous (Garland and McPherson, 1992) or exogenous (White and Hiley, 1998a) nitric oxide.

Since activators of the endogenous smooth muscle cyclic GMP system can modulate the actions of K^+ channel activating agents (White and Hiley, 1998a), we investigated the effects of cyclic AMP modulators on levcromakalim-induced relaxation. Previous studies have shown that cyclic AMP can activate K_{ATP} through activation of protein kinase A, presumably via a phosphorylation step (Miyoshi and Nakaya, 1993; Quayle et al., 1994; Kleppisch and Nelson, 1995). Furthermore, Kessler et al. (1997) showed that several activators of the cyclic AMP system induced a glibenclamide-sensitive $^{86}Rb^+$ efflux in rat aorta, indicative of activation of K_{ATP}. Results from this group have also provided evidence that activation of the cyclic AMP system potentiates the ability of K^+ channel activating agents such as P1075 (2-cyano-1-(1,1-dimethylpropyl)-3-(3-pyridyl)guanidine, a pinacidil derivative; Linde and Quast, 1995) and cromakalim (Kessler et al., 1997) to cause $^{86}Rb^+$ efflux, and that this modulatory action is dependent on activation of protein kinase A.

The effects of three cyclic AMP modulating agents on relaxation to levcromakalim have been examined in detail

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in the present study; forskolin (an activator of adenylyl cyclase), dibutyryl cyclic AMP (a cell permeable cyclic AMP analogue), and 5′-*N*-ethylcarboxamidoadenosine (NECA; a non-selective adenosine receptor agonist). We have also examined the actions of dideoxyforskolin, a forskolin analogue, which does not activate adenylyl cyclase, and 8-bromo cyclic AMP, a cell permeable cyclic AMP analogue. The interactions between vasodilator agents were examined by using protocols described previously (White and Hiley 1998a,b), whilst the role of protein kinase A and K_{ATP} in responses was examined by using their respective inhibitors, Rp-cAMPS and glibenclamide. We have recently shown that the effects of K^+ channel activating agents can both modulate (White and Hiley, 1998b) and be modulated by the endothelium (White and Hiley, 1997b); hence, all experiments were carried out in endothelium-denuded vessels in order to avoid the influence of endothelium-derived factors.

2. Materials and methods

2.1. Myograph studies

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, Essex). 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 long) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany–Halpern myograph (Model 500A, Danish Myo-technology, 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. After equilibration, vessels were normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mm Hg (Mulvany and Halpern, 1977). The mean vessel diameter under these conditions was 356 ± 5 μm ($n = 176$).

After normalisation, the endothelium was removed by rubbing the intimal layer of the vessels with a human hair. The successful removal of endothelium was then demonstrated by contracting vessels with methoxamine (10 μM) and adding carbachol (10 μM); a relaxation of $< 10\%$ was indicative of endothelial removal. The mean tension generated on exposure to 10 μM methoxamine was 14.0 $\pm$ 0.6 mN ($n = 176$).

2.2. Experimental protocol

The vasorelaxant effect of levcromakalim was tested by precontracting vessels with methoxamine (10 μM) and then cumulatively adding levcromakalim. A representative trace showing the relaxant effect of levcromakalim is given in Fig. 1. This figure also shows that the relaxant effect of levcromakalim is greatly reduced in the presence of glibenclamide (10 μM), confirming that the relaxation involves activation of K_{ATP}. Previous studies have shown that consistent concentration–response curves to levcromakalim can be generated in a single preparation (White and Hiley, 1997b).

For investigation of levcromakalim-induced relaxation, a control concentration–response curve was first generated. The arteries were then washed and left for 20–30 min, when a second, test concentration–response curve was generated in the presence of the appropriate cyclic AMP modulator. When test concentration–response curves were evaluated in the presence of Rp-cAMPS, the control response to levcromakalim was also determined in the presence of this agent.

After generating a concentration–response curve to dibutyryl cyclic AMP, forskolin or NECA, or a concentration–response curve to levcromakalim in the presence of one of these agents, vessels were subsequently discarded in order to avoid possible effects due to incomplete washout.

When used, glibenclamide (10 μM) and Rp-cAMPS (50 μM) were incubated with vessels for 30 min before construction of a concentration–response curve to the vasorelaxant under investigation.

2.3. Vasodilator interaction studies

The effect of vasodilators, such as to dibutyryl cyclic AMP, forskolin and NECA, on responses to levcromakalim were evaluated according to the ‘standard tone’ protocol described previously (White and Hiley, 1998a,b).

Briefly, arteries were first precontracted with methoxamine (10 μM). The interacting vasodilator (dibutyryl cyclic AMP, forskolin or NECA) was then added at concentrations titrated in individual arteries to produce approximately 30% relaxation of tone (near EC_{30} concentration) or 50% relaxation of tone (near EC_{50} concentration). When a stable level of tone was reached, the methoxamine concentration was increased to 15–30 μM such that tone was restored to within 10% of the level prior to addition of the relaxant. A concentration–response curve to levcromakalim was then constructed from this restored level of tone.

It was found that, in vessels challenged with a near EC_{50} concentration of dibutyryl cyclic AMP in the presence of 10 μM glibenclamide, further addition of methoxamine could not fully restore precontracted tone to the initial level. The response to levcromakalim was therefore examined from approximately 60–70% of the initial tone level, which we have previously shown causes only a modest (approximately three-fold) increase in potency of the relaxant effect of levcromakalim (White and Hiley, 1998b).

