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Basal nitric oxide release differentially modulates vasodilations by pinacidil and levcromakalim in goat coronary artery

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Abstract

In the current investigation, the role of basal nitric oxide (NO) in modulating the vasorelaxant responses to pinacidil and levcromakalim was examined in goat isolated coronary artery. Pinacidil (10^{-8}–10^{-4} M) elicited concentration-dependent relaxations of the coronary artery ring segments (with intact endothelium) constricted with 30 mM K^+ saline solution. The EC_{50} of the vasodilator was 2.57×10^{-6} M (95% CL, 1.9–3.46×10^{-6} M). The removal of endothelium by mechanical rubbing caused a rightward shift in the concentration–response curve of pinacidil with a corresponding increase in EC_{50} value (1.90×10^{-5} M; 95% CL, 1.12–3.23×10^{-5} M). Similar to endothelium removal, treatment of endothelium-intact rings either with the NO synthesis inhibitor L-NAME (N^G-nitro-L-arginine methyl ester; 3×10^{-5} M) or the guanylate cyclase inhibitor, methylene blue (3×10^{-6} M) resulted in a marked inhibition in the relaxant responses to pinacidil. Hence, the EC_{50} values of the potassium channel opener were significantly higher in tissues treated either with L-NAME (7.41×10^{-6} M; 95% CL, 6.02–9.12×10^{-6} M) or methylene blue (2.29×10^{-5} M; 95% CL, 1.58–3.31×10^{-5} M) as compared to untreated controls. The ATP-sensitive potassium (K_{ATP}) channel blocker glibenclamide, which caused a significant rightward shift in the concentration–relaxation curve of pinacidil in control tissues, was found to be less potent in antagonising the relaxant responses of the K_{ATP} channel opener in endothelium-denuded rings and in rings with intact endothelium but treated with either L-NAME or methylene blue. In contrast to the observations made with pinacidil, the vasodilator responses to another K_{ATP} channel opener, levcromakalim, were potentiated in the absence of basal NO. Thus, the EC_{50} of levcromakalim was 1.33×10^{-8} M (95% CL, 0.8–2.21×10^{-8} M) in control tissues with intact endothelium, which was significantly higher than those obtained in endothelium-denuded rings (4.81×10^{-9} M; 95% CL, 4.04–5.73×10^{-9} M) or endothelium intact rings treated either with L-NAME (2.63×10^{-9} M; 95% CL, 1.58–4.36×10^{-9} M) or methylene blue (2.82×10^{-9} M; 95% CL, 1.7–4.68×10^{-9} M). The selective modulation by basal NO of the arterial relaxations elicited with the K_{ATP} channel openers was evident from the findings that papaverine-induced relaxations were not affected in the absence of basal NO. Taken together, the results of the present study suggest that basal NO differentially modulates the interaction of pinacidil and levcromakalim with the K_{ATP} channels in goat coronary artery through a cGMP-dependent pathway. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide (NO); Pinacidil; Levcromakalim; Vasodilation; K_{ATP} channel; Coronary artery

1. Introduction

It is now becoming increasingly clear that the endothelium plays a major role in regulating the tone of the underlying vascular smooth muscles through the release of a variety of vasoactive factors like, endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF), prostanoids and endothelins (Griffith, 1994). Studies on isolated blood vessels demonstrate that the vascular endothelium also modulates the responses of the smooth muscles to various vasodilator and vasoconstrictor agents (Luscher and Vanhoutte, 1990). Recently, interest has been developing in investigating into the role of endothelium, with particular emphasis on EDRF (nitric oxide) in regulating the vascular ion channels. In view of the critical role of ATP-sensitive potassium (K_{ATP}) channels in regulating the tone and excitability of vascular smooth muscles (Nelson and Quayle, 1995), numerous studies have been conducted to examine the effects of nitric oxide (NO) on these channels. For example, Tare et al. (1990) demonstrated that NO derived from the endothelium caused hyperpolarization and relaxation of guinea pig

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coronary artery through the activation of K_{ATP} channels. In a recent report too, it was shown that NO hyperpolarized the rabbit mesenteric arteries through the opening of K_{ATP} channels, with the accumulation of cGMP as an intermediate step (Murphy and Brayden, 1995).

Potassium channel openers like pinacidil, levcromakalim and diazoxide are a group of drugs which are structurally different and yet all of them are known to activate K_{ATP} channels to produce vasodilation(Edwards and Weston, 1993). Their pharmacological actions can be inhibited by the K_{ATP} channel blocker glibenclamide (Standen et al., 1989). Although, the vasorelaxations by potassium channel openers have been shown to be independent of the vascular endothelium (Taylor et al., 1988), there are reports showing that basal release of NO from endothelial cells can modify the responses of these drugs as a result of its action on the vascular K_{ATP} channels (Randall and Griffith, 1993; Herity et al., 1994). For instance, vasorelaxant responses of cromakalim in rat isolated aorta were inhibited in arterial ring segments deprived of a functional endothelium when compared to those with an intact endothelium (Cavero et al., 1989). On the contrary, inhibition of basal NO release was shown to augment the vasodilator responses to potassium channel openers in rabbit-ear artery (Randall et al., 1994) and in rat isolated superior mesenteric arterial bed (McCulloch and Randall, 1996). Though all these findings are promising with respect to understanding the role of NO in modulating the interaction between the potassium channel openers and the K_{ATP} channels, they are somewhat discordant. Furthermore, there is little information on the influence of endogenous NO on the antagonism of the potassium channel openers—induced vasodilations by glibenclamide, a specific and potent inhibitor of K_{ATP} channels.

The present study was undertaken to investigate the role of endogenous NO release in modifying the K_{ATP} channel functions, using goat isolated coronary artery as a model of vascular smooth muscle. Pinacidil and glibenclamide were used as ligands for the functional assessment of K_{ATP} channels in coronary artery rings endowed with or deprived of endothelium. In addition, NO synthase inhibitor, L-NAME (Rees et al., 1990) and guanylate cyclase inhibitor, methylene blue (Ignarro et al., 1984) were employed to determine the influence of endogenous NO release on the vasodilations by pinacidil in the absence and in the presence of glibenclamide. For comparison, the effects of endothelium removal, L-NAME and methylene blue were investigated on the vasorelaxant responses of the coronary artery smooth muscle to another K_{ATP} channel opener, levcromakalim and a nonspecific vasodilator, papaverine that is known to relax vascular smooth muscles independent of K_{ATP} channel activation.The purpose of choosing goat coronary artery as a model of vascular smooth muscle in the present study was that the K_{ATP} channels were shown to play a major role in the dynamics of coronary blood flow in goats (Dankelman et al., 1994), as in other species of animals (Eckman et al., 1992; Imamura et al., 1992), and basal NO had a vasodilatory tone in the coronary arterial bed of goats (Garcia et al., 1992). In one of the most recent investigations, we have demonstrated that NO donors like 3-morpholinosydnonimine (SIN-1) and sodium nitroprusside relaxed the goat isolated coronary artery independent of K_{ATP} channel activation (Deka et al., 1997). However, very little is known about the role of basal NO release in modulating these channels.

