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Evidence that Ca^{2+}-activated K^+ channels play a major role in mediating the vascular effects of iloprost and cicaprost

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Abstract

The role of K^+ channels in mediating vasorelaxation induced by two prostacyclin analogues was investigated in guinea-pig aorta. Iloprost caused substantial relaxation of tissues contracted with phenylephrine or 25 mM K^+ but not 60 mM K^+. In endothelial-denuded tissues, maximal relaxations to iloprost, cicaprost or isoprenaline were inhibited by ~40–50% with tetraethylammonium or iberiotoxin, both blockers of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. In contrast, the response to forskolin, an activator of adenylate cyclase was marginally inhibited by tetraethylammonium. The K_{ATP} channel blocker, glibenclamide significantly augmented the response to iloprost but not cicaprost. These effects were largely inhibited by the EP_1 receptor antagonist, 8-chlorodibenz[*b*,*f*][1,4]oxazepine-10(11*H*)-carboxylic acid 2-[1-oxo-3(4-pyridinyl)propyl]hydrazide, monohydrochloride (SC-51089) and partially by indomethacin, suggesting that iloprost relaxation is counterbalanced by activation of EP_1 receptors, in part through a constrictor prostaglandin. We conclude that BK_{Ca} channels play an important role in mediating the effects of iloprost and cicaprost and raises the possibility that cyclic AMP-independent pathways might be involved.

Keywords: Smooth muscle, vascular; Iloprost; Cicaprost; Forskolin; Iberiotoxin; K^+ channel, Ca^{2+}-activated; Aorta, guinea-pig

1. Introduction

Prostaglandins, formed from the metabolism of arachidonic acid by the cyclooxygenase pathway, are involved in a number of diverse biological processes, including inflammation, platelet function and regulation of vascular smooth muscle tone. One of the major products of this pathway is prostacyclin, a potent inhibitor of vascular tone and platelet aggregation (Kerins et al., 1991; Grant and Goa, 1992). Prostacyclin, synthesised mainly in endothelial cells, but also in smooth muscle, is released in response to various endogenous agents such as bradykinin and arachidonic acid, as well as by mechanical or immunological stimuli (Gryglewski et al., 1986; Kerins et al., 1991; Jackson et al., 1993). In view of the relatively short half life (~3 min) of prostacyclin, a number of stable analogues have been developed for clinical use, and these appear beneficial in the treatment of peripheral vascular disease (Grant and Goa, 1992). Two such analogues are iloprost and cicaprost, which have potent hypotensive actions in vivo (Kerins et al., 1991; Grant and Goa, 1992), and relax a variety of arteries in vitro (Gryglewski et al., 1986; Siegel et al., 1989; Frolich, 1990; Rosolowsky and Campbell, 1993; Parkington et al., 1995). Several lines of evidence suggests that the major biological activity of prostacyclin and its analogues are mediated by specific cell surface prostanoid (IP) receptors which are known to be coupled to adenylate cyclase via the guanine nucleotide regulatory protein, G_S (Wise and Jones, 1996). This is consistent with the observation that these agents increase cyclic AMP in many cell types (Kerins et al., 1991; Grant and Goa, 1992; Wise and Jones, 1996). However, whilst cicaprost appears to be a highly selective IP receptor agonist, binding studies have shown that iloprost also has significant agonist activity at EP receptors (particularly at the EP_1 receptor subtype), although the distribution and function of such receptor subtypes remains unclear in vascular smooth muscle (Coleman et al., 1994; Wise and Jones, 1996).

Increasing evidence suggests that iloprost acts, in part, through the activation of K^+ channels, with vasodilatation

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presumed to occur as the result of membrane hyperpolarisation closing voltage-gated Ca^{2+} channels (Siegel et al., 1990). Consistent with this view, iloprost has been shown to cause concentration-dependent membrane hyperpolarisation and relaxation of canine carotid artery with a similar EC_{50} for both effects, and to cause substantial increases in K^+ permeability (Siegel et al., 1989). In addition, patch-clamp studies have demonstrated that iloprost activates Ca^{2+}-activated K^+ ($I_{K(Ca)}$) current in isolated vascular smooth muscle (Siegel et al., 1990; Schubert et al., 1996). However, recent evidence suggests that large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels either do not contribute to prostacyclin-induced dilation in rat mesenteric artery (Adeagbo and Malik, 1990) or only contribute a small fraction to the overall vasodilation evoked by iloprost in endothelium-intact rat tail artery (Schubert et al., 1997). Moreover, a number of studies have now concluded that ATP-sensitive K^+ (K_{ATP}) channels are the predominant K^+ channels responsible for the vasodilation induced by iloprost, since glibenclamide, a blocker of these channels substantially inhibits the effects of iloprost in both rat and rabbit blood vessels (Jackson et al., 1993; Bouchard et al., 1994; Schubert et al., 1997).

Thus, despite evidence from patch-clamp experiments, it is not clear if BK_{Ca} channels play a major role in the vascular action of iloprost, and whether this be independent of endothelial-derived nitric oxide which can also activate BK_{Ca} channels (for review see Nelson and Quayle, 1995). Moreover, no studies to date have investigated the role of K^+ channels in response to cicaprost. The aims of this study were to assess the relative importance of K_{ATP} versus BK_{Ca} channels in mediating the effects of the stable prostacyclin analogues, iloprost and cicaprost, and to investigate the role of the endothelium. The effects of K^+ channel blockers were examined against responses to iloprost and cicaprost in muscle strips isolated from guinea-pig aorta. We also studied the vasorelaxant profile of two other compounds which elevate cyclic AMP either directly (forskolin) or through specific receptors (isoprenaline). Preliminary results have appeared elsewhere in abstract form (Baloch et al., 1995; Hall et al., 1995).

