British Journal of Pharmacology (1996) 117, 1035–1040

Evidence for mediation by endothelium-derived hyperpolarizing factor of relaxation to bradykinin in the bovine isolated coronary artery independently of voltage-operated Ca^{2+} channels

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1 The role of endothelium-derived hyperpolarizing factor and voltage-operated Ca^{2+} channels in mediating endothelium-dependent, N^G-nitro-L-arginine (L-NOARG; 100 μM) -resistant relaxations to bradykinin (BK), was examined in isolated rings of endothelium-intact bovine left anterior descending coronary artery.

2 Rings of artery were contracted isometrically to approximately 40% or their respective maximum contraction to 125 mM KCl Krebs solution ($KPSS_{max}$) with the thromboxane A_2-mimetic, U46619. Relaxations to BK and the endothelium-independent NO donor, S-nitroso-N-acetylpenicillamine (SNAP), were normalized as percentages of reversal of the initial contraction to U46619. All experiments were carried out in the presence of indomethacin (3 μM).

3 BK caused concentration-dependent relaxations [sensitivity (pEC_{50}) 9.88 ± 0.05; maximum relaxation (R_{max}), 103.3 ± 0.5%] in U46619-contracted rings of bovine coronary artery. L-NOARG (100 μM) caused a significant ($P<0.01$) 3 fold reduction in the sensitivity to BK (pEC_{50}, 9.27 ± 0.11) without affecting the R_{max} (101.8 ± 2.3%). A similar, significant 3 fold reduction in sensitivity to BK with no change in R_{max} was observed after treatment with oxyhaemoglobin (20 μM; pEC_{50}, 9.18 ± 0.13, $P<0.001$) or a combination of oxyhaemoglobin (20 μM) and L-NOARG (100 μM; pEC_{50}, 9.08 ± 0.10, $P<0.001$). Oxyhaemoglobin (20 μM) either alone or in combination with L-NOARG (100 μM) caused an approximate 600 fold decrease in the sensitivity to SNAP.

4 The L-type voltage-operated Ca^{2+} channel inhibitor, nifedipine (0.3 μM – 3 μM), reduced the maximum contraction (F_{max}) to isotonic 68 mM KCl Krebs solution (103.5 ± 2.0% $KPSS_{max}$) by 85–90% ($P<0.001$); yet, the highest concentration of nifedipine (3 μM) caused only a small but significant reduction in both the sensitivity and F_{max} to U46619. By contrast, nifedipine (3 μM) had no effect on the relaxation response to BK. Furthermore, a combination of nifedipine (3 μM) and L-NOARG (100 μM) had no further inhibitory effects on relaxations to BK (pEC_{50}, 8.79 ± 0.10; R_{max}, 101.7 ± 2.4%) than did L-NOARG (100 μM) alone (pEC_{50}, 9.05 ± 0.12; R_{max}, 99.62 ± 1.19). Also, nifedipine (0.3 μM and 3 μM) had no effect on the maximum relaxation to the K^+ channel opener, levcromakalim (0.3 μM).

5 In the presence of nifedipine (0.3 μM to control contractions induced by high KCl) and isotonic 68 mM KCl Krebs solution (to inhibit K^+ channel activity), relaxations to BK (pEC_{50}, 9.42 ± 0.10; R_{max}, 93.9 ± 1.8%) were similar to those observed in normal Krebs solution (pEC_{50}, 9.58 ± 0.09; R_{max}, 98.4 ± 0.8%). However, in the presence of 68 mM KCl Krebs solution the inhibitory effect of L-NOARG (100 μM) on relaxations to BK (pEC_{50}, 8.53 ± 0.20; R_{max}, 31.0 ± 11.3%) was markedly greater than that in normal KCl Krebs solution (pEC_{50}, 9.12 ± 0.08; R_{max}, 91.5 ± 2.0%). Similar treatment with 68 mM KCl Krebs had no effect on relaxations to the NO donor, SNAP, yet abolished the response to the K^+ channel opener, levcromakalim (0.3 μM).