2. Materials and methods

2.1. Isolated tissue experiments

Goat hearts were obtained from a local abattoir within 15–30 min of slaughter and were transported to the laboratory in oxygenated (95% O_2 + 5% CO_2), cold physiological saline solution (PSS) of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 11.9 and glucose 11.1. The anterior descending branch of the left coronary artery was isolated and was cleaned of fat and connective tissues and cut into 4–5 rings of about 3 mm length and 1.5–2 mm outer diameter. The arterial rings thus obtained were individually suspended between two stainless steel angular hooks and mounted in an organ bath containing 20 ml of PSS and continuously bubbled with carbogen (95% O_2 + 5% CO_2) at 37 ± 0.5°C. The tissues were equilibrated under a resting force of 1.5 g for 90 min during which PSS was changed every 15 min. Isometric force was recorded in an ink-writing polygraph via a force transducer (Recorders and Medicare, India). In some rings endothelium was removed by gently rubbing the intimal surface of the coronary artery rings with a fine forceps. This procedure did not damage the smooth muscle of the arterial segments because of the fact that the force of contraction elicited by low K^+ (30 mM) medium of the following composition (mM: NaCl, 92.7; KCl, 30; $CaCl_2 \cdot 2H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2, $NaHCO_3$, 11.9 and glucose 11.1) was 1.13 ± 0.08 g ($n = 13$) and 1.68 ± 0.27 g ($n = 6$) in preparations with or without endothelium, respectively. An absence of functional endothelium was indicated by the failure of α_2-adrenoceptor agonists, clonidine (10^{-7}–3×10^{-6} M) or noradrenaline (10^{-5} M) to produce relaxation of the denuded coronary arterial rings precontracted with 30 mM K^+ PSS.

Following a 90-min equilibration period, coronary artery rings (either endowed with or deprived of functional endothelium) were contracted with K^+ (30 mM). When the contractions reached a steady state (normally 15–20 min after exposure to K^+), cumulative concentration–response curves to the vasodilators were elicited. This concentration of K^+ has been used previously (Deka et al., 1997) to

evoke consistent and reproducible contractions in goat isolated coronary artery and to elicit vasorelaxant responses with the K_{ATP} channel opener, pinacidil. The reversal of K^+ contracture by the vasodilators was expressed as percentage relaxation in terms of maximum vasodilation (100%) produced by papaverine (10^{-4} M). In each ring preparation, only one individual drug (challenge) and its antagonist were used.

2.2. Experimental protocol

2.2.1. Effect of endothelium removal on the responses of the tissues to pinacidil and papaverine

Cumulative concentration–relaxation responses to K_{ATP} channel opener, pinacidil (10^{-8}–10^{-4} M) and papaverine, a vasodilator which acts independently of K_{ATP} channels(10^{-7}–10^{-4} M), were elicited in preparations with or without endothelium and contracted with 30 mM K^+ saline solution. In order to examine the specificity of alterations in the K_{ATP} channel functions on endothelium removal, the antagonism of vasodilator responses of pinacidil by K_{ATP} channel blocker, glibenclamide was studied in coronary artery rings, either deprived of or endowed with endothelium. Here in this case, concentration–response curves to pinacidil were generated by adding the drug to the bath cumulatively at an increment of 0.5 log unit in the presence of glibenclamide (1 or 3×10^{-6} M).

2.2.2. Effect of L-NAME on the vasorelaxant responses of pinacidil and papaverine

In order to reduce the basal NO synthesis, coronary artery rings were pretreated with L-NAME (3×10^{-5} M) for 45 min. Then, in the continued presence of L-NAME, tissues were contracted with K^+ (30 mM) and when the contractions attained a steady state, concentration-dependent relaxations were evoked with the vasodilators by adding them to the bath at a log progression of 0.5 unit.

2.2.3. Effect of methylene blue on the responses of isolated coronary artery rings to pinacidil and papaverine

NO is known to activate guanylyl cyclase and cause accumulation of cGMP in vascular smooth muscles. So, the involvement of cGMP-dependent mechanism in modifying the vasodilator responses to pinacidil (by implication of K_{ATP} channel activation) was examined in tissues pretreated with methylene blue, a known inhibitor of guanylyl cyclase. In this series of experiments, the tissues were pretreated with methylene blue (3×10^{-6} M) for 45 min before they were contracted with K^+ (30 mM) medium. Thereafter, concentration–response curves to pinacidil or papaverine were generated by adding the vasodilators at an increment of 0.5 log unit.

2.2.4. Effect of indimethacin on relaxant responses to pinacidil

Prostacyclin is concomitantly released with EDRF by the vascular endothelium (De Nucci et al., 1988). In order to see the contribution of eicosanoid on the relaxant response to potassium channel openers, the tissues were pretreated with indomethacin (10^{-6} M), a cyclooxygenase inhibitor, for 30 min and concentration–response curve to pinacidil was elicited in the continued presence of indomethacin.

2.2.5. Effect of endothelium removal, L-NAME and methylene blue on the antagonism of pinacidil-induced vasodilations by glibenclamide

These experiments were designed to determine the role of endogenous NO in modifying the sensitivity of the coronary artery smooth muscle to glibenclamide. In K^+ (30 mM)-constricted preparations (with or without endothelium), cumulative concentration– response curves to pinacidil were generated in the presence of glibenclamide (1 or 3×10^{-6} M). To further elucidate the role of NO, similar experiments were conducted in rings with intact endothelium but pretreated for a period of 45 min either with L-NAME (3×10^{-5} M) or methylene blue (3×10^{-6} M).

2.2.6. Effect of NO donor, SIN-1 to reverse the influence of endothelium removal or L-NAME on the responses of the tissues to pinacidil

The ability of 3×10^{-7} M SIN-1 to reverse the effects of endothelium removal or L-NAME on the vasodilator responses to pinacidil in goat isolated coronary artery was examined. In a recent study, we have shown that SIN-1 at this concentration caused about 20–25% relaxation of the goat coronary artery (Deka et al., 1997) through an NO-dependent mechanism. In the present experiments, the tissues were pretreated with SIN-1 (3×10^{-7} M) for 45 min with intermittent washings at 15-min intervals. At each wash SIN-1 was freshly added. Then, the tissue was contracted with K^+ (30 mM) before cumulative concentration-dependent relaxations to pinacidil were elicited in the continued presence of SIN-1.

2.2.7. Effects of endothelium removal, L-NAME and methylene blue on the vasodilator responses of levcromakalim

Besides pinacidil, another K_{ATP} channel opener, levcromakalim, was used in the present study to ascertain the role of basal NO in modulating the vasorelaxant responses to K_{ATP} channel opening drugs specifically. Since 30 mM K^+ saline solution markedly blunted the relaxant responses to levcromakalim in goat coronary artery rings, we used a lower concentration of K^+ (20 mM) as the vasoconstrictor agent in all our studies with levcromakalim. At the plateau phase of K^+ (20 mM) contraction, levcromakalim was added cumulatively to evoke dose-dependent

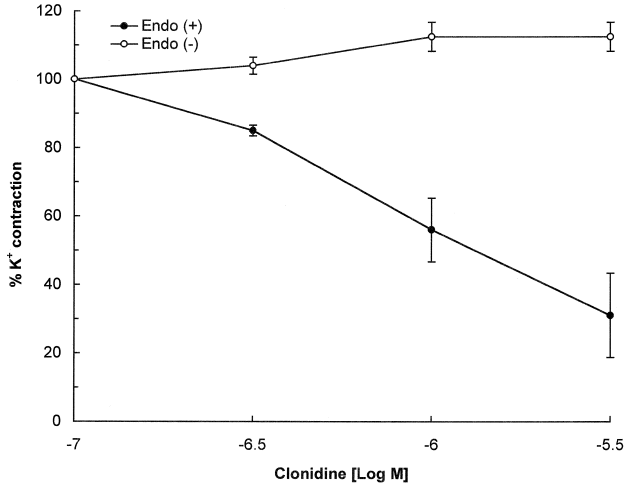

Fig. 1. Effect of endothelium removal by mechanical rubbing on the relaxant responses of clonidine (10^{-7}–3×10^{-6} M) in goat isolated coronary artery rings contracted with 30 mM K^+ saline solution. The vertical bars represent the standard error of the mean ($n = 4$).

relaxations. Some experiments were conducted in the presence of glibenclamide (10^{-6} M) to elucidate the role of K_{ATP} channels in mediating cromakalim-induced relaxations in goat coronary artery.