2. Materials and methods

2.1. Measurement of isometric tension

Male guinea-pigs (Duncan Harver, 200–250 g) were killed by cervical dislocation and the thoracic aorta removed and placed in physiological salt solution containing in mM: 112 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 glucose and 0.03 phenol red (pH 7.4 with 95% O_2/ 5% CO_2). The connective tissue was carefully removed and rings (~2 mm wide, and cut longitudinally) or helical strips (~2 mm wide, 10 mm long) of muscle cut. To remove the endothelium, the intimal surface of the muscle was gently rubbed with filter paper. Tissues were mounted in a small organ bath (0.3 ml) and tension measured using an isometric transducer (Harvard apparatus, USA) connected to a chart recorder (Labdata Instrument Services, UK). Muscles were subjected to a basal tension of 1.25 g and perfused (1.0 ml/min) at 37°C with physiological salt solution for 40–45 min before the addition of any drugs. Tissues were then precontracted with either phenylephrine (1 or 6 μM) or by raising external K^+ to either 25 or 60 mM by equimolar replacement of NaCl for KCl in the physiological salt solution. Phentolamine (0.5 μM) was included in the high K^+ solutions to block effects of any noradrenaline released from surviving nerve endings. Endothelial function was assessed by exposing tissues to either 10 μM acetylcholine or 10 μM carbachol for 1–2 min. In the presence of intact endothelium these agents produce relaxation whereas they cause contraction when the endothelium is removed. Tension was expressed as the percentage of the phenylephrine or high K^+ contraction measured just before exposure to additional agents. To minimise possible time-dependent changes in response to relaxing agents, wherever possible drugs were given randomly before or after obtaining the first concentration–response curve.

2.2. Effect of glibenclamide on iloprost-induced relaxation

Helical strips of muscle with endothelium were precontracted with phenylephrine, and concentration–response curves were obtained for iloprost (3–1000 nM) in the absence and presence of the K_{ATP} channel blocker, glibenclamide (10 μM). Glibenclamide was continuously applied 15–20 min before the lowest dose of iloprost was given and remained in the perfusion solution until after the highest dose of iloprost had been applied. Each dose of iloprost was given for 2 min with at least 15 min allowed between each application. To ensure that K_{ATP} channels were blocked in the presence of glibenclamide, tissues were also exposed to the K_{ATP} channel activator, levcromakalim (10 μM for 40s). A 1-h period was allowed between dose–response curves. In other experiments two doses of iloprost (0.1 μM for 2 min) were given ~60 min apart to tissues precontracted with 6 μM phenylephrine. Glibenclamide (10 μM) and/or indomethacin (10 μM) were given 15 or 30 min, respectively before the second application of iloprost. The majority of these experiments were carried out on strips without endothelium.

2.3. Effect of K^+ channel blockers and SC-51089 on responses to iloprost, cicaprost, isoprenaline and forskolin

Rings of thoracic aorta were opened up, denuded of the endothelium, and mounted. After an equilibration period, tissues were contracted 2–3 times with phenylephrine (2 min), with a 10 min washout in physiological salt solution between contractions. Following this procedure, muscle

segments were contracted again with phenylephrine and after 20 min, iloprost (0.001–1 μM), cicaprost (0.001–1 μM), isoprenaline (0.01–100 μM) or forskolin (0.001–1 μM) were added in a cumulative fashion, with each dose being applied for a fixed time (3–5 min) before the addition of the next dose. Tetraethylammonium (2 mM), iberiotoxin (25–50 nM), glibenclamide (10 μM) or apamin (100 nM) were given at least 10–15 min before the addition of the lowest concentration. After a 1-h wash-out period, a second concentration–response curve to iloprost was obtained, and channel blockers given to those tissues not previously exposed to them. In other experiments concentration–response curves were obtained for iloprost in the absence, presence of 30 μM SC-51089 (applied for 30 min beforehand) or presence of SC-51089 and glibenclamide (10 μM).

2.4. Chemicals / drugs

Phenylephrine, acetylcholine, carbachol, isoprenaline, phentolamine, indomethacin and sodium nitroprusside were purchased from Sigma Chemical company (Poole, Dorset, UK) and tetraethylammonium chloride was purchased from Fluka (Gillingham, Dorset, UK). Forskolin was purchased from Calbiochem-Novachem (Beeston, Nottingham, UK), SC-51089 from Affinity Research Products (Exeter, UK), iberiotoxin from Alomone Laboratories (Jerusalem, Israel) and 1H-[1,2,4]oxadiazole[4,3-*a*]quinoxaline-1-one (ODQ) and apamin from Alexis Corporation (Bingham, Nottingham, UK). ODQ, levcromakalim and forskolin were dissolved in 100% dimethylsulphoxide and glibenclamide was dissolved in 50% v/v dimethylsulphoxide/polyethylene glycol; all were stored as 10 mM stock solutions at 4°C. Iloprost and cicaprost were stored at 4°C at a concentration of 0.5 mg/ml in a buffer containing in mM: 9.9 Tris, 152 NaCl and 176 ethanol. Indomethacin was made up in 100% ethanol and used immediately. All other stock solutions were made up in distilled water. On the day of the experiment, stock solutions were diluted in physiological salt solution to give the desired concentration. Solvent controls were performed, where the appropriate solvent was applied at the highest concentration used in experiments. Under these circumstances, solvents had no visible effect on tension.

