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Synergistic interaction between endothelium-derived NO and prostacyclin in pulmonary artery: potential role for K^+_{ATP} channels

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1 The aim of the present study was to assess interactions between nitric oxide (NO) and prostacyclin (PGI_2) during endothelium-dependent relaxations evoked by bradykinin, calcium ionophore (A23187) and acetylcholine in canine isolated pulmonary artery.

2 Relaxations to low concentrations of bradykinin and A23187 were abolished by combined inhibition of NO-synthase (by N^{ω}-nitro-L-arginine methyl ester L-NAME, 30 μM) and cyclo-oxygenase (indomethacin, 10 μM), suggesting mediation by NO and PGI_2. The individual contributions of NO and PGI_2 to the dilator responses were quantified by use of areas above the separate indomethacin-insensitive and L-NAME-insensitive components of the concentration-effect curves, respectively. Individually, NO and PGI_2 accounted for only $53 \pm 5\%$ and $16 \pm 9\%$ of total bradykinin-induced relaxation, and $46 \pm 10\%$ and $20 \pm 9\%$ of total A23187-induced relaxation, suggesting that NO and PGI_2 acted synergistically to cause endothelium-dependent relaxation.

3 Relaxation to low concentrations of acetylcholine was abolished by L-NAME but not affected by indomethacin, suggesting the response was mediated solely by NO with no interaction from PGI_2.

4 Glibenclamide (1 μM), an inhibitor of ATP-sensitive potassium (K^+_{ATP}) channels, inhibited responses to bradykinin or A23187 but did not affect relaxations evoked by acetylcholine. Glibenclamide did not affect endothelium-independent relaxations to PGI_2 or the NO-donor, 3-morpholinosydnonimine (SIN-1).

5 With bradykinin, glibenclamide attenuated total relaxation by $49 \pm 8\%$, but did not alter the individual NO and PGI_2-mediated components of the response. Glibenclamide abolished the synergistic interaction between endothelium-derived NO and PGI_2.

6 At high concentrations, bradykinin, A23187 or acetylcholine caused endothelium-dependent relaxation that was insensitive to L-NAME + indomethacin. With bradykinin or A23187, this component of relaxation was inhibited by glibenclamide, whereas with acetylcholine, glibenclamide had no effect.

7 The synergistic interaction between endothelium-derived NO and PGI_2 in canine pulmonary artery is mediated by activation of K^+_{ATP} channels, presumably by an endothelium-derived hyperpolarizing factor (EDHF). The pattern of endothelial dilator mediators and the presence of this synergistic interaction is dependent on the nature of the endothelial stimulus.

Keywords: Bradykinin; A23187; acetylcholine; L-NAME; indomethacin; EDHF

Introduction

Vascular endothelial cells generate a diverse range of vasoactive substances that evoke contraction or relaxation of the underlying smooth muscle. The primary vasorelaxant mediators are endothelium-derived nitric oxide (NO) and prostacyclin (Furchgott & Vanhoutte, 1989; Flavahan & Vanhoutte, 1995). These substances are released basally in some vascular beds, and are synthesized in response to blood flow-induced shear stress and agonist activation (Furchgott & Vanhoutte, 1989; Flavahan & Vanhoutte, 1995; Loscalzo & Welch, 1995; Davies, 1995). NO causes relaxation primarily by activating soluble guanylyl cyclase in smooth muscle cells to increase intracellular guanosine 3′:5′-cyclic monophosphate (cyclicGMP), whereas prostacyclin relaxation is mediated by stimulation of adenylyl cyclase to increase adenosine 3′:5′-cyclic monophosphate (cyclicAMP) (Furchgott & Vanhoutte, 1989; Flavahan & Vanhoutte, 1995; Loscalzo & Welch, 1995). The endothelium also release diffusible factors, distinct from prostacyclin and NO, that cause smooth muscle relaxation by activating potassium channels (EDHFs, endothelium-derived hyperpolarizing factors) (Bolton *et al*., 1984; Standen *et al*., 1989; Nagao & Vanhoutte, 1992; Parkington *et al*., 1993; 1995; Vanhoutte, 1993).

Vascular smooth muscle relaxation is also mediated by interactions between endothelium-derived mediators. Interactions between NO and prostacyclin have been documented in some, but not all, blood vessels (Shimokawa *et al*., 1988; Lidbury *et al*., 1989; Maurice *et al*., 1991; Kovitz *et al*., 1993). Prostacyclin can evoke relaxation both by stimulating the release of NO, and through synergistic interactions with NO at the level of the smooth muscle (Shimokawa *et al*., 1988). The mechanisms underlying the interaction between these mediators on smooth muscle cells may be mediated in part by cyclicGMP-mediated inhibition of cyclicAMP phosphodiesterase (PDE III) (Maurice *et al*., 1991; Lugnier & Komas, 1993; Eckly & Lugnier, 1994). Relaxation of smooth muscle induced by prostacyclin can, therefore, be amplified by endothelium-derived NO. The objectives of the present study were to assess pulmonary vascular responses to agonist-induced en dothelium-dependent vasorelaxation in canine isolated pul-

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monary arteries, to assess the activities of the mediators responsible for the relaxation response to each agonist, and to assess possible interactions between those mediators.

Methods

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee.

Organ chamber experiments

The present *in vitro* studies were performed with canine pulmonary artery rings harvested from healthy male mongrel dogs weighing 20–25 kg. The dogs were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.) and fentanyl citrate (15 μg kg^{-1}, i.v.), exsanguinated by controlled haemorrhage, and killed with a bolus of saturated KCl injected into the left atrium (Flavahan *et al*., 1994). Injection at this site arrests the heart before the KCl can be perfused through the pulmonary circulation. The lower right and lower left lung lobes were removed. The main intra-lobar pulmonary artery was dissected free and immersed in cold modified Krebs-Ringer bicarbonate solution of the following composition in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, Ca-EDTA 0.016 and glucose 11.1. The arteries were cut into 0.5 cm length rings with care taken not to damage the endothelium. In some rings, the endothelium was removed by inserting forceps into the vessel lumen and rolling the ring over damp filter paper. Endothelium-removal was confirmed during the course of the experiment by lack of relaxation to acetylcholine (10^{-6} M) during a phenylephrine contraction. The rings were suspended between two stainless steel stirrups in organ chambers filled with 25 ml modified Krebs-Ringer bicarbonate (37°C), gassed with 95% O_2-5% CO_2. One of the stirrups was anchored and the other was connected to a strain gauge (Grass FT03) for the measurement of isometric tension (Grass Polygraph model 7E).