In order to examine the role of basal NO in modulating the responses of levcromakalim, concentration-dependent relaxations to the K_{ATP} channel opener were elicited in control tissues with intact endothelium and compared with those elicited in endothelium-deprived preparations or in the tissues treated with L-NAME or methylene blue. The detailed procedures have already been described earlier with respect to pinacidil.

2.3. Drugs

Glibenclamide (a gift from Hoechst, Germany) was prepared as stock solution of 10^{-2} M in dimethyl sulfoxide. L-NAME (Sigma) was dissolved in distilled water to get a stock solution of 10^{-3} M. Indomethacin (Sigma) was prepared as a 10^{-2} M stock solution in ethanol. Methylene blue (Sigma) was prepared in distilled water to get a stock solution of 10^{-3} M. A stock solution of 10^{-2} M of papaverine (Ingelheim, Germany) was prepared in distilled water. Pinacidil (a gift from Leo Pharmaceutical Products, Denmark) was prepared as stock solution of 10^{-2} M in 0.1 M HCl. 3-Morpholinosydnonimine was a generous gift from Hoechst, Germany and was freshly prepared as stock solution of 10^{-2} M in PSS every day. Clonidine (Sigma) was prepared as a stock solution of 10^{-2} M in distilled water. Levcromakalim (a gift from Smith Kline and Beecham, UK) was dissolved in dimethylsulfoxide to get a stock solution of 10^{-2} M. A stock solution of 10^{-2} M of noradrenaline (Sigma) was freshly prepared in 0.1 M HCl before the experiment was done. Prazosin (Pfizer) was dissolved in a few drops of dimethylsulfoxide and a stock solution of 10^{-2} M was prepared in distilled water. Propranolol (May & Baker) was dissolved in distilled water to prepare a stock solution of 10^{-2} M.

2.4. Statistics

The results (absolute force and % relaxation) are presented as mean + SEM. Student's *t*-test was used to measure the level of significance. The EC_{50} values of the vasodilators were determined by regression analysis and were expressed as geometric mean with their 95% confidence limits. Analysis of variance (ANOVA) was used to

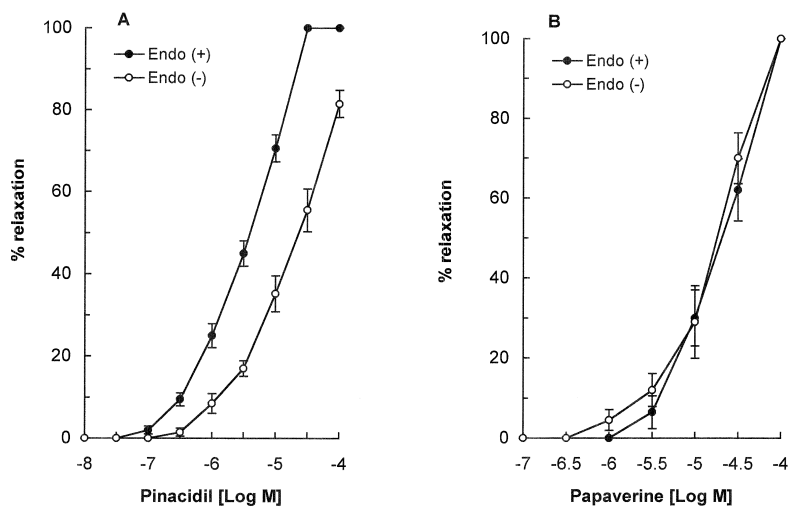

Fig. 2. Effect of endothelium removal on the concentration–relaxation curves of (A) pinacidil (10^{-8}–10^{-4} M) and (B) papaverine (10^{-7}–10^{-4} M) elicited on goat isolated coronary artery rings constricted with 30 mM K^+. The figure legends depicting Endo (+) and Endo (−) refer to the tissues endowed with or denuded of endothelium, respectively. The vertical bars represent standard error of the mean ($n = 6$–13 for pinacidil and 4 for papaverine).

Table 1
Variables for the concentration–response curves (in 30 mM K^+ PSS-induced contraction) for pinacidil in endothelium-intact (controls), endothelium-denuded preparations, in the presence of L-NAME (3×10^{-5} M), MB (3×10^{-6} M), L-NAME (3×10^{-5} M) plus SIN-1(3×10^{-7} M) and in endothelium-denuded tissues treated with SIN-1(3×10^{-7} M)

Variables	EC_{50}	n	R_{max}
Endo (+)	2.57×10^{-6} M (95% CL, 1.9–3.46×10^{-6} M)	13	100 ± 0%
Endo (−)	1.9×10^{-5} M[a] (95% CL,1.12–3.23×10^{-5} M)	6	81.5 ± 3.3%[a]
Endo (+) + L-NAME	7.41×10^{-6} M[a] (95% CL, 6.02–9.12×10^{-6} M)	4	100 ± 0%
Endo (+) + MB	2.29×10^{-5} M[a] (95% CL, 1.58–3.31×10^{-5} M)	4	85 ± 1.5%[a]
Endo (+) + L-NAME + SIN-1	2.16×10^{-5} M[a] (95% CL, 1.61–2.9×10^{-5} M)	4	79.8 ± 1.2%[a]
Endo (−) + SIN-1	2.82×10^{-5} M[a] (95% CL, 2.26–3.51×10^{-5} M)	4	83.8 ± 1.9%[a]

[a] $P < 0.001$ when compared with the control concentration–response curve.

examine the statistical significance of the EC_{50} values (Snedecor and Cochran, 1967).

3. Results

3.1. Examination of endothelial integrity

In endothelial intact coronary artery rings constricted with K^+ (30 mM), clonidine elicited concentration-dependent relaxations (Fig. 1). However, on removal of endothelium by mechanical rubbing, the vasorelaxant responses to clonidine were abolished. Similarly, noradrenaline elicited 20.8 ± 3.0% ($n = 4$) relaxation of the endothelium intact rings constricted with K^+(30 mM) in the presence of prazosin (10^{-6} M) and propranolol (10^{-6} M). Endothelium removal by mechanical rubbing abolished the relaxant responses to noradrenaline ($n = 4$). Pretreatment of endothelium-intact rings with L-NAME also abolished the relaxant responses to noradrenaline ($n = 4$).