2.5. Data analysis

All data is presented as mean ± S.E.M. of n observations. The concentration of prostacyclin analogues causing 50% relaxation of phenylephrine contractions is expressed as the log EC_{50} value. Individual EC_{50} values for control and test concentration–response curves were determined using the sigmoidal-curve fitting routine in Origin 4.1 (Microcal Software, Northampton, MA, USA). Statistical significance within groups was determined using one-way analysis of variance (ANOVA) with correction for pairwise comparisons of groups (Student–Newman–Keuls Method) or comparisons to a control group (Bonferroni t-test). For a single intervention at one concentration of an agent a paired, or unpaired Student's t-test was performed. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of iloprost on high K^+ contractions

We investigated the potency of iloprost against contractions evoked by high extracellular K^+. It was found that iloprost (0.001–1 μM) significantly relaxed muscles contracted with 25 mM K^+ in a dose-dependent manner ($P < 0.05$, one way ANOVA) with an EC_{50} of -7.14 ± 0.11 (Fig. 1). The maximal relaxation produced by iloprost was $54 \pm 7\%$ ($n = 6$) which was reduced to $13 \pm 5\%$ ($n = 8$; $P < 0.005$) in the presence of 60 mM K^+. Responses to iloprost in high K^+ were also largely unaffected by removal of the endothelium. Application of acetylcholine (10 μM) and iloprost (0.1 μM) to endothelium-intact tissues contracted with 25 mM K^+, gave average relaxations of $25 \pm 4\%$ and $51 \pm 8\%$ ($n = 5$), respectively. After removal of the endothelium, acetylcholine caused contraction ($11 \pm 5\%$) while iloprost relaxed tissues by $46 \pm 13\%$ ($P > 0.05$, Student's t-test, paired).

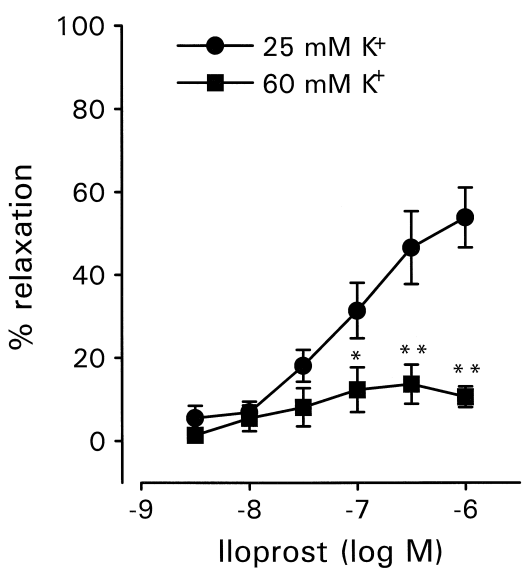

Fig. 1. Iloprost is more effective at relaxing contractions evoked by low compared to high external K^+. Concentration–response curves for iloprost (0.003–1 μM) where muscle strips of guinea-pig aorta were precontracted with either 25 (●) or 60 mM (■) external K^+. Results are expressed as the percentage relaxation of the total contraction seen prior to exposure of iloprost. Each data point represents the mean ± S.E.M. of six to eight experiments from 11 animals. * = $P < 0.05$ and ** = $P < 0.005$ (Student's t-test, unpaired) when compared to relaxations observed in 25 mM K^+. Each dose of iloprost was applied for 2 min.

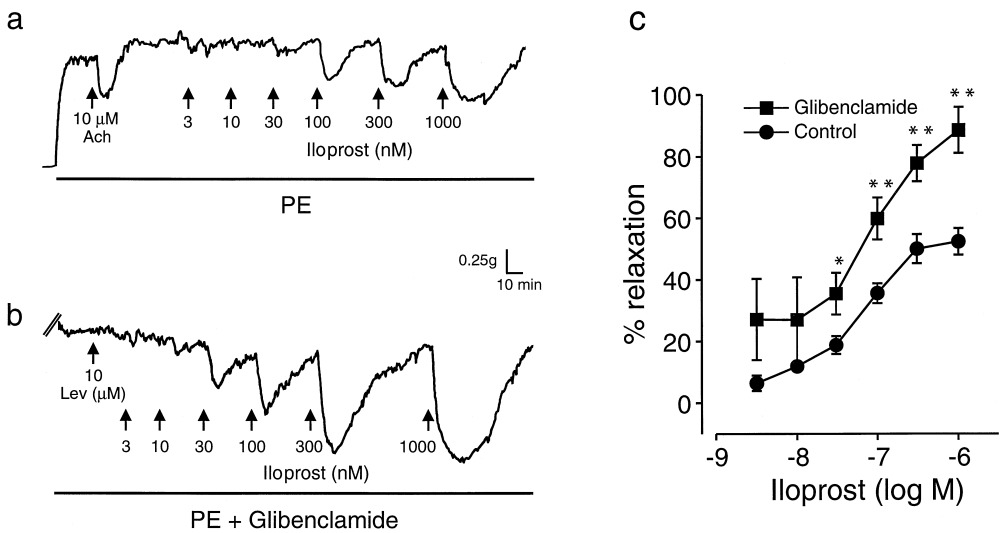

Fig. 2. Glibenclamide significantly augments the response to iloprost. (a) Control relaxations to increasing doses of iloprost (3–1000 nM) in a muscle strip precontracted with phenylephrine (6 μM). Before application of iloprost, acetylcholine (10 μM) was given for 1 min to assess endothelial function. (b) Responses to iloprost after the same tissue had been exposed to glibenclamide (10 μM) for 15 min. Initially, levcromakalim (lev; 10 μM) was applied to confirm K_{ATP} channel blockade. (c) Averaged concentration–response curves to iloprost generated from experiments like the one above under control conditions (●) and in the presence of 10 μM glibenclamide (■). Muscle strips were precontracted with 6 μM phenylephrine and each dose of iloprost applied for 2 min. All values are expressed as mean ± S.E.M. of six to nine experiments. $* P < 0.05$ and $* * P < 0.01$ compared to control (Student's *t*-test, unpaired).