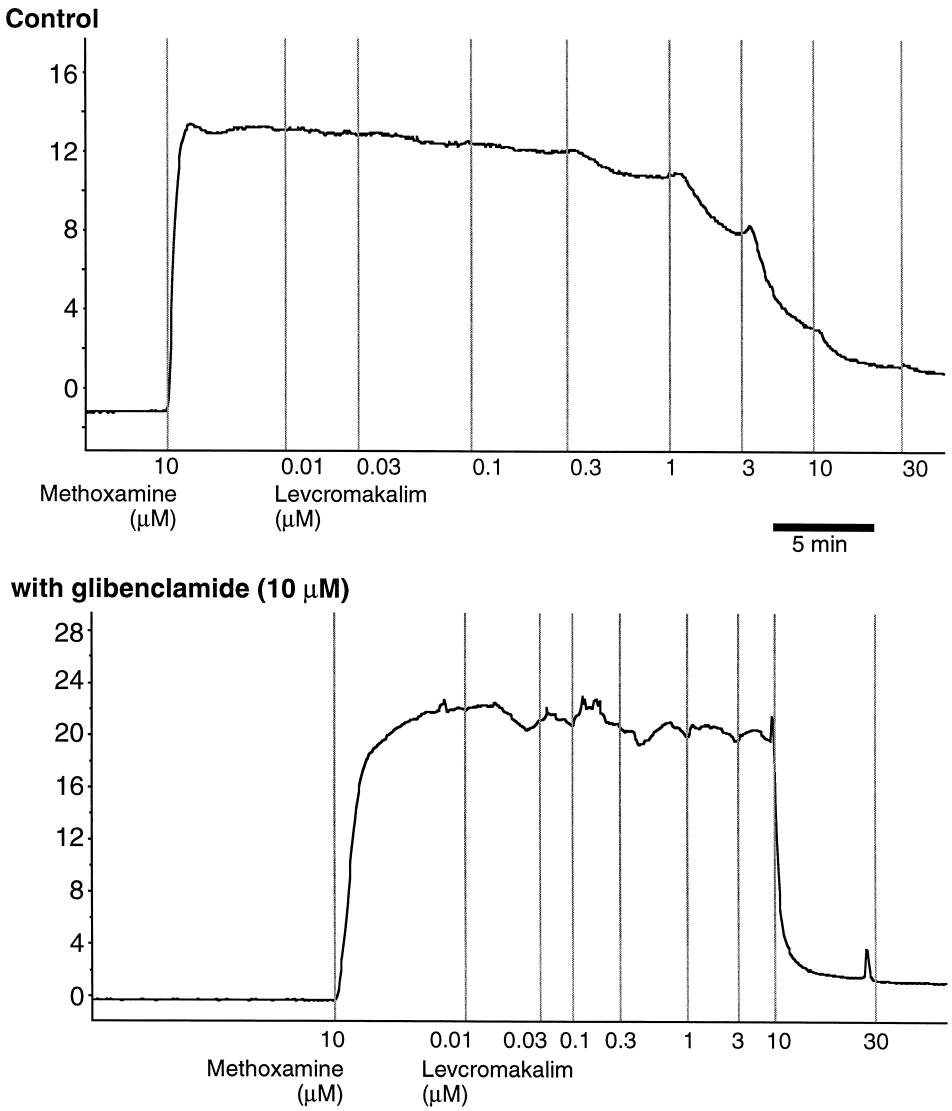

Fig. 1. Original traces showing the vasorelaxant effect of levcromakalim in methoxamine-precontracted rat mesenteric arteries in the absence of endothelium. Levcromakalim induced concentration-dependent relaxations (top panel) that were attenuated in the presence of the K_{ATP} inhibitor, glibenclamide (10 μM; bottom panel). Vertical lines denote addition of drugs at the concentrations indicated.

2.4. Drugs

Methoxamine, carbachol, 8-bromo cyclic AMP, and dibutyryl cyclic AMP (all from Sigma), and Rp-cAMPS (Research Biochemicals International) were dissolved in distilled water. Indomethacin (Sigma) was dissolved in 5% (w/v) $NaHCO_3$ solution. Levcromakalim (SmithKline Beecham) was dissolved in 100% ethanol. Forskolin and dideoxyforskolin (Sigma), and glibenclamide (Aldrich) were dissolved in 100% dimethyl sulphoxide. NECA (Sigma) was dissolved in 1 M HCl.

2.5. Statistical analysis

Relaxation responses in myograph experiments 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 cumulative responses were obtained from individual concentration–response curves by fitting the data to the logistic equation:

$$E = \frac{E_{max} A^{n_H}}{EC_{50} + A^{n_H}}$$

where E is the effect (reduction in tone), A the concentration of the agonist, E_{max} the maximum effect, n_H the slope function and EC_{50} the concentration of relaxant giving half the maximal relaxation.

Where the curve appeared to have more than one component, the data were also fitted to a two-site logistic model:

$$E = \frac{E_{max}\, aA}{EC_{50,1} + A} + \frac{E_{max}(1-a)\, A}{EC_{50,2} + A}$$

The parameters for the two-site fit were the same as those for the one-site fit, with the exception of $EC_{50,1}$ and $EC_{50,2}$, which indicate the EC_{50} values for sites 1 and 2, respectively, and *a*, the proportion of response attributable to site 1. The curve fitting was carried out using KaleidaGraph (Synergy Software, Reading, PA, USA) running on a Macintosh computer. Comparison of all data was by Student's unpaired *t*-test, whilst an *F*-test was used to determine whether the data set was fitted better by a one-site or two-site logistic equation (Otley et al., 1996). *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Relaxation of methoxamine-induced tone induced by cyclic AMP modulating agents

The relaxant effects of cyclic AMP modulators in isolated rat mesenteric arteries are shown in Fig. 2. Of the agents tested, the adenylyl cyclase activator, forskolin, was the most potent at relaxing methoxamine-induced tone in rat mesenteric arteries with an $EC_{50} = 0.11 \pm 0.02$ μM and $E_{max} = 89.4 \pm 3.3\%$ ($n = 5$). The non-selective adenosine receptor agonist, NECA, caused vasorelaxation with $EC_{50} = 0.44 \pm 0.08$ μM and $E_{max} = 98.9 \pm 2.5\%$ ($n = 6$), whilst the cell-permeable cyclic AMP analogue, dibutyryl cyclic AMP relaxed arteries with $EC_{50} = 22 \pm 5$ μM and $E_{max} = 110.5 \pm 6.7\%$ ($n = 4$).

Dideoxyforskolin, which possesses all the properties of forskolin with the exception of adenylyl cyclase activation, was approximately 100-fold less potent than forskolin at causing relaxation ($n = 4$), although the data could not be fitted to a logistic equation. Another cyclic AMP analogue, 8-bromo cyclic AMP, was found to be a very weak relaxant (maximal relaxation $14.2 \pm 2.1\%$ at a concentration of 100 μM, $n = 3$).

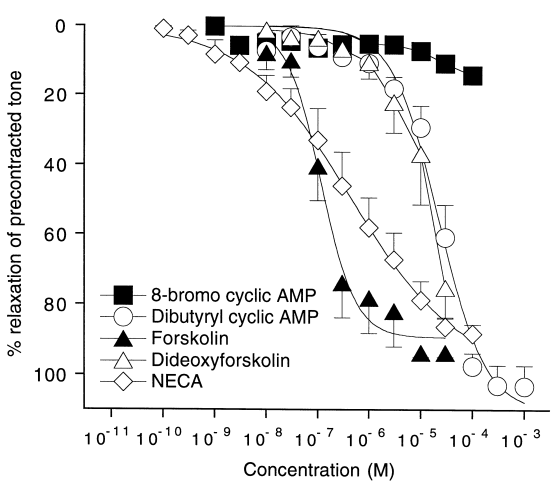

Fig. 2. Concentration–response curves for relaxation by cyclic AMP modulating agents of methoxamine-induced tone in endothelium-denuded rat mesenteric arteries. Where possible, logistic curve-fit parameters were determined as described in Materials and methods, and the parameters are given in the text. Forskolin, $n = 5$; NECA, $n = 6$; dibutyryl cyclic AMP, $n = 4$; dideoxyforskolin, $n = 4$; 8-bromo cyclic AMP, $n = 3$.