3.2. Effect of endothelium removal on the vasodilator responses of pinacidil and papaverine

Fig. 2A depicts the concentration-dependent relaxation curves elicited with pinacidil in coronary artery rings either endowed with or deprived of endothelium. Pinacidil added cumulatively at an increment of 0.5 log unit, caused concentration-related relaxation of the arterial ring segments (with intact endothelium) contracted with 30 mM K^+ saline solution (absolute force: 1.13 ± 0.08 g, $n = 13$) with complete relaxation ($R_{max} = 100\%$) occurring at 3×10^{-5} M of the vasodilator. Removal of endothelium by mechanical rubbing sensitised the tissues to K^+ contraction (absolute force: 1.68 ± 0.27 g, $n = 6$) which was significantly ($P < 0.05$) greater than that obtained in arterial rings with intact endothelium. Further, endothelium removal caused a rightward shift in the concentration–response curve of pinacidil with the maximum relaxation ($R_{max} = 100\%$) occurring at 10^{-4} M of the drug. The EC_{50} of pinacidil in endothelium-intact and endothelium-denuded preparations are presented in Table 1.

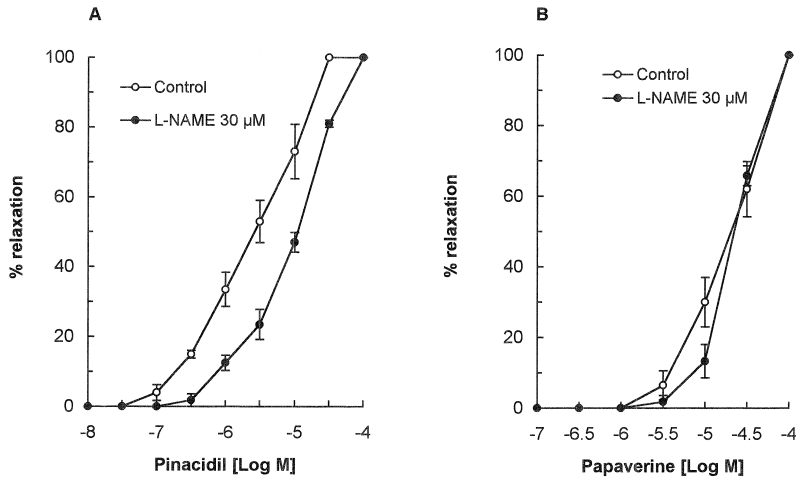

Fig. 3. Effect of pretreatment with 30 µM of L-NAME on the vasodilator responses of (A) pinacidil (10^{-8}–10^{-4} M) and (B) papaverine (10^{-7}–10^{-4} M) in the goat isolated coronary artery rings (endothelium intact) contracted with K^+ (30 mM). The vertical bars represent standard error of the mean ($n = 4$–13 for pinacidil and 4 for papaverine).

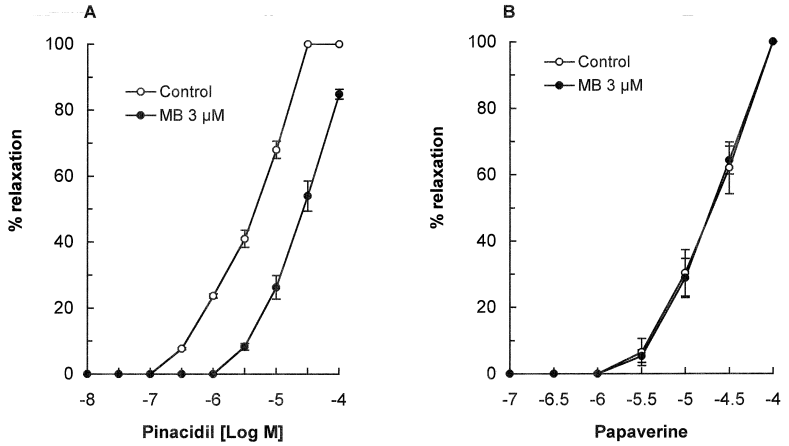

Fig. 4. Effect of methylene blue (3 μM) on the concentration-related relaxations produced by (A) pinacidil (10^{-8}–10^{-4} M) and (B) papaverine (10^{-7}–10^{-4} M) in the goat isolated coronary artery rings (endothelium intact) contracted with K^+ (30 mM). The vertical bars represent standard error of the mean ($n = 4$–13 for pinacidil and 4 for papaverine).

Under the similar experimental conditions as described above, papaverine evoked concentration-related relaxations of the coronary artery rings with or without endothelium (Fig. 2B). In both the groups the R_{max} of 100% was achieved at 10^{-4} M of papaverine. Furthermore, the potency of papaverine to relax the rings denuded of endothelium ($n = 4$) was not significantly different from those with intact endothelium ($n = 4$).

3.3. Effect of L-NAME on the vasodilator responses of pinacidil and papaverine

Concentration–response curves of pinacidil and papaverine in the presence of L-NAME are shown in Fig. 3. Tissues pretreated with L-NAME for 45 min exhibited contractions (mean absolute force: 1.28 ± 0.08 g, $n = 8$) with 30 mM K^+ saline solution which were comparable to controls (mean absolute force: 1.13 ± 0.08 g, $n = 13$). In the presence of L-NAME, the concentration–response curve of pinacidil was shifted to right with no significant change in the amplitude of the maximal relaxation (Fig. 3A). The EC_{50} value of pinacidil (Table 1) in L-NAME-treated tissues ($n = 4$) was, however significantly greater ($P < 0.01$) than that obtained in controls ($n = 4$). L-NAME, however, had no significant effect on the concentration–response curves elicited by papaverine in the coronary artery rings (Fig. 3B). R_{max} of 100% was achieved at 10^{-4} M of papaverine, both in the presence ($n = 4$) and absence of L-NAME ($n = 13$).

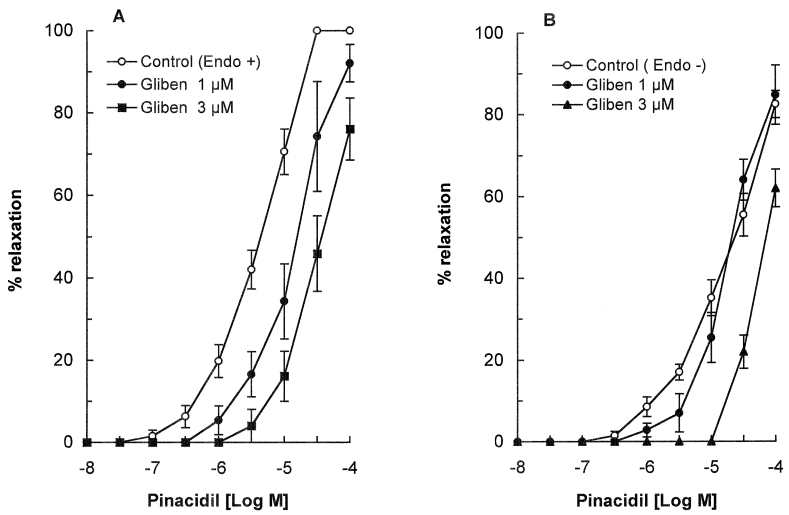

Fig. 5. Effect of glibenclamide (1 and 3 μM) on the relaxant responses of pinacidil (10^{-8}–10^{-4} M) in goat isolated coronary artery rings (A) with intact endothelium or (B) without a functional endothelium. The tissues were contracted with 30 mM K^+ saline solution prior to inducing concentration-dependent relaxations with pinacidil. Note that glibenclamide was less potent in antagonizing the responses to pinacidil in endothelium-denuded preparations. The vertical bars represent standard error of the mean ($n = 4$–13 for intact endothelium and 4–6 for denuded preparations).