3.2. Effect of glibenclamide on iloprost- and cicaprost-induced relaxation

The effect of glibenclamide, a blocker of K_{ATP} channels, was first studied against responses to iloprost in tissues contracted with 6 μM phenylephrine. Glibenclamide (10 μM), had no effect on phenylephrine contractions, nor did it inhibit relaxations to iloprost (Fig. 2). On the contrary, relaxations to iloprost were augmented at all doses in the presence of glibenclamide (Fig. 2b and c), an effect also observed in the absence of the endothelium (see later). The maximal response to iloprost was significantly increased ($P < 0.001$, unpaired Student's test) although there was no significant change in the EC_{50} (Table 1). In contrast, glibenclamide (10 μM) failed to augment the response to the selective IP receptor agonist, cicaprost, which gave a similar maximal response to that observed with iloprost in the presence of glibenclamide (Table 1). The EC_{50} for cicaprost (-7.53 ± 0.06, $n = 30$) in the absence of glibenclamide was however, greater ($P < 0.001$) than that obtained for iloprost under similar conditions (-7.23 ± 0.07, $n = 21$).

Table 1
Summary of the actions of K^+ channel blockers on relaxant concentration–response curves to iloprost and cicaprost in guinea-pig aorta

Compound	EC_{50}		Maximum relaxation (%)	
	Control	Test	Control	Test
Iloprost				
Glibenclamide	-7.22 ± 0.14 (9)	-7.32 ± 0.11 (6)	52.4 ± 4.3 (9)	88.5 ± 7.4 (6)[a]
Tetraethlyammonium	-7.24 ± 0.09 (12)	-7.11 ± 0.07 (12)	68.3 ± 5.2 (12)	31.7 ± 2.9 (12)[a]
Iberiotoxin	-7.23 ± 0.20 (4)	-7.14 ± 0.14 (4)	55.8 ± 4.0 (4)	20.4 ± 3.5 (4)[a]
Cicaprost				
Glibenclamide	-7.59 ± 0.09 (12)	-7.47 ± 0.07 (12)	91.8 ± 8.4 (12)	93.0 ± 4.9 (12)
Tetraethlyammonium	-7.30 ± 0.17 (5)	-7.09 ± 0.17 (5)	75.6 ± 3.4 (5)	42.8 ± 6.2 (5)[a]
Iberiotoxin	-7.75 ± 0.07 (5)	-7.40 ± 0.08 (5)[b]	87.3 ± 2.8 (5)	56.4 ± 6.2 (5)[a]
Apamin	-7.60 ± 0.13 (8)	-7.68 ± 0.05 (8)	78.2 ± 3.0 (8)	82.7 ± 3.0 (8)

Tissues were contracted with 6 μM phenylephrine and glibenclamide (10 μM), tetraethylammonium (2 mM), iberiotoxin (25–50 nM) or apamin (100 nM) added at least 10 min before the addition of prostacyclin analogues (0.001–1 μM). Values are the mean ± S.E.M. of *n* determinations. The concentration causing 50% relaxation of contractions is expressed as the log EC_{50} value. Individual EC_{50} values for control and test concentration–response curves were determined using a sigmoidal-curve fitting routine.
[a] $P < 0.005*$ and [b] $P < 0.05$ (paired or unpaired Student's *t*-test) compared to control.

It is unlikely that the effects of glibenclamide were related to time-dependent increases in the response to iloprost, since we failed to detect any significant ($n = 11$; $P = 0.81$ Student's *t*-test) difference in the relaxation obtained to iloprost comparing first and second applications applied up to 240 min apart. Glibenclamide also prolonged the recovery time following exposure to iloprost, increasing the time taken for the muscle strip to decline by 50% of the peak response from 10 ± 0.9 min to 16 ± 1.9 min ($n = 6$; $P < 0.02$, Student's *t*-test, paired) at a concentration of 0.1 μM. A similar increase in peak relaxation and recovery time was observed in the absence of the endothelium ($n = 7$; $P < 0.02$), suggesting that glibenclamide was interfering with the action of iloprost primarily at the level of the smooth muscle. To determine if glibenclamide was potentiating the response to iloprost by inhibiting the effect of a constricting prostaglandin, experiments were performed with the cyclooxygenase inhibitor, indomethacin. Treatment of tissues with indomethacin alone (10 μM; 30–60 min), produced a significant increase in the peak relaxation to 0.1 μM iloprost compared to time-matched controls ($46.6 \pm 2.9\%$ versus $60.7 \pm 4.0\%$, $n = 11$; $P < 0.05$, ANOVA). This response was further enhanced to $70.1 \pm 3.2\%$ ($n = 7$; $P < 0.05$, ANOVA) in the presence of both indomethacin and glibenclamide. Recovery time was also increased 2.0 fold with indomethacin from 8.3 ± 1.1 min to 17.2 ± 2.0 min ($n = 11$; $P < 0.05$, ANOVA), and similarly with indomethacin and glibenclamide together (1.9 fold; $n = 7$). These results suggest that glibenclamide enhances the response to iloprost in part through inhibition of a constricting prostanoid.