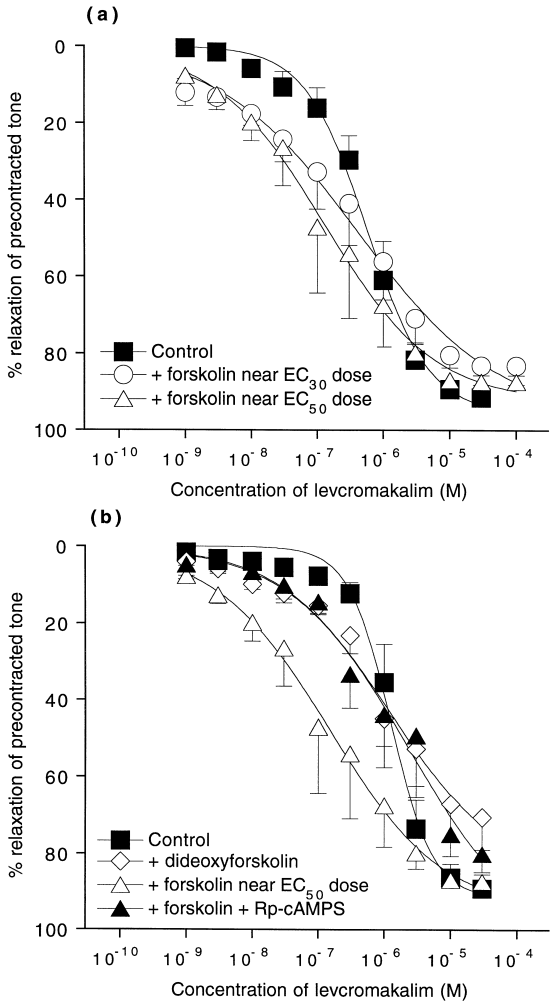

Fig. 3. Concentration–response curves for relaxation by levcromakalim of methoxamine-precontracted arteries in the absence or presence of forskolin, dideoxyforskolin and Rp-cAMPS. Levcromakalim responses were determined in the presence of (a) near EC_{30} or near EC_{50} concentrations of forskolin, or (b) dideoxyforskolin or a near EC_{50} concentration of forskolin in the presence of 50 μM Rp-cAMPS, according to the protocol described in Materials and methods. Data for the tone in the protocols are given in Table 1. Logistic curve-fit parameters are given in Table 2. Matched controls for the forskolin responses are pooled for clarity. In (a), control, $n = 10$; forskolin EC_{30}, $n = 6$; forskolin EC_{50}, $n = 4$. In (b), control, $n = 10$; dideoxyforskolin, $n = 6$; forskolin with Rp-cAMPS, $n = 4$; the curve for forskolin EC_{50} is reproduced from (a) for clarity.

3.2. Effect of forskolin, dideoxyforskolin and Rp-cAMPS on levcromakalim-induced relaxation of rat mesenteric arteries

Relaxations induced by levcromakalim under control conditions, and in the presence of either near EC_{30} or near EC_{50} concentrations of forskolin, are given in Fig. 3a.

Table 1
Drug concentrations and induced tone for standard tone protocol experiments

Treatment	Control contraction (mN)	Concentration of drug added	Relaxation (%)	Restored tone (mN)	*n*
+ Forskolin (EC_{30})	18.1 ± 1.2	20–60 nM	23.0 ± 2.4	17.9 ± 1.4	6
+ Forskolin (EC_{50})	20.1 ± 2.7	30–60 nM	57.1 ± 11.8	18.9 ± 3.0	4
+ Forskolin (EC_{50}) + Rp-cAMPS	12.9 ± 3.7	30 nM	11.5 ± 4.4[a]	11.2 ± 2.9	4
+ Dideoxyforskolin	21.0 ± 3.0	60 nM	0.8 ± 0.6	20.8 ± 2.9	6
+ Db-cAMP (EC_{30})	13.2 ± 1.7	10–20 μM	28.4 ± 3.2	11.2 ± 1.4	12
+ Db-cAMP (EC_{30}) + Rp-cAMPS	15.4 ± 2.4	10–20 μM	13.5 ± 3.7[a]	14.1 ± 2.0	13
+ Db-cAMP (EC_{50})	14.0 ± 1.5	50–110 μM	53.9 ± 5.0	10.5 ± 1.6	11
+ Db-cAMP (EC_{50}) + Rp-cAMPS	20.5 ± 2.6	14–90 μM	30.6 ± 8.1[a]	18.1 ± 2.3	6
+ Db-cAMP (EC_{50}) + Rp-cAMPS + glibenclamide	20.4 ± 2.7	90–140 μM	54.4 ± 3.6	13.2 ± 2.1[b]	6
+ NECA (EC_{30})	22.8 ± 2.5	60–70 nM	20.7 ± 2.7	21.7 ± 3.0	6
+ NECA (EC_{50})	8.4 ± 1.9	7–30 μM	42.9 ± 5.4	7.8 ± 1.7	7
+ NECA (EC_{50}) + Rp-cAMPS	17.3 ± 3.8	10 μM	14.2 ± 3.4[a]	15.9 ± 3.5	5

EC_{30} and EC_{50} indicate the concentrations of drug added to give 30% or 50% relaxation of methoxamine-induced tone, respectively. The concentrations added were titrated in individual vessels and the range of concentrations used is shown in the table. Restored level of tone indicates the tone established after addition of the vasorelaxant by further addition of methoxamine. *n* indicates the number of animals studied.

[a] Significant difference from level of relaxation in the absence of Rp-cAMPS, $P < 0.05$.

[b] Significant difference from control level of tone, $P < 0.05$.

Values for vessel tone before addition of levcromakalim in the interactions protocol are given in Table 1, whilst concentration–response data for levcromakalim-induced relaxations are given in Table 2.

It can be seen from Table 2 that a near EC_{30} concentration of forskolin did not alter the potency or maximum relaxation to levcromakalim, but significantly decreased the Hill slope of the concentration–response curve. The higher, near EC_{50} concentration of forskolin also decreased the Hill slope of the levcromakalim response curve, but now also enhanced the potency of levcromakalim by 4.4-fold.

Fig. 3b and Table 2 show that, in the presence of 60 nM dideoxyforskolin (equating to the EC_{50} concentration of forskolin used), the Hill slope for a subsequent levcromakalim response curve was decreased without effects on other parameters. In the presence of Rp-cAMPS, the relaxant effect of the near EC_{50} dose of forskolin was reduced (Table 1), and the agent now no longer increased the potency of levcromakalim. However, the Hill slope of the levcromakalim response was still reduced (Fig. 3b, Table 2).