Table 2
Variables for the concentration–response curves (in 30 mM K^+ PSS-induced contraction) for pinacidil in endothelium-intact (controls), in the presence of glibenclamide (1 or 3×10^{-6} M), glibenclamide (3×10^{-6} M) plus MB (3×10^{-6} M) and glibenclamide (3×10^{-6} M) plus L-NAME (3×10^{-5} M)

Variables	EC_{50}	n	R_{max}
Endo (+)	2.57×10^{-6} M (95% CL, 1.9–3.46×10^{-6})	13	$100 \pm 0\%$
Endo (+) + Glib (10^{-6} M)	1.44×10^{-5} M[a] (95% CL, 0.52–3.98×10^{-5} M)	4	$92.1 \pm 4.6\%$
Endo (+) + Glib (3×10^{-6} M)	5.04×10^{-5} M[a] (95% CL, 2.47–10.3×10^{-5} M)	4	$70.1 \pm 5.8\%$[a]
Endo (+) + Glib + MB	2.04×10^{-5} M[a] (95% CL, 1.41–2.95×10^{-5} M)	4	$96.5 \pm 3.5\%$
Endo (+) + GLIB + L-NAME	1.38×10^{-5} M[a] (95% CL, 0.95–1.99×10^{-5} M)	4	$100 \pm 0\%$

[a] $= P < 0.001$ when compared with the control concentration–response curves.

3.4. Effect of indomethacin on relaxant responses to pinacidil

Pretreatment of the tissues for 30 min with indomethacin 1×10^{-6} M had no effect on the isometric tension of the endothelium intact arterial rings ($n = 3$) contracted with K^+ (30 mM) PSS. Further, the concentration–response curve of pinacidil was not altered in the presence of indomethacin. Thus, the EC_{50} of pinacidil in the presence and absence of indomethacin were 2.69×10^{-6} M (95% CL:$1.41–5.13 \times 10^{-6}$ M, $n = 3$) and 2.57×10^{-6} M (95% CL: $1.9–3.46 \times 10^{-6}$ M), respectively.

3.5. Effect of methylene blue on the concentration-dependent relaxation elicited by pinacidil and papaverine

In coronary arterial rings with intact endothelium, preexposure to methylene blue caused a rise in basal tone (0.1 ± 0.04 g, $n = 4$) and the contractions evoked by 30 mM K^+ (mean absolute force: 1.38 ± 0.13 g, $n = 4$) were not significantly different from controls (1.13 ± 0.08, $n = 13$). In the presence of methylene blue, the concentration–response curve of pinacidil was shifted to the right with a decrease in the maxima (Fig. 4A). The EC_{50} values of the K^+ channel opener in the presence and absence of methylene blue are shown in Table 1. On the contrary, methylene blue had no significant effect on papaverine-induced concentration–response curves in the coronary arteries (Fig. 4B).

3.6. Effect of endothelium removal, L-NAME and methylene blue on the antagonism of pinacidil responses by glibenclamide

Glibenclamide caused a concentration-dependent rightward shift in the concentration–response curves of pinacidil in coronary artery rings with an intact endothelium (Fig. 5A and Table 2). Following endothelium removal, not only the potency of pinacidil to relax the tissues was reduced but the tissue sensitivity to glibenclamide was also inhibited significantly ($P < 0.001$, $n = 4$) (Table 2; Fig. 5B).

Glibenclamide was significantly ($P < 0.001$, $n = 4$) less potent in antagonising the responses of pinacidil in the tissues (endothelium intact) pretreated with L-NAME for 45 min when compared with the controls. Nevertheless, concentration–response curve to pinacidil was shifted to

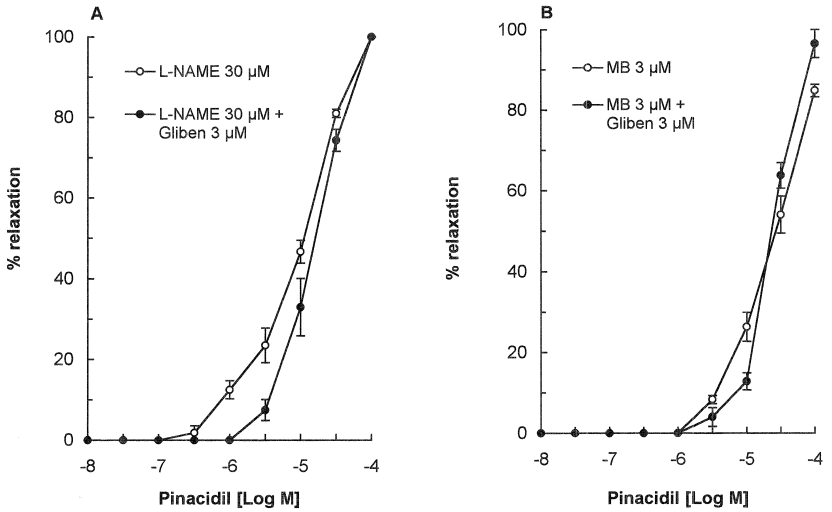

Fig. 6. Effect of glibenclamide (3 μM) on the vasodilator responses of pinacidil (10^{-8}–10^{-4} M) in coronary artery rings (endothelium intact) pretreated with either (A) 30 μM of L-NAME or (B) 3 μM of methylene blue. The tissues were constricted with 30 mM K^+ before eliciting concentration-dependent relaxations with pinacidil. The vertical bars represent standard error of the mean ($n = 4$).

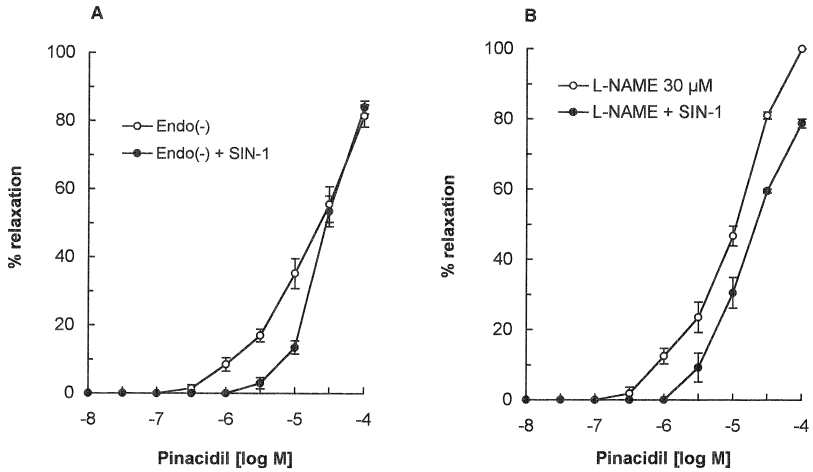

Fig. 7. Effect of nitric oxide donor SIN-1 (3×10^{-7} M) on the reversal of decreased vasorelaxant responses of pinacidil in (A) coronary arteries denuded of endothelium or (B) arterial segments endowed with endothelium but pretreated with the NO synthase inhibitor, L-NAME (30 μM). Note that the exogenous NO donor caused a small rightward shift in the concentration–response curve of pinacidil in both the groups. The vertical bars represent standard error of the mean ($n = 4$–6).

the right in the presence of glibenclamide in L-NAME-treated coronary artery rings (Fig. 6A; Table 2).

As illustrated in Fig. 6B and Table 2, in coronary artery rings (endothelium intact) pretreated with methylene blue, glibenclamide had no significant effect on the concentration-dependent relaxations elicited by pinacidil.