6 In summary, this study has shown that (1) NO synthesis in response to BK in bovine coronary artery endothelial cells *in situ* is likely to be abolished by L-NOARG, (2) NO-independent relaxations to BK are markedly attenuated by 68 mM KCl-containing Krebs, which, in the absence of L-NOARG, had no effect, (3) nifedipine blocked contractions to a maximum-depolarizing stimulus (KCl) yet had no effect on NO-independent relaxations to BK, and (4) maximum relaxations to levcromakalim were abolished by 68 mM KCl Krebs but were not affected by nifedipine. Therefore, we hypothesize that if smooth muscle hyperpolarization is involved in non-NO-, endothelium-dependent relaxation in bovine coronary arteries contracted with U46619, then it can accomplish this via a mechanism which does not involve closure of voltage-operated Ca^{2+} channels.

Keywords: Bradykinin; endothelium-derived hyperpolarizing factor; L-type voltage-operated Ca^{2+} channels; nifedipine; N^G-nitro-L-arginine-resistant relaxations

Introduction

In addition to the release of prostacyclin (PGI_2) and nitric oxide (NO), the endothelium can initiate relaxation of vascular smooth muscle via the release of a third factor termed endothelium-derived hyperpolarizing factor (EDHF; see Taylor & Weston, 1988; Komori & Vanhoutte, 1990; Beny & von der Weid, 1991; Garland *et al.*, 1995). Whilst most investigations have been directed towards determining the types of K^+ channels activated by EDHF (see Garland *et al.*, 1995), few studies have examined the mechanism by which the resultant hyperpolarization mediates smooth muscle relaxation. Rather, it is generally accepted that endothelium-dependent hyperpolarization results in closure of voltage-operated Ca^{2+} channels

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and thus relaxation (Beny & von der Weid, 1991; Garland *et al.*, 1995; Godfraind & Govoni, 1995). EDHF-mediated relaxations, however, are observed in arteries contracted with agonists such as thromboxane-mimetics like U46619 (Holzmann *et al.*, 1994; Kilpatrick & Cocks, 1994), which are believed to raise intracellular Ca^{2+} via a means which is largely independent of voltage-operated Ca^{2+} channels (Loutzenhiser & van Breemen, 1981; Angus & Brazenor, 1983). Therefore, the aim of the present study was to examine the effects of the L-type voltage-operated Ca^{2+} channel inhibitor, nifedipine, on non-NO-, endothelium-dependent relaxation responses to bradykinin (BK) in bovine isolated coronary arteries (see Drummond & Cocks, 1995). Our results suggest that if EDHF accounts for most of the non-NO-mediated relaxation to BK in bovine coronary arteries contracted with U46619 in the presence of nifedipine, then it probably does so via a mechanism which does not involve inhibition of voltage-dependent Ca^{2+} influx.

Methods

Preparation of the assay tissue

Sections of bovine myocardium containing the left anterior descending coronary artery were obtained from a local abattoir and immediately placed in cold (4°C) Krebs solution (composition in mM; Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2, glucose 11.0 and ethylenediaminetetraacetic acid 0.1). The coronary artery was then isolated, cleared of connective tissue and cut into ring segments 3 mm in length. Each artery ring was then suspended between two stainless steel wire hooks, one of which was connected to a force displacement transducer (model FT03C, Grass, Quincy, MA, U.S.A.) and the other to a micrometer-adjustable support leg. Preparations were subsequently immersed in water-jacketed 30 ml organ baths containing carbogenated (95% O_2, 5% CO_2) Krebs solution (pH 7.4) maintained at 37°C. Changes in isometric, circumferential force were amplified and displayed on dual-channel, flat-bed recorders (W & W Scientific Instruments, Basel, Switzerland).