Experimental protocol

Arterial rings were stretched at 10 min intervals in increments of 0.5 g to reach optimal resting tension. Optimal resting tension was the minimum amount of stretch required to achieve the largest contractile response to KCl (20 mM) and was determined in preliminary experiments to be 4 g for the size of arteries used in these studies (2–4 mm i.d.). After the arterial rings had been stretched to their optimal resting tension, the contractile response to 60 mM KCl was measured. After washout of KCl from the organ chamber and return of contractile tone to prestimulation values, a concentration-response curve to phenylephrine was performed in each ring. This was achieved by increasing the concentration of phenylephrine in half-log increments (from 10^8 to 3×10^{-5} M) after the response to each preceding concentration had reached a steady state. Initial experiments showed that phenylephrine caused β-adrenoceptor-mediated relaxation in addition to α-adrenoceptor-mediated contraction in these arteries. Thus, the rings were pretreated with the β-adrenoceptor antagonist, propranolol (5×10^{-6} M; incubated for 30 min), before phenylephrine for all protocols. After washout of phenylephrine from the organ chamber and return of contractile tone to baseline values, the rings were again pretreated with propranolol and contracted to 50% of their maximal response to phenylephrine (ED_{50} level of tension). When the contractile response stabilized, concentration-response curves to the endothelial activators bradykinin, calcium ionophore (A23187) or acetylcholine was generated. Some vascular rings were exposed to two endothelium-dependent vasorelaxants separated by thorough washouts. For these arterial rings the sequence for exposure was either bradykinin followed by A23187, or acetylcholine followed by A23187. Responses to the endothelium-independent dilators, prostacyclin and the NO-donor SIN-1, were determined in rings without endothelium.

In another series of experiments, after completion of the concentration-response curve to phenylephrine and the return of contractile tone to baseline values, arterial rings were incubated with one or more of the following pharmacological antagonists: N^{ω}-nitro-L-arginine methyl ester (L-NAME, 3×10^{-5} M), an inhibitor of NO synthase, indomethacin (10^{-5} M), an inhibitor of cyclo-oxygenase, or glibenclamide (10^{-6} M), an inhibitor of ATP-sensitive potassium (K^{+}_{ATP}) channels. Arterial rings were treated with these inhibitors for 30 min before contraction to the ED_{50} level of tension with phenylephrine. The inhibitors remained in the bath solution for the duration of the exposure to the vasorelaxants. Vascular responses to vasorelaxant stimuli in antagonist-treated arterial rings were compared to responses measured in untreated arterial rings that were size- and position-matched (right vs left lung lobe). The responsiveness of pulmonary arteries to phenylephrine (control log ED_{50}: -6.87 ± 0.08, $n=11$) was not significantly influenced by L-NAME (-7.00 ± 0.07, $n=10$), indomethacin (-7.01 ± 0.05, $n=10$) or by glibenclamide (-6.97 ± 0.07, $n=8$). Combined inhibition of NOS and cyclo-oxygenase (L-NAME plus indomethacin) caused a small, but significant, increase in sensitivity to phenylephrine (log ED_{50}: -7.15 ± 0.03, $n=10$). This was equivalent to a 2 fold shift in the concentration-effect curve (log shift of 0.29 ± 0.05, $n=10$), which was the same as that produced by endothelial-denudation (log shift of 0.29 ± 0.07, $n=5$). Glibenclamide did not alter the influence of L-NAME and/or indomethacin on phenylephrine-induced contractions. None of the inhibitors had any effect on baseline tone.

Drugs and solutions

The following drugs were obtained from Sigma (St. Louis, MO) unless stated otherwise: A23187, acetylcholine chloride, bradykinin (Calbiochem, CA), glibenclamide, indomethacin, L-NAME, phenylephrine, propranolol, prostacyclin and 3-morpholinosydnonimine (SIN-1, Casella AG, Frankfurt, Germany). All drug concentrations are expressed as the final molar concentration in the organ chamber. Stock solutions were prepared each day and kept on ice during the course of the experiment. Unless stated otherwise, drugs were dissolved in distilled water. Indomethacin was dissolved in a $NaHCO_3$ solution (final bath concentration of $NaHCO_3$; 0.2 mM), A23187 was dissolved in DMSO followed by dilution in distilled H_2O (final bath concentration of DMSO: 0.00004–0.013% v/v), glibenclamide was dissolved in methanol followed by dilution in distilled H_2O (final bath concentration of methanol: 0.16% v/v). At these concentrations, the solvents had no effect on endothelium-dependent relaxations or on constrictor responses to phenylephrine.

Data analysis

Results are expressed as means $\pm$ s.e., and n equals the number of animals from which blood vessels were taken. Responses to the vasorelaxants are expressed as a percentage of phenylephrine contraction. The effect of antagonists on concentration-response curves to vasorelaxant stimuli were evaluated by comparing the concentration of vasorelaxant causing 50% relaxation of the contraction to phenylephrine (inhibitory concentration: IC_{50}). This value was interpolated from the linear portion of the vasorelaxant concentration-effect curve by regression analysis and is presented as log IC_{50}. The method used to quantitate the contribution of individual mediators (e.g. NO or prostacyclin) to the total relaxation response to an agonist involved calculating the areas above the individual concentration-response curves. The area above the control vasorelaxant concentration-response curve, representing the total response to the agonist, was assigned a value of 100%. The area above the vasorelaxant curve after each antagonist was then determined and expressed as a percentage of the total response to that agonist. Statistical evaluation of the data was

performed by Student's t test for either paired or unpaired observations. When more than two means were compared, analysis of variance was used. If a significant F value was found, Scheffe's test for multiple comparisons was employed to identify differences between groups. Values were considered to be statistically different when P was less than 0.05.

Results

Interactions between mediators are most apparent at low or threshold concentrations of agonists (Flavahan & Vanhoutte, 1988). Therefore, responses evoked by low concentrations of dilator agonists will be described separately from the full relaxant concentration-effect relationship.

Low concentrations of endothelial activators

The 'low concentration-range' was restricted to the range in which combined inhibition of NO synthase (L-NAME) and cyclo-oxygenase (indomethacin) abolished relaxation. The component of relaxation that was resistant to L-NAME + indomethacin will be discussed in the next section.