3.7. Effect of NO donor, SIN-1 to reverse the effects of endothelium removal and L-NAME on the vasorelaxant responses of the tissues to pinacidil

The effects of SIN-1 on the concentration–response curves of pinacidil in endothelium-denuded preparations and in endothelium-intact tissues pretreated with L-NAME are shown in Fig. 7. In the presence of SIN-1, the concentration–response curve to pinacidil was not significantly influenced in the coronary artery rings deprived of endothelium (Fig. 7A; Table 1). Pretreatment of endothelium-intact rings with SIN-1 in the continued presence of L-NAME however, inhibited the relaxant responses of pinacidil thereby, causing a rightward shift in the concentration–response curve of the vasodilator (Fig. 7B; Table 1).

3.8. Effect of endothelium removal on the concentration–responses of levcromakalim in goat coronary artery

Fig. 8A illustrates the comparative relaxant responses to levcromakalim of goat coronary artery segment constricted

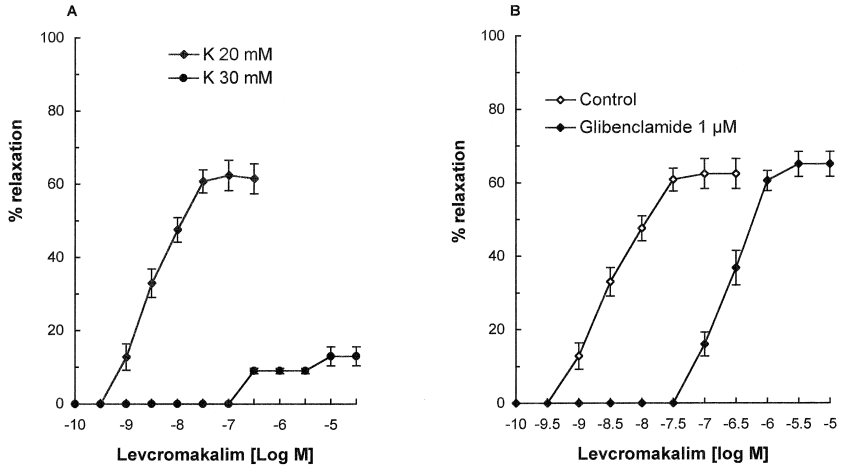

Fig. 8. Characteristics of the vasorelaxant responses of the K_{ATP} channel opener, levcromakalim in goat isolated coronary artery segments with intact endothelium. (A) illustrates the concentration-dependent relaxations evoked by levcromakalim (10^{-10}–$\times 10^{-5}$ M) in tissues contracted either with 20 or 30 mM K^+ saline solution. The vertical bars represent standard error of the mean ($n = 4$–5). (B) Effect of glibenclamide (1 μM) on the concentration-related relaxations elicited by levcromakalim (10^{-10}–10^{-5} M) in arterial rings constricted with K^+ (20 mM) saline solution.

Table 3
Variables for the concentration–response curves (in 20 mM K^+ PSS-induced contraction) for levcromakalim and pinacidil in endothelium-intact (controls), endothelium-denuded tissues, in the presence of glibenclamide (10^{-6} M), L-NAME (3×10^{-5} M) and MB (3×10^{-6} M)

Variables	EC_{50}	n	R_{max}
Levcromakalim			
Endo (+)	1.33×10^{-8} M (95% CL, 0.8–2.21×10^{-8} M)	5	$62.4 \pm 4.1\%$
Endo (−)	4.81×10^{-9} M[a] (95% CL, 4.04–5.73×10^{-9} M)	5	$80.0 \pm 2.9\%$[b]
Endo (+) + Glib	6.16×10^{-7} M[a] (95% CL, 3.71–12.58×10^{-7} M)	4	$65 \pm 3.4\%$
Endo (+) + L-NAME	2.63×10^{-9} M[a] (95% CL, 1.58 − 4.36×10^{-9} M)	5	$82.3 \pm 3.3\%$[b]
Endo (+) + MB	2.82×10^{-9} M[a] (95% CL, 1.7–4.68×10^{-9} M)	4	$79 \pm 2.6\%$[b]
Pinacidil			
Endo (+)	1.55×10^{-7} M (95% CL, 1.15–2.09×10^{-7} M)	3	$100 \pm 0\%$
Endo (−)	4.81×10^{-7} M[a] (95% CL, 2.34–9.33×10^{-7} M)	3	$100 \pm 0\%$
Endo (+) + L-NAME	5.95×10^{-7} M[a] (95% CL, 5.55–6.38×10^{-7} M)	4	$100 \pm 0\%$
Endo (+) + MB	5.24×10^{-7} M[a] (95% CL, 3.38–8.12×10^{-7} M)	4	$100 \pm 0\%$

[a] $P < 0.001$ and [b] $P < 0.01$ when compared with the corresponding control concentration–response curves

with 20 mM and 30 mM K^+ PSS. At K^+ (30 mM), the responses to levcromakalim were blunted. However, levcromakalim, evoked concentration-dependent relaxations of the goat isolated coronary artery (endothelium intact) precontracted with 20 mM K^+ saline solution (mean absolute force: 0.86 ± 0.13 g, $n = 5$). The maximum relaxation (R_{max}, $62.4 \pm 4.1\%$) was achieved at 10^{-7} M of levcromakalim. Glibenclamide caused a parallel rightward shift in the concentration–response curve of levcromakalim with a corresponding increase in EC_{50} value ($P < 0.001$, $n = 5$). However, glibenclamide had no effect on the maximal response of the vasodilator. The results are illustrated in Fig. 8B and Table 3.

Removal of endothelium augmented the vasorelaxant responses to levcromakalim (Fig. 9A) in arterial segments constricted with 20 mM of K^+ (mean absolute force: 1.18 ± 0.06 g, $n = 5$). The EC_{50} values of the potassium channel opener in the presence and absence of endothelium are presented in Table 3. Further, there was a significant ($P < 0.001$, $n = 5$) increase in the maximal relaxation by levcromakalim in endothelium denuded preparations as compared to tissues with intact endothelium. Since the influence of endothelium removal was studied on pinacidil-induced relaxations in 30 mM K^+-contracted tissues, some experiments were done with pinacidil using 20 mM K^+ as a constrictor agent for comparison with the results obtained with levcromakalim under similar conditions. As observed with K^+ (30 mM), the relaxations elicited by pinacidil in 20 mM K^+ saline solution were also inhibited significantly ($P < 0.001$, $n = 3$) following endothelium removal (Fig. 9B; Table 3).