3.3. Effect of the EP_1 receptor antagonist, SC-51089 on iloprost responses

To test the possibility that the vasorelaxant effects of iloprost may be counterbalanced by activation of EP_1 receptors, either directly or indirectly through the release of a constricting prostanoid, we investigated the effects of SC-51089, a highly selective EP_1 receptor antagonist (Hallinan et al., 1993). In the absence of iloprost, SC-51089 (30 μM) had no visible effect on phenylephrine (6 μM) contractions in endothelium-denuded tissues. It did however, significantly increase the size of iloprost relaxations ($n = 7$; $P < 0.05$; one way ANOVA with Bonferroni correction), which was associated with a leftward but not significant shift in the EC_{50} compared to control (Fig. 3). A small, but not significant, enhancement of the iloprost response was observed in the presence of both SC-51089 (30 μM) and glibenclamide (10 μM), but the EC_{50} was now significantly shifted to the left ($P < 0.05$ one way ANOVA with Bonferroni correction) compared to control (Fig. 3). These results are consistent with the idea that glibenclamide enhancement of iloprost relaxations may be largely mediated through its blockade of EP_1 receptors.

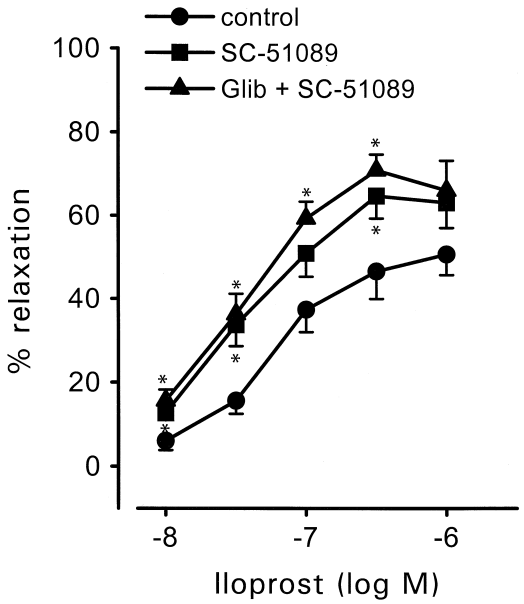

Fig. 3. The effects of the selective EP_1 receptor antagonist, SC-51089 on iloprost responses. Cumulative concentration–response curves to iloprost (0.01–1 μM) measured in the absence (Control ●), in the presence of 30 μM SC-51089 (■) or in the presence of 30 μM SC-51089 and 10 μM glibenclamide (Glib + SC-51089 ▲). Values for EC_{50}'s were -7.22 ± 0.09, -7.46 ± 0.07 and -7.50 ± 0.07, respectively. Strips of aorta were denuded of endothelium and precontracted with 6 μM phenylephrine. SC-51089 and glibenclamide were applied for 30 or 10 min, respectively before the application of iloprost. Data are expressed as the mean ± S.E.M. of six to seven experiments from seven animals. $* P < 0.05$ compared to control (ANOVA, with Bonferroni correction).

3.4. Influence of Ca^{2+}-activated K^+ channel blockers on responses to iloprost and cicaprost

In tissues denuded of the endothelium, the relaxant effects of iloprost or cicaprost on phenylephrine contractions were substantially inhibited by 2 mM tetraethylammonium (Table 1 and Fig. 4a), a known blocker of BK_{Ca} channels at this concentration. The maximum responses were reduced by ~54% ($n = 12$; $P < 0.005$, paired Student's *t*-test) and 43% ($n = 5$; $P < 0.005$, paired Student's *t*-test), respectively. In these experiments tetraethylammonium produced on average a 23% increase in tension in the presence of phenylephrine. The effect of tetraethylammonium was also studied on tissues contracted by 25 mM K^+ (Fig. 4b). It was found that iloprost (0.1 μM) relaxations were inhibited from $52.6 \pm 4.8\%$ under control conditions to $22.9 \pm 5.3\%$ and 17.1 ± 5.5 ($n = 5$; $P < 0.05$, ANOVA with Bonferroni correction) in the presence of 2 and 10 mM tetraethylammonium, respectively. In contrast, relaxations evoked by the K^+ channel activator, levcromakalim (10 μM) were not inhibited by 2 mM tetraethylammonium although at 10 mM they were significantly inhibited ($n = 5$; $P < 0.05$, one way ANOVA). In order to further confirm our results with tetraethylammonium, we investigated the effects of iberiotoxin (25–50 nM), a highly selective blocker of BK_{Ca} channels. In