Table 2
Effects of forskolin, dideoxyforskolin and Rp-cAMPS on levcromakalim-induced relaxation

Treatment	EC_{50} (μM)	E_{max} (%)	n_H	*n*
Control	0.57 ± 0.08	95.5 ± 3.2	0.9 ± 0.1	10
+ Forskolin (EC_{30})	0.41 ± 0.18	96.5 ± 6.1	0.4 ± 0.1[a]	6
+ Forskolin (EC_{50})	0.13 ± 0.02[b]	92.5 ± 2.5	0.5 ± 0.1[b]	4
Control	1.26 ± 0.16	91.6 ± 3.6	1.4 ± 0.2	10
+ Dideoxyforskolin	1.21 ± 0.67	86.0 ± 9.5	0.5 ± 0.1[a]	6
+ Forskolin (EC_{50}) + Rp-cAMPS	2.30 ± 1.85	104.5 ± 16.8	0.5 ± 0.1[b]	4

EC_{30} and EC_{50} indicate concentrations of drug added to give 30% or 50% relaxation of methoxamine-induced tone, respectively. Curve fit parameters were obtained from the logistic model as described in Materials and methods. *n* indicates the number of animals studied.

[a] Significant differences from controls, $P < 0.01$.

[b] Significant differences from controls, $P < 0.05$.

3.3. Effect of Rp-cAMPS on dibutyryl cyclic AMP-induced relaxation of rat mesenteric arteries

Rp-cAMPS (50 μM) caused a significant rightward shift of 4.2-fold in the dibutyryl cyclic AMP concentration–response curve (Control $EC_{50} = 22 \pm 5$ μM, $n = 4$; in the presence of Rp-cAMPS, $EC_{50} = 93 \pm 23$ μM, $n = 7$; Fig. 4) without altering the maximum response. Although dibutyryl cyclic AMP caused small relaxations at low

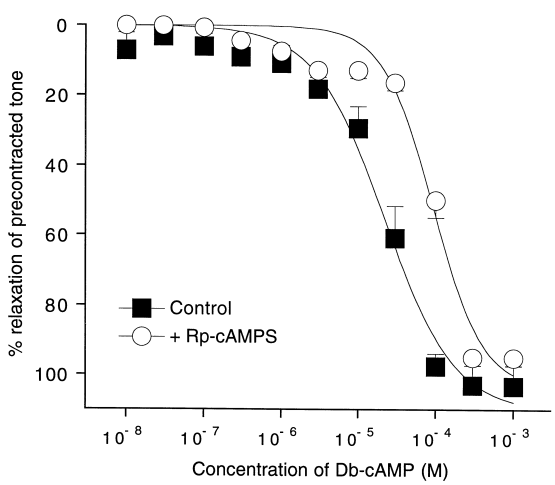

Fig. 4. Concentration–response curves for relaxation by dibutyryl cyclic AMP of methoxamine-precontracted arteries in the absence or presence of 50 μM Rp-cAMPS. Control, $n = 4$; with Rp-cAMPS, $n = 7$.

concentrations, the data were found to fit better to a single-site logistic model than the two-site model.

3.4. Effect of glibenclamide on relaxation of rat mesenteric arteries induced by forskolin, dibutyryl cyclic AMP and NECA

Fig. 5 shows that glibenclamide (10 μM) had no effect on relaxation of precontracted arteries induced by either forskolin, dibutyryl cyclic AMP or NECA. There were no significant changes in either EC_{50}, maximum response or Hill slopes.

3.5. Effect of dibutyryl cyclic AMP, Rp-cAMPS and glibenclamide on levcromakalim-induced relaxation of rat mesenteric arteries

Fig. 6 shows original traces of experiments investigating the effects of dibutyryl cyclic AMP on levcromakalim-induced relaxation. The upper panel shows that dibutyryl cyclic AMP alone causes relaxation, but that subsequent additional methoxamine can re-contract the vessel to a sustained level of tone. In the middle panel, it can be seen that an EC_{30} concentration of the cyclic AMP analogue causes levcromakalim to subsequently induce relaxation in the sub-nanomolar range of concentrations. This was also observed with an EC_{50} concentration of dibutyryl cyclic AMP, however the lower panel is representative of six of the 17 experiments carried out in which levcromakalim, administered after the cyclic AMP analogue, caused almost complete relaxation at sub-nanomolar concentrations, as described below in detail.

Fig. 7a shows quantitatively the effects of the EC_{30} concentration of dibutyryl cyclic AMP on levcromakalim-induced relaxation, and the curve fit data are given in Table 3. The normal monophasic relaxation to levcromakalim was found to become biphasic in the presence of dibutyryl cyclic AMP, although the relaxations revealed in the sub-nanomolar concentration range were slower in onset than those in the normal, approximately micromolar