Bradykinin

At low concentrations of bradykinin (10^{-10} to 3×10^{-9} M), L-NAME or indomethacin each significantly attenuated the vasorelaxation (Figure 1). The component of relaxation that was insensitive to L-NAME (i.e. the stippled area above the L-NAME concentration-effect curve, Figure 1a) was abolished by the additional presence of indomethacin (Indo + L-NAME) suggesting that the L-NAME-insensitive component of relaxation was mediated by prostacyclin. Conversely, the component of relaxation that was insensitive to indomethacin (i.e. the shaded area above the indomethacin concentration-effect curve, Figure 1b) was abolished by L-NAME, suggesting that it was mediated by NO. Combined administration of L-NAME and indomethacin totally abolished the relaxation to bradykinin (10^{-10} to 3×10^{-9} M) (Figure 1a and b). Therefore, the response to low concentrations of bradykinin appears to be mediated entirely by NO and prostacyclin. In order to assess potential interactions between these mediators, the action of each mediator was assessed when the response to the other mediator was blocked. The total relaxation response to bradykinin for this concentration range (10^{-10} to 3×10^{-9} M) was quantified as the area above the control concentration-effect curve and assigned a value of 100% (Figure 1). The individual components of bradykinin-induced vasorelaxation mediated by prostacyclin (L-NAME-resistant, but indomethacin-sensitive component: stippled area, Figure 1a) and NO (indomethacin-resistant, but L-NAME-sensitive component: shaded area, Figure 1b) were quantified and expressed as a percentage of the total relaxation response. These data are summarized in Figure 2a. The total relaxation response to bradykinin (due to the combined effect of NO and prostacyclin) was greater than the sum of the individual effects of NO and prostacyclin (i.e. NO accounts for $53 \pm 5\%$ of total relaxation; and prostacyclin accounts for $16 \pm 9\%$ of total relaxation, $n = 5$). Therefore, the total relaxation response to low concentrations of bradykinin is mediated in part by a synergistic interaction between NO and prostacyclin.

A23187 At low concentrations of A23187 (10^{-9} to 3×10^{-8} M), L-NAME or indomethacin each significantly attenuated the vasorelaxation (Figure 3). The L-NAME- insensitive component of relaxation (stippled area, Figure 3a) was abolished by indomethacin, whereas the indomethacin-insensitive component of relaxation (shaded area, Figure 3b) was abolished by L-NAME. Combined administration of L-NAME and indomethacin totally abolished the vasorelaxant response (Figure 3a and b). Therefore, the relaxation response to low concentrations of A23187 appears to be mediated entirely by NO and prostacyclin. As with bradykinin, a potential interaction between these mediators was evaluated by determining the activity of each mediator once the other mediator had been inhibited. The individual components of A23187-induced vasorelaxation, calculated as a percentage of the total relaxation response, are summarized in Figure 2b. Like bradykinin, the total vasorelaxant response to A23187 was greater than the sum of the individual NO ($46 \pm 10\%$, $n = 5$) and prostacyclin ($20 \pm 9\%$, $n = 5$) components, suggesting a synergistic interaction between these mediators.

Acetylcholine At low concentrations of acetylcholine (10^{-9} to 10^{-8} M), L-NAME abolished acetylcholine-induced relaxation (Figure 4a). Indomethacin did not significantly alter the vasorelaxant response to acetylcholine (Figure 4b). Thus, in contrast to bradykinin and A23187, the vasorelaxant response to low concentrations of acetylcholine appears to be mediated exclusively by NO, with little or no contribution from prostacyclin (Figure 2c).

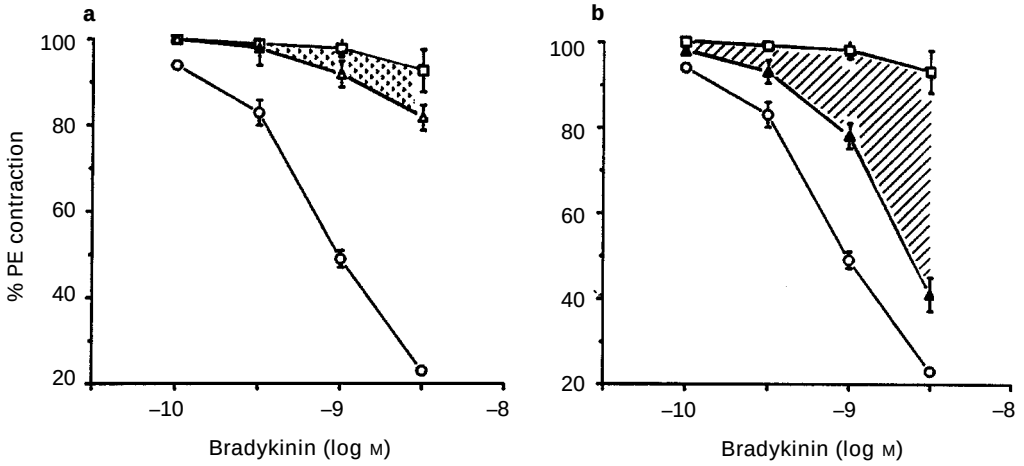

Figure 1 Effect of inhibition of nitric oxide synthase (L-NAME, 3×10^{-5} M) and/or cyclo-oxygenase (indomethacin, Indo, 10^{-5} M) on vasorelaxation evoked by bradykinin (10^{-10} to 3×10^{-9} M) in canine pulmonary arteries. Responses to bradykinin were determined during contractions to the ED_{50} level of tension with phenylephrine (PE). Relaxations are expressed as % of phenylephrine contraction and are presented as means ($n = 5$); vertical lines show s.e.mean. Prostacyclin-mediated relaxation (stippled area, a) accounts for $16 \pm 9\%$ of the total control relaxation, whereas NO-mediated relaxation (shaded area, b) accounts for $53 \pm 5\%$ of total relaxation (calculated by use of areas above the concentration-effect curves). Symbols: (○) control; (△) L-NAME; (▲) indomethacin; (□) indomethacin + L-NAME.