After a 25 min equilibration period, rings were stretched to 5 g passive force and allowed to equilibrate for 25 min, after which time they were again stretched to 5 g. After a further 25 min, rings of artery were contracted with an isotonic, high potassium physiological salt solution (KPSS) in which all of the NaCl of normal Krebs was replaced with KCl (composition of KPSS in mM; K^+ 124.9, Na^+ 25.0, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2, glucose 6.1 and ethylenediaminetetraacetic acid 0.1). When the tissue contracted to a steady plateau the bathing solution was changed back to normal Krebs solution and the tissues allowed to return to resting levels of passive force. All tissues were then treated with the cyclo-oxygenase inhibitor, indomethacin (3 μM) for the remainder of the experiment.

Effects of N^G-nitro-L-arginine (L-NOARG) and oxyhaemoglobin

Rings of artery were either left untreated, or were treated with L-NOARG (100 μM), oxyhaemoglobin (10 μM) or a combination of L-NOARG (100 μM) and oxyhaemoglobin (10 μM), 20 min after the addition of indomethacin. After a further 20 min, all tissues were contracted to aproximately 40% of their respective maximum contraction to KPSS ($KPSS_{max}$) with titrated concentrations of the thromboxane A_2-mimetic, U46619 (1–30 nM). Once a stable level of active force was reached, tissues that had been treated with oxyhaemoglobin (10 μM; either alone or in combination with L-NOARG), were treated with a further concentration of oxyhaemoglobin (10 μM) to compensate for any denaturing of the protein that may have occured during the precontraction process. All tissues were then exposed to cumulatively increasing half log molar concentrations of either BK or S-nitroso-N-acetylpenicillamine (SNAP).

Effect of nifedipine on KCl contractions

In studies designed to determine the concentration of nifedipine required for maximum inhibition of L-type Ca^{2+} channel activity, tissues were either left untreated, or were treated with nifedipine at concentrations of 0.1 μM, 0.3 μM, 1 μM or 3 μM, 20 min after the addition of indomethacin. In all preparations in which nifedipine was used, tissues were light-protected by wrapping the organ bath with black plastic. Tissues were left for a further 20 min before the organ bath contents were progressively replaced with Krebs solution containing increasing (isotonic) concentrations of KCl (i.e. 14 mM, 21 mM, 37 mM and 68 mM) together with indomethacin (3 μM) and the appropriate concentration of nifedipine, to construct cumulative concentration-contraction curves to KCl.

Effect of nifedipine on responses to U46619, BK and levcromakalim

Tissues were either untreated or treated with nifedipine (0.3 μM or 3 μM) 20 min after the addition of indomethacin (3 μM). After 20 min some of these tissues were exposed to L-NOARG (100 μM) and left to incubate for a further 20 min. Tissues were then contracted with U46619, either by its addition in cumulative half log molar concentrations to generate concentration-contraction curves, or in titrated concentrations until tissues reached a level of active force that corresponded to approximately 40% of their respective $KPSS_{max}$. Upon reaching a steady plateau, rings of artery contracted to 40% $KPSS_{max}$ were treated with either cumulative half log molar additions of BK or a single concentration of levcromakalim (0.3 μM).

Effect of high KCl on responses to BK and SNAP

In this group of experiments, all tissues were treated with nifedipine (0.3 μM) 20 min after the addition of indomethacin (3 μM). Tissues were then either left untreated or were treated with L-NOARG (100 μM), 68 mM KCl Krebs or 68 mM KCl Krebs containing L-NOARG (100 μM). After a further 20 min, tissues were contracted to approximately 40% of their respective $KPSS_{max}$ with U46619, and upon reaching a stable plateau were relaxed with cumulative half log molar additions of either BK or SNAP or by a single concentration of levcromakalim (0.3 μM).