3.9. Effect of L-NAME and methylene blue on the vasorelaxant responses to levcromakalim in coronary arteries with intact endothelium

Fig. 10 illustrates the influence of L-NAME and methylene blue on the vasodilation caused by levcromakalim in tissues endowed with endothelium. Pretreatment with L-NAME had no effect on the basal tone of the tissues. The

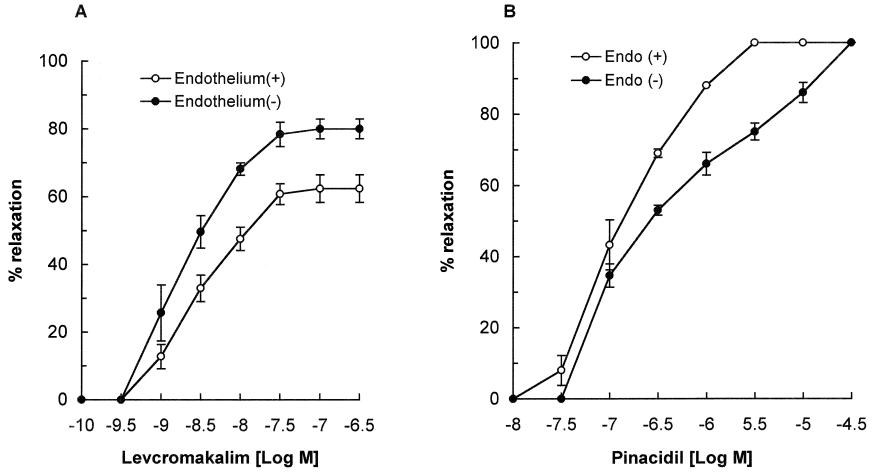

Fig. 9. Effect of endothelium removal on the vasorelaxant responses of (A) levcromakalim (10^{-10}–3×10^{-7} M) and pinacidil (10^{-8}–3×10^{-5} M) in the coronary arterial segments contracted with 20 mM K^+ saline solution. Note that the removal of endothelium potentiated the vasorelaxant responses of levcromakalim but inhibited those of pinacidil. The vertical bars represent standard error of the mean ($n = 3$–5).

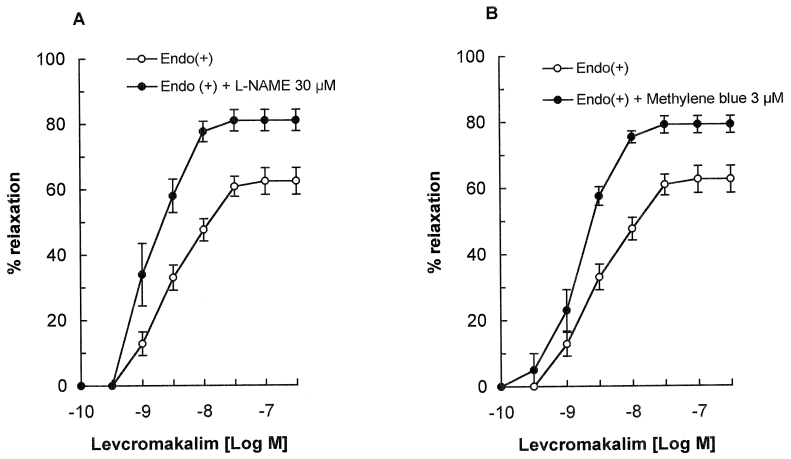

Fig. 10. Effects of (A) 30 μM of L-NAME or (B) 3 μM of methylene blue on the vasodilator responses to levcromakalim (10^{-10}–3×10^{-7} M) in endothelium-endowed coronary artery rings constricted with 20 mM K^+ saline solution. The vertical bars represent standard error of the mean ($n = 4$–5).

contractions caused by 20 mM of K^+ in the presence of L-NAME (1.12 ± 0.15 g, $n = 5$) were comparable to controls (absolute force 0.86 ± 0.13 g, $n = 5$). Fig. 10A and Table 3 show that levcromakalim was more potent in relaxing the arterial rings in the presence of L-NAME as compared to the controls. Further, pretreatment of tissues with L-NAME caused a significant increase in the maximal relaxation to levcromakalim, thereby yielding a significantly higher R_{max} value of $81.0 \pm 3.3\%$ ($n = 5$) as compared to the controls ($R_{max} = 62.4 \pm 4.1$, $n = 5$). Contrary to the results obtained with levcromakalim, pinacidil was less potent in relaxing coronary arterial rings constricted with K^+ (20 mM) (Table 3).

The concentration–response curves of levcromakalim in coronary artery segments (endothelium intact) pretreated with methylene blue are shown in Fig. 10B. The vasorelaxant responses elicited by levcromakalim in 20 mM K^+ constricted arteries were enhanced following pretreatment of the tissues with methylene blue (Fig. 10B, Table 3). Similar to endothelium removal and L-NAME, pretreatment of the coronary arteries with methylene blue increased the maximal relaxation by levcromakalim. On the contrary, the relaxations caused by pinacidil were inhibited following methylene blue treatment of the tissues constricted with K^+ (20 mM) (Table 3).

4. Discussion

The results of the present investigation provide evidence that basal NO differentially modulates the vasodilation caused by the K_{ATP} channel openers pinacidil and levcromakalim in goat isolated coronary artery. The fact that indomethacin had no effect on endothelium-dependent relaxations to pinacidil in goat coronary artery suggests that prostacyclin and other prostanoids do not contribute to the vasodilation caused by the potassium channel openers. The major findings are that: (1) Endothelium removal by mechanical rubbing or inhibition of NO-synthesis by L-NAME attenuated the vasorelaxant responses of pinacidil. (2) The guanylate cyclase inhibitor, methylene blue, also markedly inhibited the vasodilator responses of pinacidil. (3) The K_{ATP} channel blocker, glibenclamide, was less potent in antagonising the coronary artery relaxations elicited by pinacidil in tisssues deprived of functional endothelium and in arterial rings endowed with endothelium but treated with L-NAME or methylene blue. (4) Contrary to the observations with pinacidil, a more specific K_{ATP} channel agonist, levcromakalim, was found to be more potent in relaxing coronary arterial ring segments wherein basal NO was removed. (5) However, the absence of endothelium or treatment of endothelium intact preparations with L-NAME or methylene blue had little effect on arterial relaxations elicited by papaverine, an agent known to relax vascular smooth muscles independent of K_{ATP} channel activation. The pharmacological implications of the above findings and the strength of evidence in support of the role of the basal NO in modulating the vasorelaxant responses of pinacidil and levcromakalim are discussed below.

Since the discovery of the role of EDRF/NO in acetylcholine-induced endothelium dependent vascular relaxations (Furchgott and Zawadzki, 1980), several other vasoactive substances, such as, clonidine and bradykinin, have been reported to release NO from the vascular endothelium. In the present investigation, mechanical rubbing of the intimal surface of the coronary arterial rings abolished the relaxant responses of the goat coronary artery to clonidine and noradrenaline, a finding which is consistent with the ability to cause endothelium-dependent relaxation through alpha-2 adrenoceptor stimulation in other vascular preparations (Pepke-Zaba et al., 1993; Bockman et al., 1996). Further, this procedure determined the selective loss of functional endothelial integrity of the goat coronary artery smooth muscle. Basal NO has been

shown to blunt the vasoconstrictor responses to a number of both exogenous and endogenous vasoactive substances (Umans and Levi, 1995). The present observation that the endothelium denudation enhanced the vasoconstrictor responses to 30 mM K^+ indirectly suggests that basal NO has an inhibitory influence in vitro on the responses of goat coronary artery smooth muscle to vasoconstrictor substances. This is consistent with the reported basal vasodilator tone of endogenous NO on goat coronary artery circulation in vivo (Garcia et al., 1992).