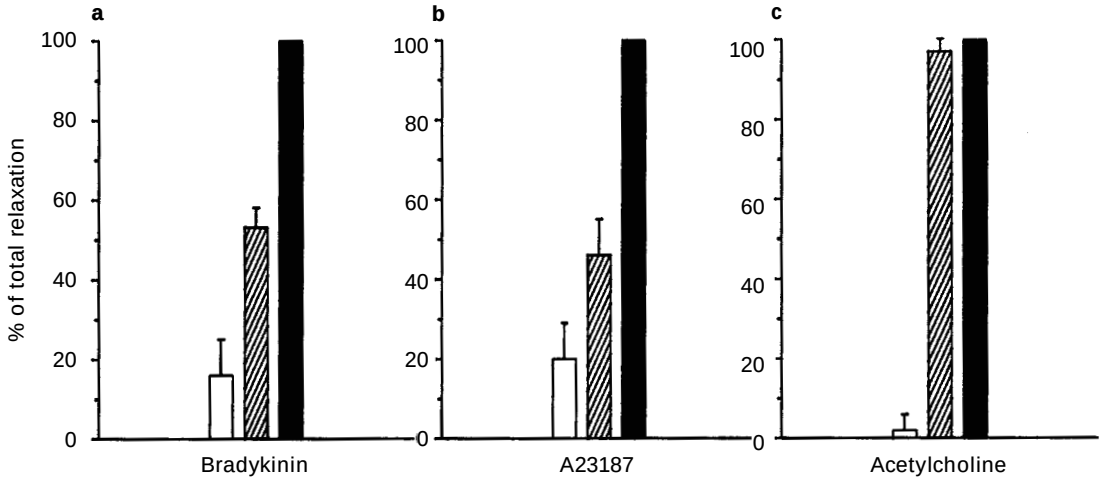

Figure 2 Nitric oxide (NO) and prostacyclin (PGI_2)-mediated components of relaxation evoked by bradykinin (a), A23187 (b) or acetylcholine (c) at low concentrations of each agonist (derived from Figures 1,3 and 4). The NO and PGI_2 components of relaxation were calculated (using Figures 1,3 and 4) from areas above the Indo-insensitive and L-NAME-insensitive concentration-effect curves, respectively. Areas are expressed as a % of the total relaxation and are presented as means ± s.e. ($n=5$). Symbols: solid columns, total relaxation response (=100%); open columns, prostacyclin-mediated component of relaxation; hatched columns, NO-mediated component of relaxation.

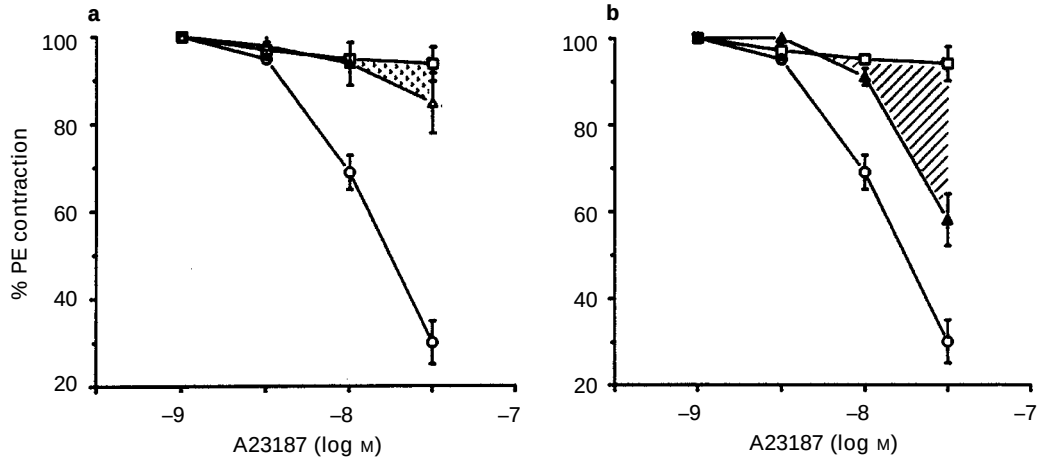

Figure 3 Effect of inhibition of nitric oxide synthase (L-NAME, 3×10^{-5} M) and/or cyclo-oxygenase (indomethacin, Indo, 10^{-5} M) on vasorelaxation evoked by A23187 (10^{-9} to 3×10^{-8} M) in canine pulmonary arteries. Responses to A23187 were determined during contractions to the ED_{50} level of tension with phenylephrine. Relaxations are expressed as % of phenylephrine (PE) contraction and are presented as means ($n=5$); vertical lines show s.e.mean. Prostacyclin-mediated relaxation (stippled area, a) accounts for 20±9% of total control relaxation, whereas NO-mediated relaxation (shaded area, b) accounts for 46±10% of total relaxation (calculated by use of areas above the concentration-effect curves). Symbols: (○) control; (△) L-NAME; (▲) indomethacin; (□) indomethacin + L-NAME.

K^+_{ATP} channels and the interaction between NO and prostacyclin

Glibenclamide (10^{-6} M) significantly attenuated the relaxation responses to bradykinin and A23187, but had no effect on the relaxation response to acetylcholine (Figure 5). Bradykinin was used as a representative agonist to evaluate further the role of K^+_{ATP} channels in the relaxation response to endothelium-dependent vasorelaxants. Although glibenclamide attenuated the total relaxation response (49±10% versus 100%, $n=5$, $P<0.05$) (Figure 6a vs b), it had no effect on the individual prostacyclin-mediated (16±6% versus 16±9%, $n=5$) or NO-mediated (42±7% versus 53±5%, $n=5$) components of bradykinin-induced vasorelaxation (Figure 6c). Furthermore, in the presence of glibenclamide, the total vasorelaxant response to bradykinin was no longer greater than the sum of the individual NO and prostacyclin components, suggesting that K^+_{ATP} channel inhibition abolished the synergistic interaction between these mediators (Figure 6c).

Glibenclamide (10^{-6} M) did not inhibit vasodilator responses to prostacyclin or the NO donor, SIN-1 (Table 1).

Higher concentrations of endothelial activators

Bradykinin The concentration-effect curve to bradykinin was depressed and shifted to the right by L-NAME or by indomethacin (Table 2). At concentrations of bradykinin $>3\times10^{-9}$ M, there was a component of relaxation that was resistant to combined inhibition with L-NAME plus indomethacin (Figure 7a). Endothelium-dependent relaxations that are resistant to inhibition of cyclo-oxygenase and nitric oxide synthase are generally ascribed to EDHF (Nagao & Vanhoutte, 1992; Vanhoutte, 1993). Indeed, glibenclamide further attenuated, but did not abolish, this L-NAME/indomethacin-resistant relaxation (Figure 7a).