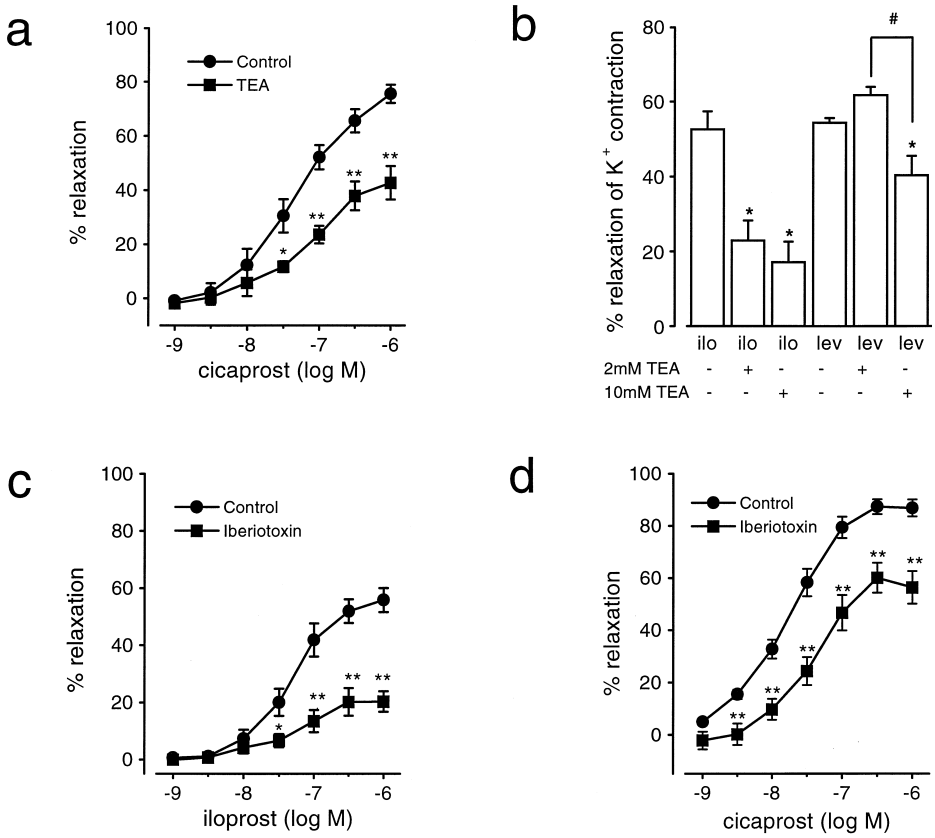

Fig. 4. Blockers of BK_{Ca} channels inhibit cicaprost- and iloprost- induced vasorelaxation. (a) Effect of tetraethylammonium (TEA) on concentration–response curves for cicaprost in arterial segments denuded of endothelium. Experiments were carried out in the absence (Control ●) and presence of 2 mM tetraethylammonium (■) and tissues were precontracted with 6 μM phenylephrine. (b) Effect of 2 and 10 mM tetraethylammonium on iloprost (ilo; 0.1 μM for 2 min) and levcromakalim (lev; 10 μM for 40s) evoked relaxation of contractions elicited by 25 mM K^+. Effect of iberiotoxin (IBTX; 25–50 nM) on responses to iloprost (c) and cicaprost (d). Experiments were carried out in the absence (Control ●) or presence of iberiotoxin (■). Protocol as in a. All data points are expressed as mean ± S.E.M. of 12 experiments (a), 5 experiments (b and d) and 4 experiments (c). $* = P < 0.05$ and $** = P < 0.005$ when compared to control values (Student's *t*-test for a, c and d and ANOVA for b). $^{\#} = P < 0.05$ when compared to response in 2 mM tetraethylammonium.

the absence of either cicaprost or iloprost, iberiotoxin did not have any significant effect on phenylephrine contractions. It did however, substantially inhibit the relaxant effects of both iloprost (Fig. 4c) and cicaprost (Fig. 4d), significantly reducing the maximum response (Table 1). In the case of cicaprost, iberiotoxin also significantly shifted the EC_{50} to the right ($P < 0.02$). The effect of apamin (100 nM), a blocker of small conductance Ca^{2+}-activated K^+ channels was also investigated and it was found not to inhibit responses to cicaprost over the whole concentration range (Table 1).

It is unlikely that the underlying action of iloprost was due to the basal release of nitric oxide from surviving endothelial cells activating BK_{Ca} channels through the cyclic GMP pathway, since in a parallel set of experiments, responses to iloprost were unaffected at any concentration ($n = 12$; $P > 0.15$) by 10 μM ODQ, a recently developed guanylate cyclase inhibitor. In other experiments, ODQ was effective at almost completely inhibiting the response to the nitric oxide-donor, sodium nitroprusside (0.001–10 μM) in guinea-pig aorta ($n = 5$; data not shown).

3.5. Influence of BK_{Ca} blockers on responses to cyclic AMP elevating agents

As shown in Fig. 5a, isoprenaline (0.01–100 μM) caused dose-dependent relaxations of endothelium-denuded tissues contracted with phenylephrine (6 μM), which were substantially blocked by iberiotoxin (25 nM) over the entire concentration range. Maximum relaxation to isoprenaline (100 μM) was reduced from 50.8 ± 7.8% to 19.8 ± 6.4% following pre-treatment with iberiotoxin. Isoprenaline (1 μM) relaxations of 25 mM K^+ contractions were also significantly reduced by tetraethylammonium being 34.6 ± 6.8%, 10.6 ± 3.2% and 7.0 ± 1.8% ($n = 5$; $P < 0.005$, ANOVA) under control conditions and in the presence of 2 or 10 mM tetraethylammonium, respectively. By contrast, forskolin-evoked relaxations were not significantly affected by tetraethylammonium (Fig. 5b) except at

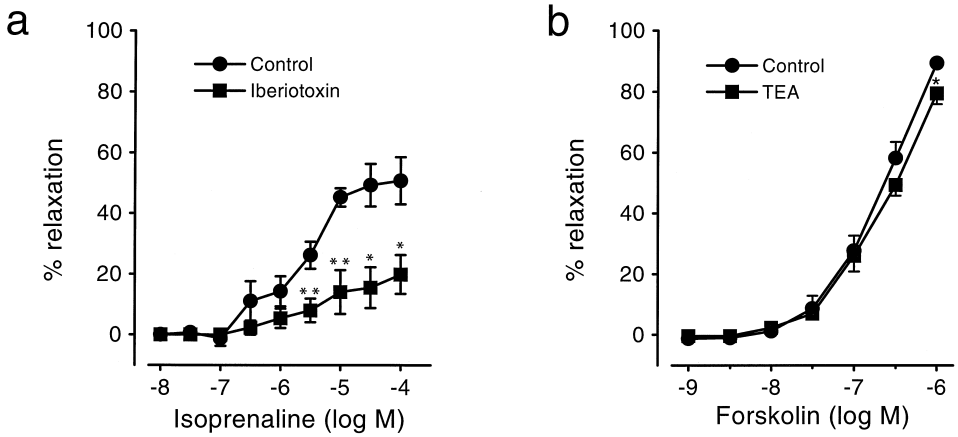

Fig. 5. Effect of iberiotoxin (25 nM) on isoprenaline (a) and tetraethylammonium (2 mM) on forskolin (b) evoked relaxation in arterial segments denuded of endothelium. Concentration–response curves were obtained in the absence (Control ●) and presence of K^+ channel blockers (■) and tissues were precontracted with 6 μM phenylephrine. Data points are expressed as mean ± S.E.M. of 4 (a) and 5 (b). $* = P < 0.05$ or $** P < 0.01$ compared to control values (paired, Student's *t*-test).

the highest concentration where relaxations to forskolin were reduced by ~11% ($n = 7$; $P < 0.05$).