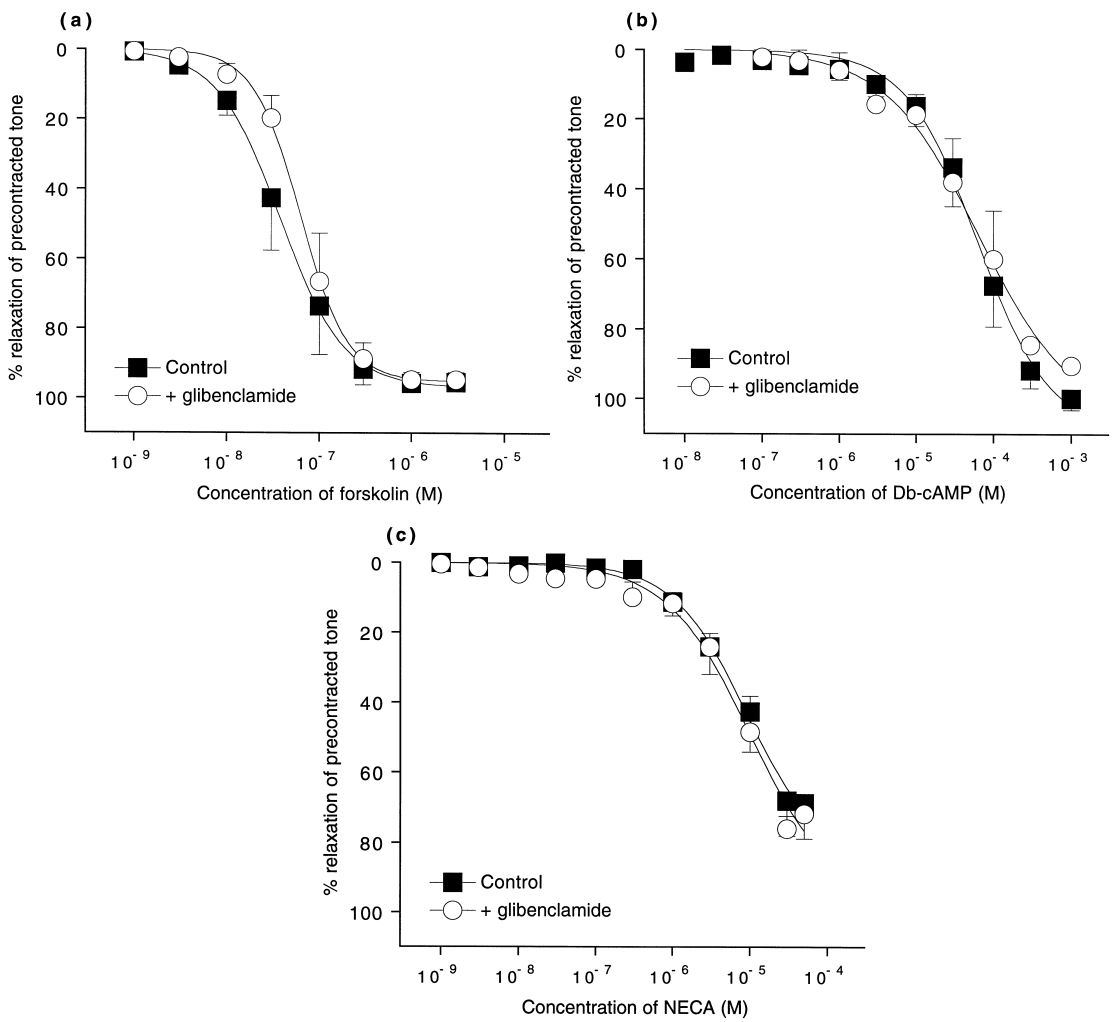

Fig. 5. Concentration–response curves for relaxation of methoxamine-precontracted arteries by (a) forskolin, (b) dibutyryl cyclic AMP and (c) NECA in the presence or absence of 10 μM glibenclamide. Curve fit parameters are given in the text. In (a) and (c), $n = 4$ for all curves. In (b), control, $n = 8$ and in the presence of glibenclamide, $n = 3$.

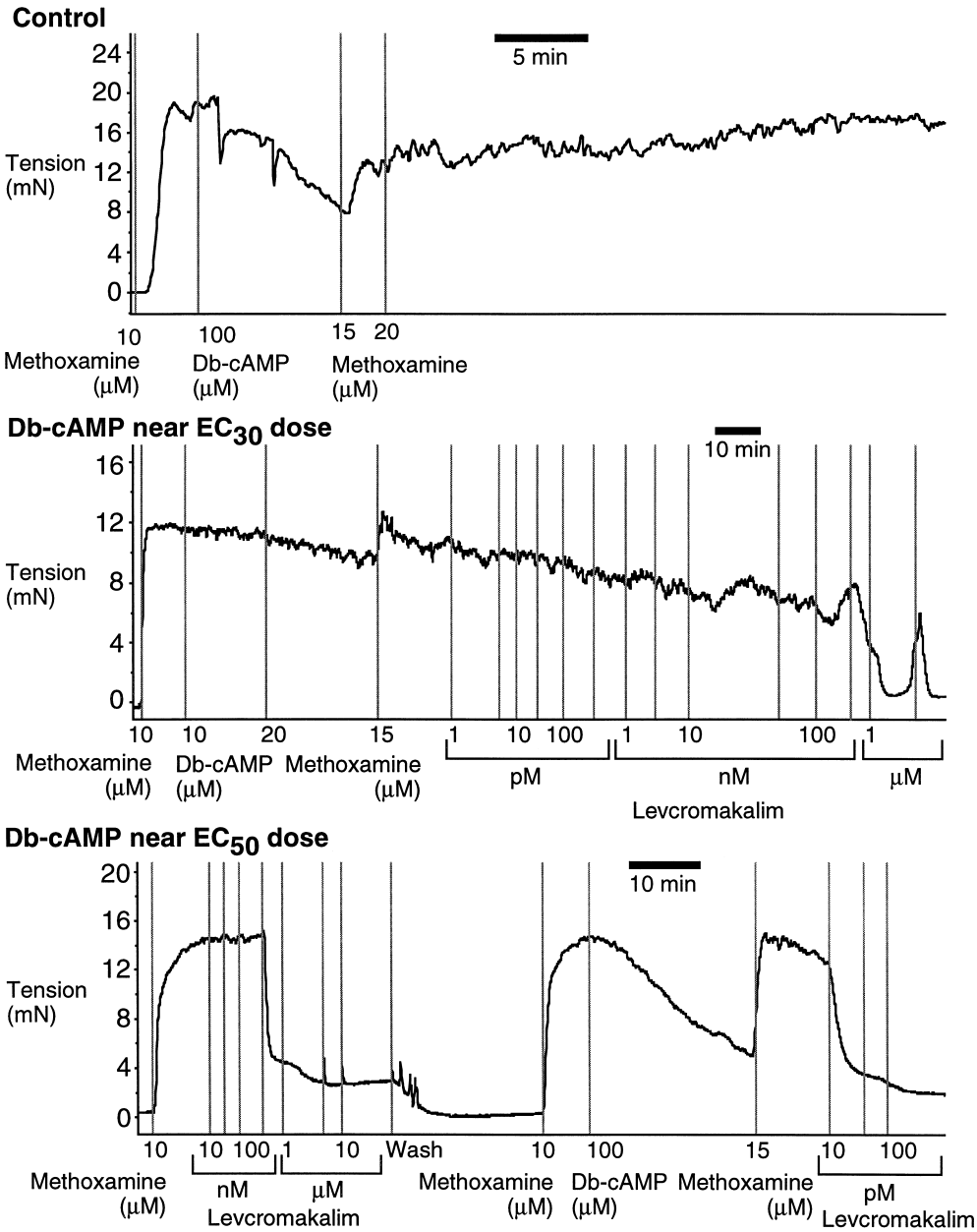

Fig. 6. Original traces showing the effects of dibutyryl cyclic AMP (db-cAMP) on levcromakalim-induced relaxation of methoxamine-induced tone. Vertical lines denote addition of drugs at the concentrations indicated.

range (Fig. 6). Dibutyryl cyclic AMP also potentiated by five-fold the relaxant effect of levcromakalim in its normal, around micromolar concentration range. However, neither the presence of the protein kinase A inhibitor, Rp-cAMPS, nor the presence of the K_{ATP} inhibitor, glibenclamide, significantly attenuated the relaxant effects of levcromakalim at concentrations below 10 nM (unpaired *t*-test, $P > 0.05$). Since these effects are therefore likely to be protein kinase A- and K_{ATP}-independent, curve fit parameters are only presented for the normally observed micromolar effects of levcromakalim (Table 3). The potentiation by dibutyryl cyclic AMP of levcromakalim relaxation in this concentration range was significantly inhibited by Rp-cAMPS, and also by glibenclamide (Fig. 7a, Table 3).

Fig. 7b shows that a higher, near EC_{50} concentration of dibutyryl cyclic AMP caused a greater (approximately 15-fold) potentiation of the effects of levcromakalim in its normal concentration range. Preincubation of vessels with Rp-cAMPS significantly reduced the relaxant effect of dibutyryl cyclic AMP (Table 3) and also caused a small, but statistically significant, inhibition of the potentiating effect of the dibutyryl analogue on levcromakalim relaxation (Fig. 7b and Table 3).