A23187 The concentration-effect curve to A23187 was depressed and shifted to the right by L-NAME or by in-

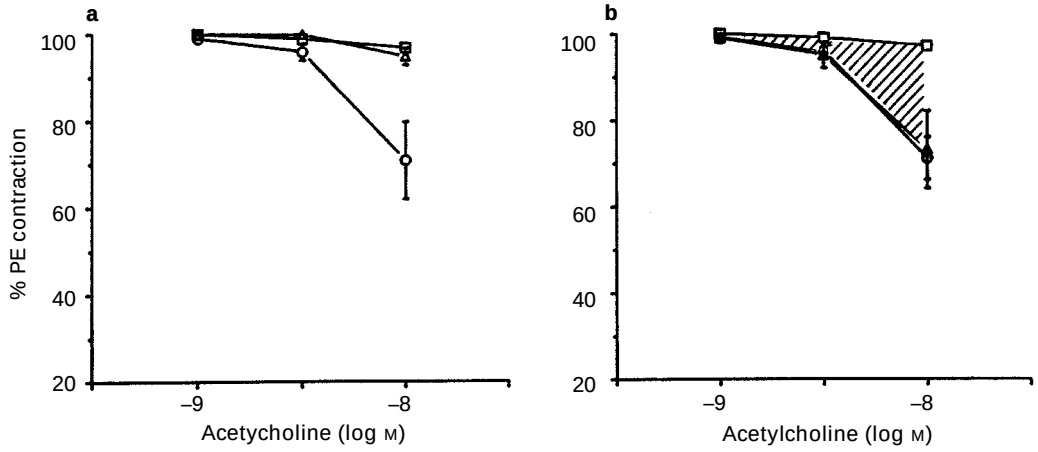

Figure 4 Effect of inhibition of nitric oxide synthase (L-NAME, 3×10^{-5} M) and/or cyclo-oxygenase (indomethacin, Indo, 10^{-5} M) on vasorelaxation evoked by acetylcholine (10^{-9} to 10^{-8} M) in canine pulmonary arteries. Responses to acetylcholine were determined during contraction to the ED_{50} level of tension with phenylephrine (PE). Relaxations are expressed as % of phenylephrine contraction and are presented as means ($n=5$); vertical lines show s.e.mean; NO-mediated relaxation (shaded area, b) accounts for 100% of total control relaxation (calculated by use of areas above the concentration-effect curves). Symbols: (○) control; (△) L-NAME; (▲) indomethacin; (□) indomethacin + L-NAME.

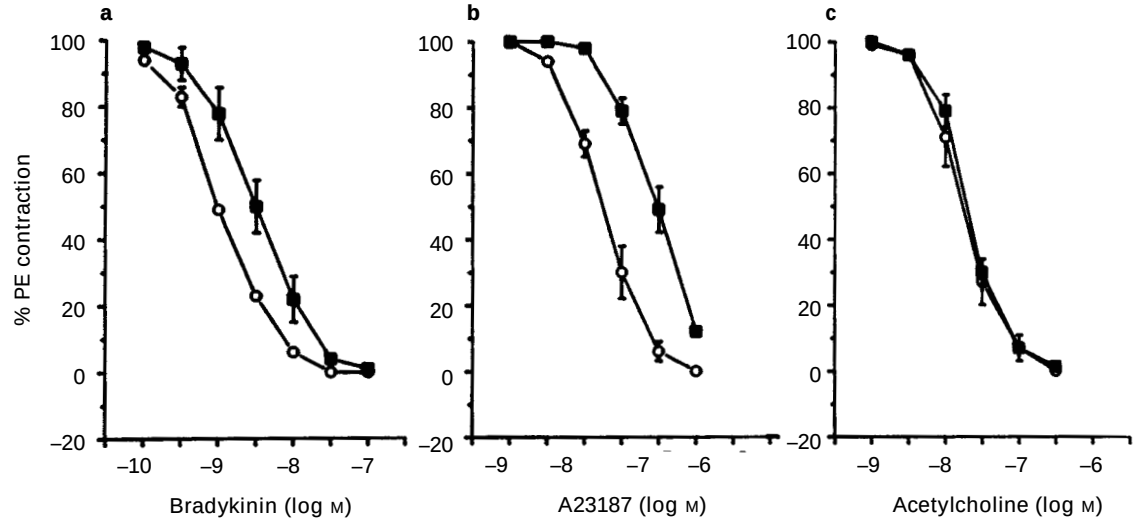

Figure 5 Effect of K^+_{ATP} channel inhibition with glibenclamide (10^{-6} M) on relaxations evoked by bradykinin (a). A23187 (b) or acetylcholine (c) in canine pulmonary arteries. Relaxations were determined during contractions to the ED_{50} level of tension with phenylephrine. Responses are expressed as % of phenylephrine contraction and are presented as means ($n=5$); vertical lines show s.e.mean. Glibenclamide caused a significant rightward shift ($P<0.05$) in the relaxation responses to bradykinin and A23187, but had no effect on the response to acetylcholine. Symbols: (○) control; (■) glibenclamide.

domethacin (Table 2). At concentrations of A23187 > 3×10^{-8} M, there was a component of relaxation that was resistant to combined inhibition with L-NAME plus indomethacin (Figure 7b). As with bradykinin, glibenclamide further attenuated, but did not abolish, this L-NAME/indomethacin-resistant relaxation (Figure 7b).

Acetylcholine The concentration-effect curve to acetylcholine was depressed and shifted to the right by L-NAME but was not affected by indomethacin (Table 2). At concentrations of acetylcholine > 10^{-8} M, there was a component of relaxation that was resistant to L-NAME or indomethacin plus L-NAME (Figure 7c). In contrast to bradykinin and A23187, this residual relaxation was not affected by glibenclamide (Figure 7c).

Discussion

Endothelium-dependent relaxation results from the release of multiple mediators from the endothelium that act and interact on vascular smooth muscle cells to cause relaxation. At least three mediators have been found to contribute to endothelial responses: NO, prostacyclin and EDHF(s) (Furchgott & Vanhoutte, 1989; Vanhoutte, 1993; Flavahan & Vanhoutte, 1995). Interactions between endothelium-derived dilators have been observed previously, with prostacyclin acting to stimulate NO release from endothelial cells as well as to enhance the dilator activity of NO on vascular smooth muscle (Shimokawa *et al*., 1988). The results of the present study demonstrate that synergistic interactions between NO and prostacyclin occur during endothelium-dependent relaxation in canine isolated pulmonary arteries. Acetylcholine, bradykinin and A23187 were equally effective in causing endothelium-dependent relaxation. Although synergistic interactions between endothelial mediators were evident during relaxations evoked by bradykinin and A23187, no interactions were observed during responses to acetylcholine. This suggests that synergistic interactions are specific to certain endothelial cell activators. The synergistic interaction between endothelium-derived NO and prostacyclin was inhibited by glibenclamide and appears to be mediated by activation of K^+_{ATP} channels.