4. Discussion

The data presented in this paper provides good evidence that BK_{Ca} channels make a major contribution to vasorelaxation induced by iloprost and cicaprost and that activation of these channels occurs through IP receptors independently of the endothelium. These conclusions are based on the following findings. (1) The responses to iloprost were substantially smaller when tissues were contracted with high (60 mM) compared to low (25 mM) extracellular K^+. This is at least consistent with an agent having an action on K^+ channels since at high elevated K^+ concentrations, the driving force on K^+ would be considerably reduced, thereby decreasing K^+ efflux and membrane hyperpolarization. (2) Iberiotoxin, a highly specific blocker of large conductance BK_{Ca} channels (Galvez et al., 1990), markedly attenuated the vasorelaxant effects of both iloprost and cicaprost, significantly reducing the maximum response. To date, this agent has not been found to block any other type of K^+ channel. (3) Responses to both agents were also inhibited by tetraethylammonium, a compound known to inhibit BK_{Ca} channels at or below 2 mM (Nelson and Quayle, 1995). The fact that we observed no further inhibition of iloprost responses at 10 mM tetraethylammonium, where other types of smooth muscle K^+ channels would start to become significantly blocked (e.g., K_{ATP} and delayed rectifier channels) (Nelson and Quayle, 1995), supports the idea that the major effect of tetraethylammonium was likely to result from blockade of BK_{Ca} channels. This is consistent with our observation that 2 mM tetraethylammonium did not inhibit relaxation to the K_{ATP} channel activator, levcromakalim and that apamin, a blocker of small conductance Ca^{2+}-activated K^+ channels did not inhibit the response to cicaprost. (4) Large inhibitory effects of BK_{Ca} blockers were observed in tissues where the endothelium had first been removed, indicating that K^+ channels were probably activated by a direct action of iloprost or cicaprost on the smooth muscle, rather than indirectly through increased endothelial nitric oxide release by iloprost (cf., Dumas et al., 1997). This is further supported by the observation that iloprost-induced relaxations of phenylephrine (compare Fig. 2c with Fig. 4c) and high K^+ contractions were essentially unaltered by endothelium removal or by the guanylate cyclase inhibitor, ODQ. These results therefore suggest that stimulation of guanylate cyclase is not involved in mediating relaxation induced by iloprost in our experiments, and shows that the newly-developed inhibitor has specificity for the cGMP-dependent pathway.

At this stage, we cannot rule out the possibility that the inhibitory effects of K^+ channel blockers on prostacyclin analogues resulted from either functional antagonism due to increased contraction (cf., Huang et al., 1993) or from a non-specific action of these agents on smooth muscle. However, we consider these possibilities unlikely for the following reasons. Firstly, although tetraethylammonium caused contraction in the presence of phenylephrine, iberiotoxin did not, and yet both had similar inhibitory effects on either iloprost or cicaprost. Secondly, 2 mM tetraethylammonium did not inhibit the response to the K^+ channel opener, levcromakalim in fact it caused a small, but significant potentiation ($P < 0.05$; paired *t*-test). This might be explained on the basis that tetraethylammonium would cause membrane depolarisation through blockade of K^+ channels (but not K_{ATP} channels), resulting in a greater driving force on K^+ ions and an increased K^+ efflux in response to levcromakalim. Thirdly, tetraethylammonium had a minimal effect on forskolin-induced relaxation also

supporting the idea that tetraethylammonium was not producing a general reduction in vasorelaxation.

The residual relaxation seen after exposure to tetraethylammonium or iberiotoxin indicates that additional mechanisms are more than likely responsible for smooth muscle relaxation. Recent data suggests that iloprost may inhibit smooth muscle contraction in part through decreasing the Ca^{2+}-sensitivity of the contractile elements (Ozaki et al., 1996). Alternatively, prostacyclin analogues might cause relaxation through blockade of voltage-dependent Ca^{2+} channels. Such a mechanism could account for the residual effects of iloprost observed in 60 mM K^+, although this idea is not supported by patch-clamp data showing iloprost to have no effect on voltage-gated Ca^{2+} current in isolated vascular myocytes (Siegel et al., 1990).