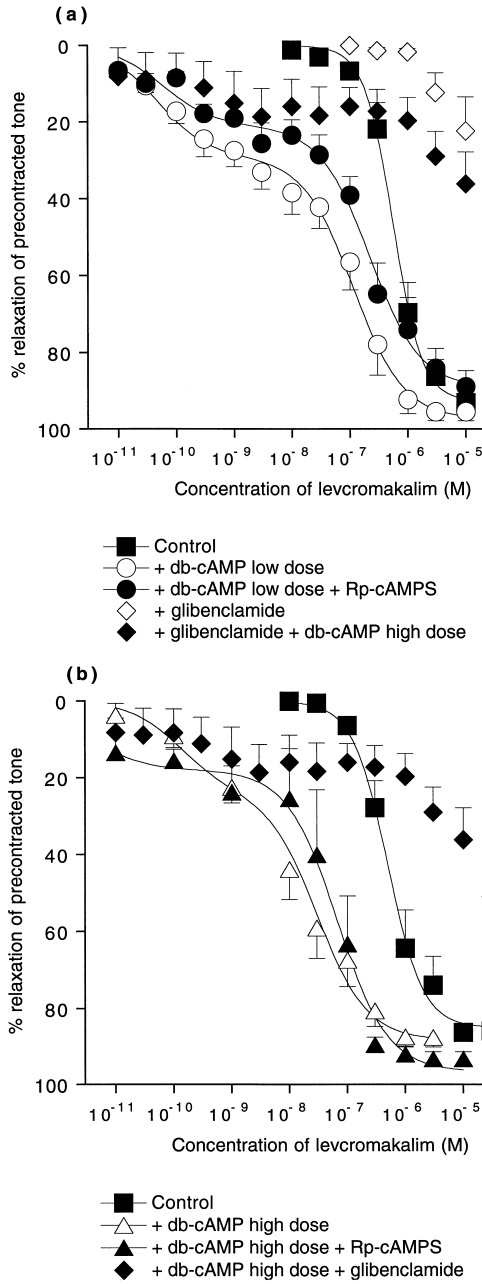

Fig. 7. (a) Effects of a near EC_{30} concentration of dibutyryl cyclic AMP (db-cAMP), Rp-cAMPS (50 μM) and glibenclamide (10 μM) on levcromakalim-induced relaxation, and (b) effects of a near EC_{50} concentration of dibutyryl cyclic AMP, Rp-cAMPS (50 μM) and glibenclamide (10 μM) on relaxation to levcromakalim. Levcromakalim responses were determined according to the protocol described in Materials and methods. Data for the protocols are given in Table 1, and the logistic one-site or two-site curve fit parameters are given in Table 3. Matched controls for the test responses are pooled for clarity. In (a), control, $n = 12$; dibutyryl cyclic AMP EC_{30}, $n = 12$; dibutyryl cyclic AMP EC_{30} with Rp-cAMPS, $n = 13$; with glibenclamide, $n = 10$; with dibutyryl cyclic AMP EC_{50} and glibenclamide, $n = 4$–6. In (b), control, $n = 10$; dibutyryl cyclic AMP EC_{50}, $n = 11$; dibutyryl cyclic AMP EC_{50} with Rp-cAMPS, $n = 6$. The curve for the near EC_{50} concentration of dibutyryl cyclic AMP with glibenclamide (10 μM) is reproduced from (a) for clarity.

It should be noted that in six out of 17 vessels exposed to the EC_{50} concentration of dibutyryl cyclic AMP, vessels relaxed completely at sub-nanomolar concentrations of levcromakalim (example trace in Fig. 6). However, the basis of this effect is not clear since it was also observed in the presence of Rp-cAMPS (data not shown). It may represent a non-specific action of dibutyryl cyclic AMP. These data are therefore not included in Fig. 7b. Similarly, although levcromakalim responses in the presence of the EC_{50} concentration of dibutyryl cyclic AMP were again biphasic, the first phase (occurring at sub-nanomolar concentrations) was not inhibited by glibenclamide or Rp-cAMPS, indicating that K_{ATP} and protein kinase A activation are probably not involved in the relaxations induced at these very low concentrations. The curve fit parameters presented in Table 3, therefore, only include the K_{ATP}-dependent effects of levcromakalim.

3.6. Effect of NECA on levcromakalim-induced relaxation of rat mesenteric arteries

The effects of either near EC_{30} or near EC_{50} concentrations of NECA are shown in Fig. 8a. Values for vessel tone in the interactions protocol are given in Table 1, whilst concentration–response data for levcromakalim-induced relaxations are given in Table 3.

As with dibutyryl cyclic AMP, a near EC_{30} dose of NECA caused the levcromakalim concentration–response curve to become biphasic, with a site in the sub-nanomolar range being revealed in addition to the normal, around micromolar site (Fig. 8a). Nevertheless, the maximum response to levcromakalim and the EC_{50} were not affected.

Table 3
Effects of NECA, dibutyryl cyclic AMP and Rp-cAMPS on levcromakalim-induced relaxation

Treatment	EC_{50} (μM)	E_{max} (%)	*n*
Control	0.41 ± 0.02	96.1 ± 1.2	13
+ NECA (EC_{30})	0.49 ± 0.10	104.7 ± 3.8	6
+ NECA (EC_{50})	0.06 ± 0.02[a]	73.2 ± 2.9[a]	7
+ NECA (EC_{50}) + Rp-cAMPS	0.31 ± 0.08[b]	84.5 ± 3.1[c]	5
Control	0.56 ± 0.04	92.8 ± 2.1	12
+ Db-cAMP (EC_{30})	0.11 ± 0.04[a]	97.4 ± 1.8	12
+ Db-cAMP (EC_{30}) + Rp-cAMPS	0.22 ± 0.04[c]	89.4 ± 2.6	13
Control	0.50 ± 0.05	85.1 ± 2.1	10
+ Db-cAMP (EC_{50})	0.03 ± 0.01[a]	88.4 ± 1.9	11
+ Db-cAMP (EC_{50}) + Rp-cAMPS	0.07 ± 0.01[c]	96.7 ± 2.6	6

EC_{30} and EC_{50} indicate concentrations of drug added to give 30% or 50% relaxation of methoxamine-induced tone, respectively. Curve fit parameters were obtained from the logistic model as described in Materials and methods. Where appropriate, data were fitted to the two-site logistic model. Db-cAMP indicates dibutyryl cyclic AMP. *n* indicates the number of animals studied.

[a] Significant difference from controls, $P < 0.01$.

[b] Significant differences from experiments in the absence of Rp-cAMPS, $P < 0.01$.

[c] Significant differences from experiments in the absence of Rp-cAMPS, $P < 0.05$.