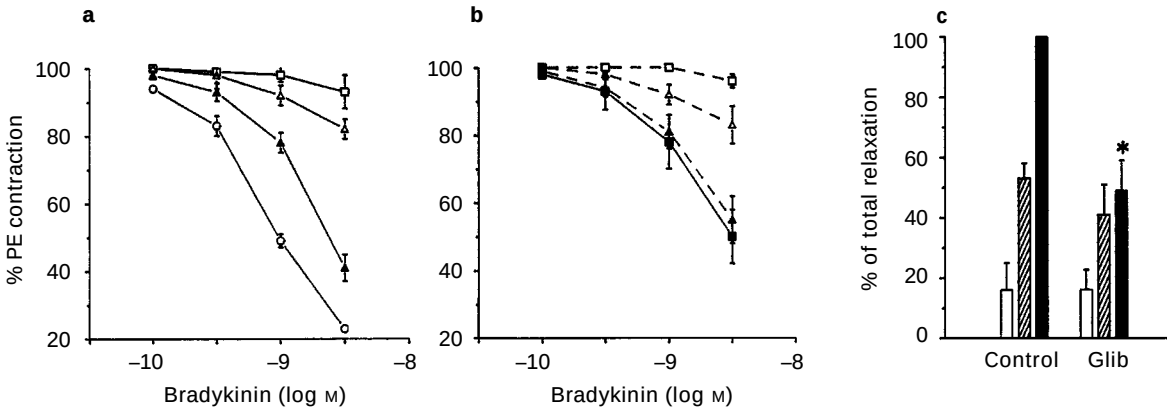

Figure 6 Effect of inhibition of nitric oxide synthase (L-NAME, 3×10^{-5} M) and/or cyclo-oxygenase (indomethacin, Indo, 10^{-5} M) on relaxations evoked by bradykinin (10^{-10} to 3×10^{-9} M) in the absence (control, a) and presence (Glib-treated, b) of the K^{+}_{ATP} channel inhibitor, glibenclamide (Glib) (10^{-6} M). Pulmonary arterial rings were contracted to the ED_{50} level of tension with phenylephrine. Relaxations are expressed as % of phenylephrine (PE) contraction and are presented as means ($n=5$); vertical lines show s.e.mean. (c) A summary of the effect of Glib on the NO and PGI_2 components of bradykinin-induced relaxation compared to control. The data in (a) were presented in Figure 1a and b. The NO and PGI_2 components were calculated from the areas above the Indo-insensitive and L-NAME-insensitive concentration-effect curves, respectively. Areas are expressed as % of total relaxation to bradykinin in control arterial rings and are expressed as means ($n=5$); vertical lines show s.e.mean. Symbols for (a) and (b): (○) control; (△) L-NAME; (▲) indomethacin; (□) indomethacin + L-NAME; (■) glibenclamide. In (c); solid columns, total relaxation response (control = 100%); open columns, prostacyclin-mediated component of relaxation; hatched columns, NO-mediated component of relaxation.

Table 1 Effect of glibenclamide on relaxations to prostacyclin or SIN-1 in canine pulmonary artery (without endothelium)

		Control	*Glibenclamide*
Prostacyclin	log IC_{50}	-6.48 ± 0.08 (4)	-6.64 ± 0.10 (4)
	Maximal response#	$76.9 \pm 4.6\%$ (4)	$79.9 \pm 3.6\%$ (4)
SIN-1	log IC_{50}	-7.49 ± 0.11 (5)	-7.58 ± 0.08 (5)
	Maximal response#	$105.9 \pm 3.0\%$ (5)	$105.5 \pm 1.1\%$ (5)

#Maximal responses are expressed as % relaxation of phenylephrine-evoked contraction. Maximal response to prostacyclin is the response evoked by 10^{-6} M of the agonist.

Table 2 Effects of L-NAME or indomethacin on endothelium-dependent relaxations in canine pulmonary arteries

		Control	*L-NAME*	*Indomethacin*
Bradykinin	log IC_{50}	-7.99 ± 0.03 (5)	-6.89 ± 0.09 (5)*	-7.60 ± 0.05 (5)*
	Maximal response#	$100.0 \pm 0\%$ (5)	$82.4 \pm 4.0\%$ (5)*	$100.0 \pm 0\%$ (5)
A23187	log IC_{50}	-7.72 ± 0.12 (5)	-6.79 ± 0.16 (5)*	-7.41 ± 0.05 (5)*
	Maximal response#	$100.0 \pm 0\%$ (5)	$61.0 \pm 10.0\%$ (5)*	$96.5 \pm 3.0\%$ (5)
Acetylcholine	log IC_{50}	-7.77 ± 0.10 (5)	-7.05 ± 0.11 (5)*	-7.75 ± 0.10 (5)
	Maximal response#	$100.0 \pm 0\%$ (5)	$72.2 \pm 6.0\%$ (5)*	$100.0 \pm 0\%$ (5)

#Maximal response represents % relaxation of phenylephrine-induced contraction evoked by 10^{-7} M bradykinin, 3×10^{-7} M A23187, or 3×10^{-7} M acetylcholine. *significantly different from control response ($P<0.05$).

The endothelium-dependent relaxations induced by bradykinin, A23187 and acetylcholine were the result of distinct patterns of release of endothelium-derived relaxing factors. At low concentrations, acetylcholine-induced relaxation was markedly reduced by L-NAME, but was not affected by either indomethacin or glibenclamide. This suggests that at low concentrations, acetylcholine-induced relaxation is mediated predominantly by NO, with no effective role for either prostacyclin or a glibenclamide-sensitive EDHF. In contrast, relaxations to bradykinin or A23187 were inhibited by L-NAME, indomethacin or glibenclamide, suggesting that NO, prostacyclin and a glibenclamide-sensitive EDHF all contributed to the response. The involvement of distinct patterns of mediators in the relaxation response to different endothelium-dependent vasodilators probably reflects differences in the signal transduction pathways activated by the different agonists (Luckhoff *et al*., 1988; Parsaee *et al*., 1992; Flavahan & Vanhoutte, 1995).