In one of the recent investigations, we have shown that pinacidil relaxes goat isolated coronary artery through the activation of K_{ATP} channels (Deka et al., 1997), a finding that is consistent with the K_{ATP} channel opening action of this vasodilator in several other vascular smooth muscles (Quast et al., 1994). Thus, the inhibition of vasorelaxant responses of pinacidil following removal of endothelium or inhibition of NO synthesis by L-NAME indicates that the interaction between pinacidil and K_{ATP} channels is mediated by endothelium-derived NO in goat isolated coronary artery smooth muscle. These results further suggest that pinacidil-induced vasodilations involving K_{ATP} channels are mediated by NO. Although, there have been reports that NO can activate K_{ATP} channels to cause vasodilation/hyperpolarization of vascular smooth muscles (Miyoshi et al., 1994; Murphy and Brayden, 1995), we found no evidence for the involvement of K_{ATP} channels in the relaxant responses of the nitric oxide donors, such as 3-morpholinosydnonimine and sodium nitroprusside in goat isolated coronary artery (Deka et al., 1997). Therefore, it is possible that NO, instead of directly opening the K_{ATP} channels, is facilitating the interaction between pinacidil and K_{ATP} channels in this smooth muscle through a mechanism which is not clear at present. This is in accordance with some of the previous reports wherein the role of basal NO in modulating the interaction between potassium channel openers and K_{ATP} channels has been suggested (Randall et al., 1994; McCulloch and Randall, 1996). However, the results of the present study in relation to modulation by basal NO of the responses to pinacidil are at variance with the observations made in other vascular smooth muscles. For instance, the inhibition of basal NO had either no effect on pinacidil-induced relaxations in rabbit ear artery (Randall et al., 1994) or it augmented the relaxant responses to the potassium channel opener in rat isolated superior mesenteric arterial bed (McCulloch and Randall, 1996). Differences between vascular smooth muscle preparations may account for the differential modulation of pinacidil-induced relaxations by basal NO.

Further evidence in support of a key role of endogenous NO in mediating K_{ATP} channel activation by pinacidil comes from the observations that the K_{ATP} channel blocker, glibenclamide, was less potent in antagonising the vasorelaxant responses of pinacidil in tissues denuded of endothelium or treated with L-NAME. The reduction in sensitivity to glibenclamide was expected for the reason that an inhibition of basal NO had significantly shifted the concentration–response curves of pinacidil to the right. These results, therefore, suggest that on removal of basal NO, relaxant responses to pinacidil are not primarily mediated by K_{ATP} channel activation; rather, mechanisms other than K_{ATP} channel opening are involved. As reported in the literature, such mechanisms may involve a redistribution of intracellular Ca^{2+} (Erne and Hermsmeyer, 1991) and/or inhibition of intracellular Ca^{2+} refilling by pinacidil (Greenwood and Weston, 1993) in vascular smooth muscles.

Many of the biological effects of NO are mediated through cGMP production as a result of stimulation of guanylate cyclase (Griffith, 1994). Accordingly, in a recent study from our laboratory, we have shown that methylene blue inhibited the relaxant responses of an NO donor 3-morpholinosydnonimine in goat coronary artery. Therefore, we sought to determine the role of cGMP-dependent pathway in the modulation by endogenous NO of the vasodilator responses to pinacidil in this tissue. Consistent with the results of endothelium removal and L-NAME treatment, methylene blue also selectively inhibited the vasorelaxant responses of pinacidil without having any effect on the relaxant responses of papaverine. Using a different experimental protocol, McCulloch and Randall (1996) also demonstrated that cGMP was involved in the modulation of vasodilator responses to pinacidil and levcromakalim by basal NO in isolated rat mesenteric bed.

If NO is critical in mediating pinacidil-induced vasodilation in goat coronary artery, then the effects of endothelium removal or L-NAME could be reversed with exogenous NO released from an NO donor such as 3-morpholinosydnonimine. Surprisingly, 3-morpholinosydnonimine had either no significant effect on the attenuated vasodilator responses of pinacidil in endothelium-denuded preparations or it caused further inhibition in the responses to the potassium channel opener in L-NAME-treated tissues with intact endothelium. The current studies do not provide an answer to the question why exogenous NO released from 3-morpholinosydnonimine could not substitute for endogenous NO. Nevertheless, it is worthwhile to note that in a similar type of study on rat mesenteric artery (McCulloch and Randall, 1996), the spontaneous NO-donor, sodium nitroprusside had no effect on the augmented responses to pinacidil in the presence of L-NAME.

Contrary to the results obtained with pinacidil, the vasodilator responses to another K_{ATP} channel opener, levcromakalim were potentiated in tissues denuded of endothelium or treated with L-NAME. Furthermore, pretreatment of tissues with methylene blue augmented the vasorelaxant responses of levcromakalim in endothelium intact preparations. While these results suggest that the modulation by basal NO of an interaction between levcromakalim and K_{ATP} channels in goat coronary artery in accordance with similar observations made in other blood vessels (Randall et al., 1994; McCulloch and Randall, 1996), they

clearly point to a difference in pharmacology of pinacidil and levcromakalim in relation to modulation of K_{ATP} channels by endogenous NO. Randall et al. (1994) have also shown that removal of basal NO differentially modulated the vasodilator responses of pinacidil and cromakalim in rabbit ear artery. In their studies, removal of endothelium or treatment with L-NAME augmented the vasorelaxant responses of levcromakalim while the responses to pinacidil were not affected under similar conditions. There were several other reports showing differences in the pharmacology of these two potassium channel openers (Cook and Quast, 1990). For example, Lawson et al. (1992) have proposed that pinacidil and levcromakalim can act at different sites on the K_{ATP} channels to mediate vasodilation. Their hypothesis was based on the finding that endothelin discriminated between the pharmacological actions of these two drugs. Radioligand studies further provide evidence that the major potassium channel openers bind to the same target but possibly to the different sites at this target (Quast et al., 1994). Thus, the differences in the comparative pharmacology of pinacidil and cromakalim, noted in the present study, could possibly explain why their vasorelaxant responses were differentially modulated by basal NO. As discussed earlier, a component of the vasorelaxant response of pinacidil is mediated by mechanisms that are independent of K_{ATP} channel activation (rat aorta, Anabuki et al., 1990; rabbit mesenteric artery and rabbit aorta, Meisheri et al., 1991; Deka et al., 1997). This could also account for the differential modulation by basal NO of the relaxant responses to levcromakalim and pinacidil in goat coronary artery.

The data obtained from the present study cannot explain the mechanism of potentiation of vasodilator responses to levcromakalim in the absence of basal NO. Nevertheless, if the endogenous NO has a hyperpolarising influence on the vascular smooth muscles of goat coronary artery, then, removal of basal NO would cause a depolarising shift in the membrane potential with a resultant potentiation of the responses to levcromakalim. As discussed earlier, NO has been reported to activate either K_{ATP} (Tare et al., 1990; Miyoshi et al., 1994; Murphy and Brayden, 1995) or K_{Ca} (Khan et al., 1993; Bolotina et al., 1994) channels in a variety of vascular smooth muscles. Recently, we too observed that the NO donor sodium nitroprusside relaxed goat coronary artery through the activation of K_{Ca} channels (unpublished observation).

In conclusion, the results of the present study demonstrate that endogenous NO, derived from vascular endothelium, can differentially modulate the vasodilator responses to the K_{ATP} channel openers pinacidil and levcromakalim in goat coronary artery. The involvement of a cGMP-dependent mechanism in the modulatory action of NO is suggested as the vasorelaxant responses to the potassium channel openers were affected by methylene blue, an inhibitor of guanylate cyclase. The observations of the present study, that the vasorelaxant responses to pinacidil were attenuated and those of levcromakalim were potentiated in the absence of NO, are of clinical significance, particularly with respect to the diseased conditions such as hypertension and atherosclerosis which are associated with endothelial dysfunction.

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