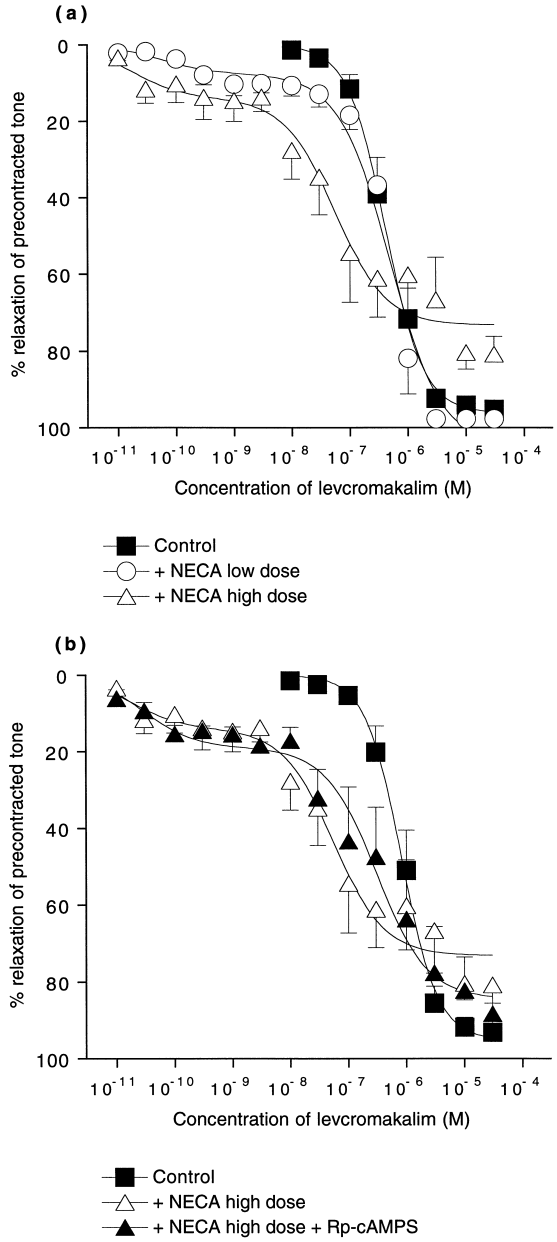

Fig. 8. (a) Effects of near EC_{30} or near EC_{50} concentrations of NECA on levcromakalim-induced relaxation, and (b) effect of a near EC_{50} concentration of NECA in the presence of Rp-cAMPS (50 μM). Levcromakalim responses were determined according to the protocol described in Materials and methods. Data for the protocols are given in Table 1, and the logistic one-site or two-site curve fit parameters are given in Table 3. Matched controls for the test responses are pooled for clarity. In (a), control, $n = 13$; NECA EC_{30}, $n = 6$; NECA EC_{50}, $n = 7$. In (b) control, $n = 12$, NECA EC_{50} with Rp-cAMPS, $n = 5$, and the NECA EC_{50} responses are reproduced from (a) for clarity.

A higher, near EC_{50} dose of NECA also caused subsequent levcromakalim concentration–response curves to become biphasic. The potency of levcromakalim in the normal, higher concentration range (i.e. sub-micromolar) was increased compared to control arteries, whereas the relaxations in the sub-nanomolar range were not significantly altered. The overall maximum response to levcromakalim, however, was reduced (Table 3).

Fig. 8b shows that incubation of vessels with Rp-cAMPS did not significantly alter the ability of a near EC_{50} concentration of NECA to induce levcromakalim relaxation in the sub-nanomolar range. However, the potentiating effect of NECA on the normal, approximately micromolar range of action of levcromakalim was inhibited. Furthermore, the ability of NECA to depress the maximum response to levcromakalim was also reduced (Table 3).

4. Discussion

The major finding of the present study is that although the cyclic AMP modulating agents forskolin, dibutyryl cyclic AMP and NECA cause vasorelaxation through K_{ATP}-independent mechanisms, each can modify the vasorelaxant effects of the K_{ATP} activating agent, levcromakalim in the rat isolated mesenteric artery. All three agents increased the potency of levcromakalim at its normal site (approximately micromolar sensitivity) through an Rp-cAMPS-sensitive mechanism (most likely activation of protein kinase A). These results indicate that vasodilator agents considered to have the same mechanism of action may show different ‘silent’ modulatory effects on other vasorelaxant mechanisms, in this case an enhancement, that do not contribute to the relaxation induced by these agents themselves.

Forskolin (an adenylyl cyclase activator), dibutyryl cyclic AMP (a cell-permeable analogue of cyclic AMP) and NECA (a non-selective adenosine receptor agonist), all caused vasorelaxation of methoxamine-induced tone. Dideoxyforskolin, a forskolin derivative, which does not activate adenylyl cyclase, also relaxed mesenteric arteries, but its potency was approximately 100-fold lower than forskolin. It seems likely that dideoxyforskolin caused relaxation through a mechanism unrelated to adenylyl cyclase activation, for example voltage-operated Ca^{2+} channel blockade (Abe and Karaki, 1992). Another cyclic AMP analogue, 8-bromo cyclic AMP, was found to be a very weak relaxant; the reason for this is unclear, but may be due to poor tissue penetration.

It is clear from the results of the interaction studies that cyclic AMP-modulating agents increase the potency of levcromakalim at relaxing precontracted arteries in its normal, approximately micromolar range of action, in a concentration-dependent fashion. However, dibutyryl cyclic AMP and NECA, but not forskolin, also caused levcromakalim to induce relaxation at much lower concentrations (sub-nanomolar) than is usually the case (approximately micromolar). Indeed, with the near EC_{50} concentration of dibutyryl cyclic AMP, some vessels (about one third of those tested) relaxed completely to levcromakalim at these very low concentrations. However, this potentiating effect of the cyclic AMP modulating agents was not sensitive to Rp-cAMPS, whilst the relaxant effect of levcromakalim in

this concentration range was apparently not inhibited by glibenclamide. It also showed little concentration-dependence. Hence, although the underlying mechanism for the sub-nanomolar effects is not clear, it is not likely to involve protein kinase A or K_{ATP} channels and therefore may reflect other actions of the agents in question. This study therefore focuses on the effects of the cyclic AMP modulating agents on levcromakalim relaxation in the normal, approximately micromolar concentration range.

It seems likely that the effect of the lower concentration of forskolin to decrease the Hill slope of the levcromakalim response occurs through a mechanism independent of adenylyl cyclase activation, as it was shared by dideoxyforskolin and not inhibited by Rp-cAMPS. However, the action of the near EC_{50} concentration of forskolin to increase the potency of levcromakalim is likely to occur through activation of adenylyl cyclase and protein kinase A, since it was inhibited by Rp-cAMPS, and was not shared by dideoxyforskolin. It is notable that Kessler et al. (1997) reported a similar cyclic AMP-dependent, potentiating action of forskolin on cromakalim relaxation and $^{86}Rb^+$ efflux in rat aorta. The magnitude of the potentiation found by Kessler et al. (1997), 3.4-fold, is similar to the effect of the near EC_{50} concentration used in the present study (4.4-fold).