At low concentrations of bradykinin and A23187, relaxation was abolished by the combination of L-NAME and indomethacin, suggesting that the response was mediated entirely by NO and prostacyclin. However, the inhibitory effects of L-NAME and indomethacin overlapped to a significant extent, with each antagonist virtually abolishing relaxation to threshold concentrations of the agonists. This pattern of inhibition would be consistent with a synergistic interaction between endothelium-derived NO and prostacyclin. It could also result from a lack of selectivity of the respective antagonists. However, at the concentrations used, indomethacin and L-NAME act as selective inhibitors of cyclo-oxygenase and NO synthase in pulmonary arteries (Tseng *et al*., 1993; Flavahan *et al*., 1994). Although some inhibitors of NO synthase and cyclo-oxygenase may directly inhibit K_{ATP}-channel activity at relatively low concentrations (Grover *et al*., 1994; Kontos & Wei, 1996), L-NAME and indomethacin, at the concentrations used in the present study, do not directly

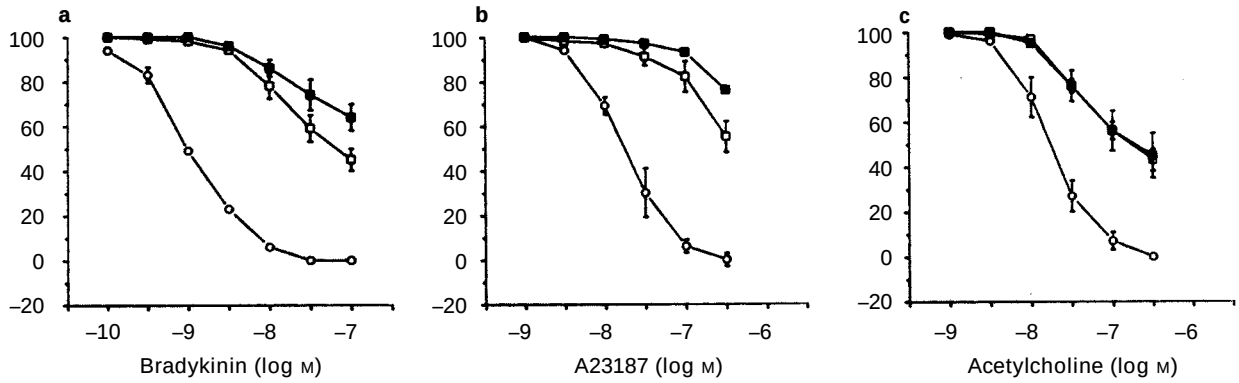

Figure 7 Effect of the K^+_{ATP} channel inhibitor, glibenclamide (10^{-6} M) on the component of relaxation to bradykinin, A23187 or acetylcholine that was resistant to indomethacin (10^{-5} M) + L-NAME (3×10^{-5} M). Relaxations were determined in canine pulmonary arteries contracted to the ED_{50} level of tension with phenylephrine. Responses are expressed as % of phenylephrine contraction and are presented as means ($n=5$); vertical lines show s.e.mean. In the presence of L-NAME and indomethacin, glibenclamide further attenuated ($P<0.05$) the relaxation response to bradykinin (a) or A23187 (b), but not to acetylcholine (c). Symbols: (○) control; (□) Indo + L-NAME; (■) Indo + L-NAME + glibenclamide.

inhibit these channels (Muller *et al*., 1992; Krippeit-Drews *et al*., 1996; McCulloch & Randall, 1996; Xu & Lee, 1996; Liu & Flavahan, 1997). To investigate a possible interaction between endothelium-derived mediators, the action of each mediator was assessed in the absence of a potential interaction with the other mediator, i.e. when the response to the other mediator was blocked. This analysis confirmed that relaxations caused by bradykinin and A23187, but not acetylcholine, were mediated by a synergistic interaction between NO and prostacyclin.

K^+_{ATP} channel inhibition also markedly attenuated relaxations evoked by bradykinin and A23187. In fact, at low concentrations of these agonists, glibenclamide attenuated the relaxation response that appeared to be mediated entirely by NO and prostacyclin. However, glibenclamide did not inhibit endothelium-dependent relaxation to acetylcholine, indicating that the antagonist does not act in a non-specific manner to depress endothelium-dependent relaxation. Previous studies have demonstrated that prostacyclin or NO can activate K^+_{ATP} channels (Jackson *et al*., 1993; Kubo *et al*., 1994; Murphy & Brayden, 1995), although this response may not contribute significantly to vasodilatation (e.g. Parkington *et al*., 1995). In the present study, glibenclamide did not inhibit relaxation evoked by exogenous administration of prostacyclin or of the NO donor, SIN-1. However, when assessed during endothelium-dependent relaxation to bradykinin, glibenclamide reduced the inhibitory effect of L-NAME (in the absence of indomethacin) which might suggest that it is inhibiting NO activity (Figure 6). Glibenclamide also reduced the inhibitory effect of indomethacin (in the absence of L-NAME) which might suggest that it is inhibiting prostacyclin activity (Figure 6). A comparison of Figure 6a and b shows that glibenclamide did not alter the position of the Indo, L-NAME, or Indo + L-NAME curves. The K_{ATP}-channel antagonist only shifted the untreated concentration-effect curve to bradykinin (i.e. Control and Glib curves in Figure 6). Therefore, glibenclamide only exerted an inhibitory effect when NO and prostacyclin were both active, suggesting that glibenclamide may have inhibited the interaction between NO and prostacyclin during bradykinin-induced relaxation. The individual NO- and prostacyclin-mediated responses were, therefore, assessed in the absence of any interaction, i.e. when the action of the other mediator was blocked. Indeed, glibenclamide did not reduce the magnitude of the prostacyclin-mediated component (L-NAME-resistant but indomethacin-sensitive component) or of the NO-mediated component (indomethacin-resistant but L-NAME-sensitive component). However, in the presence of glibenclamide, the total relaxation to bradykinin was no longer greater than the sum of the individual NO and prostacyclin components. Therefore, glibenclamide abolished the synergistic interaction between endothelium-derived NO and prostacyclin during bradykinin-induced relaxation. Because gliben-clamide inhibited this interaction, endothelium-derived prostacyclin was no longer able to amplify the NO-dependent component of the response (or vice-versa) and the inhibitory effect of indomethacin (or L-NAME) when given alone was reduced. These results support the concept that the synergistic interaction between NO and prostacyclin is mediated by activation of K^+_{ATP} channels.