Previous studies have concluded that iloprost dilates endothelium-intact blood vessels in the rat tail artery (Schubert et al., 1997) and perfused lung (Dumas et al., 1997) in part through activation of BK_{Ca} channels. However, its contribution to overall relaxation was small in the former study, with only a minimal shift in the dose–response curve to iloprost seen after exposure to iberiotoxin and no effect on the maximal response, and probably largely mediated by nitric oxide in the latter study. Thus, this is the first study to directly show a major role for BK_{Ca} channels in mediating the vasorelaxant effects of prostacyclin analogues independent of the endothelium. It confirms previous patch clamp studies with iloprost in isolated cells from rat portal vein (Siegel et al., 1990) and rat tail artery (Schubert et al., 1996) showing it to activate a Ca^{2+} and voltage-dependent current, presumed to be carried by BK_{Ca} channels. In this present study, we also investigated the effect of BK_{Ca} blockers against two other dilator agents known to increase cyclic AMP. Like iloprost or cicaprost, relaxations to the β-adrenoceptor agonist, isoprenaline were markedly blocked over the whole concentration range by inhibitors of BK_{Ca} channels. In contrast, relaxations to forskolin, a direct activator of adenylate cyclase, were essentially unaffected by tetraethylammonium, except at the highest dose where a small inhibition was observed. Thus, this raises the possibility that cyclic AMP-independent pathways may be involved in mediating IP or β adrenoreceptor mediated activation of BK_{Ca} channels in guinea-pig aorta. Indeed, cyclic AMP-dependent and -independent pathways have been demonstrated for β-adrenoreceptor activation in coronary artery and airway smooth muscle, where BK_{Ca} channels can be activated either through PKA-dependent phosphorylation or through a direct action of G_S (Scornik et al., 1993; Kume et al., 1994). Consistent with this idea, Hiramatsu et al. (1994) showed in tracheal muscle that BK_{Ca} channel blockers were more effective at inhibiting isoprenaline-induced relaxation compared to forskolin-induced relaxation. Our conclusion is not however, supported by recent data showing that iloprost-evoked dilation or activation of $I_{K(Ca)}$ was inhibited by blockers of PKA in rat tail artery (Schubert et al., 1996; Schubert et al., 1997). In contrast, lack of correlation between cyclic AMP and K^+ channel activation has been observed in the hamster cheek pouch, where vasodilation to isoprenaline, carbacyclin (a stable prostacyclin analogue) and adenosine were significantly blocked by glibenclamide, whereas vasodilation to forskolin and dibutyrl adenosine 3′,5′-cyclic monophosphate (membrane permeant analogue of cyclic AMP) were not significantly inhibited (Jackson, 1993). It therefore remains to be determined whether prostacyclin analogues stimulate K^+ channels through a direct G-protein coupling. In view of recent findings that IP receptors can couple to multiple G-proteins, including G_i and G_p and other, as yet, unidentified G-protein-linked pathways (Schwaner et al., 1995; Wise and Jones, 1996), clearly the role of these additional pathways need also to be evaluated.

Our results with glibenclamide clearly demonstrate that K_{ATP} channels are not involved in the vasorelaxant effects of iloprost or cicaprost in guinea-pig aorta. This is in contrast to many other studies where K_{ATP} channels appear to play a major role in mediating the effects of prostacyclin and iloprost (Jackson, 1993; Jackson et al., 1993; Bouchard et al., 1994; Schubert et al., 1997; Dumas et al., 1997). Thus it would appear that prostacyclin analogues, depending on the vascular bed, can activate different K^+ channel types. However, we did find that glibenclamide significantly augmented the peak relaxation and significantly prolonged recovery time from iloprost, although this effect was not observed with the highly selective IP receptor agonist, cicaprost (Coleman et al., 1994). This difference in the response to glibenclamide may be related to the observations that iloprost is known to have additional vascular effects related to activation of constricting prostanoid receptors (see below), whilst cicaprost has little or no activity at any other prostanoid receptors (Coleman et al., 1994).

Our observation that the cyclooxygenase inhibitor, indomethacin also enhanced iloprost-induced vasorelaxation in endothelium denuded tissues and partially inhibited the effects of glibenclamide, suggests that iloprost may release a constricting prostaglandin from the smooth muscle, which glibenclamide is capable of inhibiting. Certainly, previous studies have shown that indomethacin can enhance contractions to prostacyclin in rat aorta (Van Dam et al., 1986). Moreover, it is known that glibenclamide can inhibit vascular contractions elicited by prostaglandin E_2, prostaglandin $F_{2\alpha}$ and thromboxane A_2 by acting as a receptor antagonist (Cocks et al., 1990; Delaey and Van de Voorde, 1995). We favour the involvement of prostaglandin E_2 since one, the highly selective EP_1 receptor antagonist, SC-51089 (Coleman et al., 1994) enhanced the response to iloprost and two, prostaglandin E_2 is the only endogenous prostanoid known to activate this EP receptor subtype. Obviously this conclusion relies on the specificity of SC-51089, but recent results show that it does not block the effects of prostaglandin E_1 in vivo (Khasar et al.,

1994). That glibenclamide produced no significant enhancement of iloprost relaxations in the presence of SC-51089, is consistent with the idea that glibenclamide is also inhibiting EP_1 receptors. Moreover, since glibenclamide still augmented iloprost-induced vasorelaxation, albeit to a lesser extent in the presence of cyclo-oxygenase inhibition, suggests that vasorelaxation to iloprost may in part be counterbalanced by direct activation of the EP_1 receptor. This is consistent with data showing iloprost to potently bind to EP_1 receptors (Coleman et al., 1994), and functional studies showing EP_1 receptor antagonists to be effective at inhibiting contractions to iloprost in guinea-pig ileum (Botella et al., 1995). However, the existence of this EP receptor subtype has not to our knowledge been described in vascular muscle before. We cannot at this stage rule out that other prostanoids receptors might be activated by iloprost and inhibited by glibenclamide. Iloprost does have some affinity at EP_3 receptors, although thromboxane A_2 (TP) and prostaglandin $F_{2\alpha}$ (FP) receptors are either not affected or only weakly activated by iloprost (Coleman et al., 1994).

In summary, it appears from our data that in guinea-pig aorta, BK_{Ca} but not K_{ATP} channels, play a major role in vasorelaxation induced by the IP agonists, iloprost and cicaprost. Isoprenaline, also appears to cause vasorelaxation through activation of BK_{Ca} channels, although these channels do not make a major contribution to the vasorelaxant action of forskolin. This latter observation questions the pivotal role of cyclic AMP in mediating responses to receptors known to be coupled to adenylate cyclase. The vasorelaxant effects of iloprost (but not cicaprost) appear to be modulated by prostaglandin release from the smooth muscle, and we postulate that relaxation may also be limited by counter-activation of prostanoid EP_1 receptors.

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