Dibutyryl cyclic AMP, a cell-permeable cyclic AMP analogue, caused concentration-dependent relaxations of rat mesenteric arteries that were attenuated by Rp-cAMPS, and are therefore probably mediated through activation of protein kinase A. Dibutyryl cyclic AMP also potentiated the effects of the K_{ATP} activator in its normal, approximately micromolar range of action, in a concentration-dependent manner (5- and 15-fold increases in levcromakalim potency with the near EC_{30} and near EC_{50} concentrations of dibutyryl cyclic AMP, respectively). Notably, Linde and Quast (1995) showed that dibutyryl cyclic AMP potentiated levcromakalim-induced $^{86}Rb^+$ efflux in rat aorta by approximately two-fold through a protein kinase A-dependent mechanism.

The non-selective adenosine receptor agonist, NECA, is thought to cause relaxation of the rat mesenteric artery through activation of adenosine A_{2B} receptors, with the possible involvement of an additional, xanthine-insensitive site (Prentice et al., 1997). Adenosine A_2 receptors are coupled to G_s, and therefore stimulate adenylyl cyclase. As with dibutyryl cyclic AMP and forskolin, NECA potentiated the relaxations to levcromakalim in its approximately micromolar concentration range, and this effect was concentration-dependent, since the near EC_{50} concentration, but not the EC_{30} concentration, was effective.

The vasorelaxant effects of dibutyryl cyclic AMP, and also of forskolin and NECA, were reduced in the presence of Rp-cAMPS, but were unaffected by the K_{ATP} inhibitor, glibenclamide. These results suggest that cyclic AMP induces relaxation through activation of protein kinase A, but not of K_{ATP}, in rat mesenteric arteries. This is a surprising finding, as both forskolin and cyclic AMP analogues similar to dibutyryl cyclic AMP (which activate protein kinase A) have been shown to hyperpolarise rat mesenteric arteries through activation of glibenclamide-sensitive K_{ATP} (Prieto et al., 1997). Hence, it seems likely that cyclic AMP can modulate, and indeed even activate, K_{ATP} without this contributing to the relaxant effects of this agent. We have recently shown that *S*-nitroso-*N*-acetylpenicillamine and cyclic GMP exert similar effects in rat mesenteric arteries (White and Hiley, 1998a), and the general observation that activation of K_{ATP} might be dissociated from the relaxation induced by K^+ channel activating agents such as levcromakalim is consistent with many previous studies (see Quast, 1993, for review).

Electrophysiological studies have provided convincing evidence that cyclic AMP activates K_{ATP} through activation of protein kinase A, presumably via a phosphorylation step (Miyoshi and Nakaya, 1993; Quayle et al., 1994; Kleppisch and Nelson, 1995). Vascular smooth muscle K_{ATP} channels are thought to consist of a complex of SUR2B and Kir6.1 (Yamada et al., 1997), and it is notable that both components possess potential phosphorylation sites for protein kinase A (Isomoto et al., 1996; Inagaki et al., 1995). It therefore seems possible that the increase in sensitivity to levcromakalim relaxation caused by forskolin, dibutyryl cyclic AMP and NECA might represent different phosphorylation states of K_{ATP} with different sensitivity to levcromakalim.

It should be noted, however, that although the modulatory effects of the agents under investigation were apparently mediated by protein kinase A, the target site need not necessarily be the K_{ATP} channel itself. Indeed, the potentiation observed with dibutyryl cyclic AMP (up to 10-fold) is somewhat greater than the increases in ion efflux observed in the studies by Linde and Quast (1995) and Kessler et al. (1997). Indeed, the primary target for phosphorylation by protein kinase A with regard to relaxation of vascular smooth muscle is myosin light chain kinase, thus leading to decreased Ca^{2+} sensitivity of the contractile proteins (Yamagishi et al., 1994), which might thence alter the potency of other vasorelaxants.

A key finding of the present study is that forskolin, dibutyryl cyclic AMP and NECA show some differences in their modulation of levcromakalim vasorelaxation. For example, the higher, near EC_{50} concentration of dibutyryl cyclic AMP potentiated levcromakalim relaxation to a greater extent (approximately 15-fold) than similarly effective concentrations of forskolin (4.4-fold) or NECA (6.8-fold), and also acted in a manner that was rather less sensitive to the inhibitory effects of Rp-cAMPS (the potentiating effect of the EC_{50} concentrations of forskolin and NECA were essentially abolished by Rp-cAMPS, whereas the effect of dibutyryl cyclic AMP was only slightly reduced). It is of note that that dibutyryl cyclic AMP and NECA, but not forskolin, caused levcromakalim to induce relaxation at a sub- or low nanomolar concentration range.

However, as these effects were insensitive to Rp-cAMPS and glibenclamide, they are probably non-specific and beyond the scope of the present study.

It may be important that, although each agent ultimately acts through generation of cyclic AMP, this is achieved by different mechanisms; whereas dibutyryl cyclic AMP simply mimics cyclic AMP in activating protein kinase A, forskolin directly stimulates adenylyl cyclase to produce cyclic AMP, whilst NECA activates adenylyl cyclase through G-protein coupled adenosine receptors. Therefore, the relative importance of protein kinase A activation and the ability of these agents to activate K_{ATP} may vary between the three agents investigated. Clearly measurement of intracellular cyclic AMP and Ca^{2+} levels, and examination of the phosphorylation state and activity of individual K_{ATP} channels, will be required to clarify the exact reasons for the differences observed, and we aim to achieve these aims in future studies.

It may be of note that dibutyryl cyclic AMP is hydrolysed by cyclic AMP-specific phosphodiesterases more slowly than is cyclic AMP (Miller et al., 1973); hence, the lower sensitivity to Rp-cAMPS of the potentiation by dibutyryl cyclic AMP may be a consequence of this more stable cyclic AMP analogue being a better competitor towards Rp-cAMPS than the endogenous cyclic AMP stimulated by forskolin and NECA. This factor could be important given that cyclic AMP-specific phosphodiesterases are thought to be localised to regions adjacent to the plasma membrane (Jin et al., 1998), where the K_{ATP} channels are located.

Another possibility is that there may be specific compartments for cyclic AMP or protein kinase A within the smooth muscle cells, and that these may be differentially modulated by the agents investigated in this study. Indeed, previous studies have provided evidence for such compartmentalisation in both cardiac (Jurevicius and Fischmeister, 1996) and vascular smooth muscle (Eckly-Michel et al., 1997).

In summary, the present study has demonstrated that cyclic AMP modulating agents may modulate K_{ATP}, even though this channel does not seem to contribute to the direct relaxant effects of these agents. The modulatory effect is likely to involve activation of protein kinase A, and the nature of the modulatory effect depends to some extent on the mechanism by which the cyclic AMP system is activated. It is clear that the 'silent' modulatory actions of these vasodilator agents are likely to be crucial in determining the overall profile of responses to a variety of vasoactive stimuli.

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