The activation of K^+_{ATP} channels was most likely mediated by a glibenclamide-sensitive EDHF. The presence of K^+_{ATP}-dependent dilator activity was apparent not only at low concentrations of bradykinin and A23187, but throughout the full concentration-effect relationship for these agonists. Although at low concentrations of the endothelial stimuli, the K^+_{ATP}-dependent mechanism was dependent on the activity of NO and prostacyclin, at higher agonist concentrations a distinct K^+_{ATP}-dependent component of relaxation was observed. At high concentrations of bradykinin and A23187, the relaxation remaining after combined administration of L-NAME and indomethacin was further attenuated by glibenclamide, suggesting the presence of a K^+_{ATP}-dependent EDHF. At lower concentrations of these endothelial stimuli, threshold levels of this EDHF may, therefore, interact with NO and prostacyclin to augment vasorelaxation. In contrast, endothelium-dependent relaxation to acetylcholine was not characterized by an interactive effect between NO and prostacyclin, and the response to the agonist that remained after L-NAME + Indo was not mediated by a K^+_{ATP}-dependent EDHF. Therefore, relaxations to acetylcholine in the pulmonary artery were associated with decreased activity of two mediators (K^+_{ATP}-dependent EDHF, prostacyclin) that are crucial for synergy. Because both of these mediators may be derived from phospholipase A_2 activity (Hecker *et al*., 1994), their production may be regulated in a similar manner and be coupled to signalling mechanisms different from those regulating NO production (e.g. Luckhoff *et al*., 1988; Parsaee *et al*., 1992). The component of relaxation remaining after blockade of NO, prostacyclin and the K^+_{ATP}-dependent EDHF could reflect activity of K^+_{Ca}-dependent EDHF (e.g. Venhoutte, 1993) or of other endothelial dilators (e.g. CO, Zakhary *et al*., 1996).

Although synergism between NO and prostacyclin has previously been described in vascular smooth muscle (Shimokawa *et al*., 1988; Maurice *et al*., 1991), the mechanisms responsible for the synergy have not been fully defined. Maurice and co-workers determined the molecular basis of synergy between nitrovasodilators and activators of adenylate cyclase in platelets (Maurice & Haslam, 1990), and extended these studies to vascular smooth muscle (Maurice *et al*., 1991). These and other investigators have suggested that one of the underlying mechanisms of synergy between exogenous NO and

prostacyclin involves the inhibition of cyclicAMP phosphodiesterase (PDE III) by NO-activated cyclicGMP (Maurice & Haslam, 1990; Maurice *et al*., 1991; Lugnier & Komas, 1993; Eckly & Lugnier, 1994). Our data suggest that the synergistic interaction between endothelium-derived NO and prostacyclin may also involve K^+_{ATP} channels. This mechanism could also explain the increased ability of endogenous NO (released during endothelial stimulation) compared to exogenous NO to cause K^+_{ATP}-mediated hyperpolarization of smooth muscle in guinea-pig coronary artery and rabbit basilar artery (Rand & Garland, 1992; Parkington *et al*., 1993; 1995).

ATP-sensitive K^+-channels are regulated by cyclicAMP-dependent kinase with phosphorylation leading to stabilization or activation of the channel (Honore & Lazdunski, 1993; McNicholas *et al*., 1994; Kleppisch & Nelson, 1995; Xu *et al*., 1996). Indeed, cyclicAMP-dependent kinase activity appears to be closely associated with K^+_{ATP} channels, suggesting that they may be co-localized at the plasma membrane (Wang & Giebisch, 1991; Honore & Lazdunski, 1993; McNicholas *et al*., 1994; Coghlan *et al*., 1995). In the smooth muscle of rat aorta, stimulation of the cyclicAMP system did not activate K^+_{ATP} channels directly but potentiated the response to K^+_{ATP} channel openers (Linde & Quast, 1995). A similar mechanism may mediate the synergistic interaction in the present study with endothelium-derived prostacyclin amplifying the vasodilator response to an endogenous K^+_{ATP} channel opener (EDHF). However, the synergistic interaction appeared to require the concurrent activity of NO, prostacyclin and the K^+_{ATP} channel, because inhibition of any one of these signalling systems was sufficient to abolish the interaction. This interdependence would occur if endothelium-derived NO was amplifying the activity of endothelium-derived prostacyclin, for example by cyclicGMP-mediated inhibition of cyclicAMP phosphodiesterase (PDE III) as previously suggested (Maurice *et al*., 1991; Lugnier & Komas, 1993; Eckly & Lugnier, 1994). Because glibenclamide abolished the interaction between endothelium-derived NO and prostacyclin, this interaction appears to be directed selectively at the K^+_{ATP} channel rather than the K^+_{ATP}-independent vasodilator activity of prostacyclin. As with the K^+_{ATP} channel and cyclicAMP-dependent kinase (Wang & Giebisch, 1991; Honore & Lazdunski, 1993; McNicholas *et al*., 1994; Coghlan *et al*., 1995), type III cyclicAMP phosphodiesterase may be localized to the plasma membrane in pulmonary artery (Ivorra *et al*., 1992; Rabe *et al*., 1994). If the components of the signalling pathway (K^+_{ATP} channel, cyclicAMP-dependent kinase, type III cyclicAMP phosphodiesterase) were co-localized, then the modulatory effect of cyclicAMP on the K^+_{ATP} channel may require the concomitant activity of cyclicGMP to inhibit type III phosphodiesterase activity.

Certain endothelial cells express K_{ATP}-channels (Janigro *et al*., 1993; Katnik & Adams, 1995). If present on pulmonary artery endothelium, these channels could potentially contribute to the responses observed in the present study. However, the role of these channels in regulating the production of endothelial mediators has not been fully defined. Indeed, activation of the channels by K_{ATP}-channel openers was insufficient to produce a change in endothelial cell calcium levels (e.g. Katnik & Adams, 1995). However, even if activation of these channels increased endothelial mediator release, this may not be sufficient to induce the synergistic effect observed in the present study. Because the synergistic response was dependent on the concurrent activity of NO, prostacyclin and the K_{ATP}-channel, this interaction would be consistent with endothelial K_{ATP}-channels only if channel activation enabled endothelium-derived NO and prostacyclin to interact synergistically.

In summary, relaxation responses to bradykinin and A23187 in canine isolated pulmonary arteries involve a synergistic interaction between NO and prostacyclin that is mediated by activation of K^+_{ATP}-channels. The activation of K^+_{ATP}-channels may, in turn, be provided by threshold levels of an EDHF. Although acetylcholine also caused endothelium-dependent relaxation, the response was mediated primarily by NO with little apparent contribution from prostacyclin or a K^+_{ATP}-dependent EDHF. Therefore, the pattern of endothelial dilator mediators and the presence of a synergistic endothelial interaction is dependent on the nature of the endothelial stimulus.

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