Statistics

All cumulative concentration-relaxation curves were normalized as percentages of relaxation from the initial U46619-induced precontraction level. Concentration-dependent contractions as well as U46619-induced levels of precontraction force were expressed as percentages of the respective $KPSS_{max}$ for that tissue. Each normalized curve was then computer-fitted (Graphpad Prism, version 1.00) with a sigmoidal regression curve of the following equation,

$$Y=BOTTOM+(TOP-BOTTOM)/1+10^{(pD_2-X).Hill\ slope}$$

where X is the logarithm of the agonist concentration and Y is the response. BOTTOM is the lower response plateau, TOP is the upper response plateau and pD_2 is the X value when the response is halfway between BOTTOM and TOP. The variable Hill slope controls the slope of the curve. Mean pEC_{50} values, maximum relaxations (R_{max}) and maximum contractions (F_{max}) and their standard errors were then calculated for each response curve. Values of n represent number of rings of artery, each from different animals. Differences in mean pEC_{50}, R_{max} and F_{max} values were tested for significance by means of

one way analysis of variance (ANOVA) with multiple comparisons via Tukey Kramer's modified t statistic. All differences were accepted as significant at the $P<0.05$ level.

Drugs and their sources

U46619 (1,5,5-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid; Upjohn, Kalamazoo, U.S.A.), bovine haemoglobin, bradykinin triacetate, indomethacin, N^G-nitro-L-arginine (Sigma, MO, U.S.A.), nifedipine, S-nitroso-N-acetylpenicilamine (Sapphire Bioscience, N.S.W., Australia) and levcromakalim (kind gift from Dr Grant McPherson).

Stock solutions of nifedipine (10 mM) and U46619 (1 mM) were made up in absolute ethanol, while those of indomethacin (100 mM) and L-NOARG (100 mM) were made up in Na_2CO_3 (1 M) and $NaHCO_3$ (1 M), respectively. Stock solutions of levcromakalim (10 mM) were made up in dimethyl sulphoxide. Haemoglobin was dissolved in 0.9% NaCl to make up a 1 mM stock solution. The stock solution was subsequently reduced to oxyhaemoglobin by the addition of a small amount (i.e. less than 0.1 g) of sodium dithionite. Any excess sodium dithionite was removed by running the solution through a sephadex (PD-10) column equilibrated with 0.9% NaCl. All subsequent dilutions of these drug stocks were in distilled water. All other drugs were made up in distilled water.

Results

Effect of L-NOARG and oxyhaemoglobin

BK caused concentration-dependent relaxations (pEC_{50}, 9.88 ± 0.05; R_{max}, $103.3\pm0.5\%$; $n=5$) in endothelium-intact rings of bovine coronary artery precontracted to approximately 40% $KPSS_{max}$ with the thromboxane A_2-mimetic, U46619 (Figure 1a). L-NOARG (100 μM) caused a significant ($P<0.01$) 3 fold reduction in the sensitivity of relaxations to BK (pEC_{50}, 9.27 ± 0.11; $n=5$) without affecting R_{max} (Figure 1a). A similar 3 fold reduction in sensitivity with no effect on R_{max} to BK was observed following treatment with oxyhaemoglobin (20 μM) (pEC_{50}, 9.18 ± 0.13; $n=5$; $P<0.001$) or a combination of oxyhaemoglobin (20 μM) and L-NOARG (100 μM) (pEC_{50}, 9.08 ± 0.10; $n=5$; $P<0.001$) (Figure 1a). The NO-donor, SNAP, also caused concentration-dependent relaxations (pEC_{50}, 8.60 ± 0.15; R_{max}, $100.7\pm0.6\%$; $n=5$) of precontracted rings of artery, however, these were unaffected by L-NOARG (100 μM; $n=3$) (Figure 1b). By contrast, oxyhaemoglobin (20 μM) decreased the sensitivity of SNAP approximately 600 fold without affecting the R_{max} both when used alone (pEC_{50}, 5.83 ± 0.09; $n=4$; $P<0.001$) or in combination with L-NOARG (pEC_{50}, 5.73 ± 0.06; $n=4$; $P<001$) (Figure 1b).

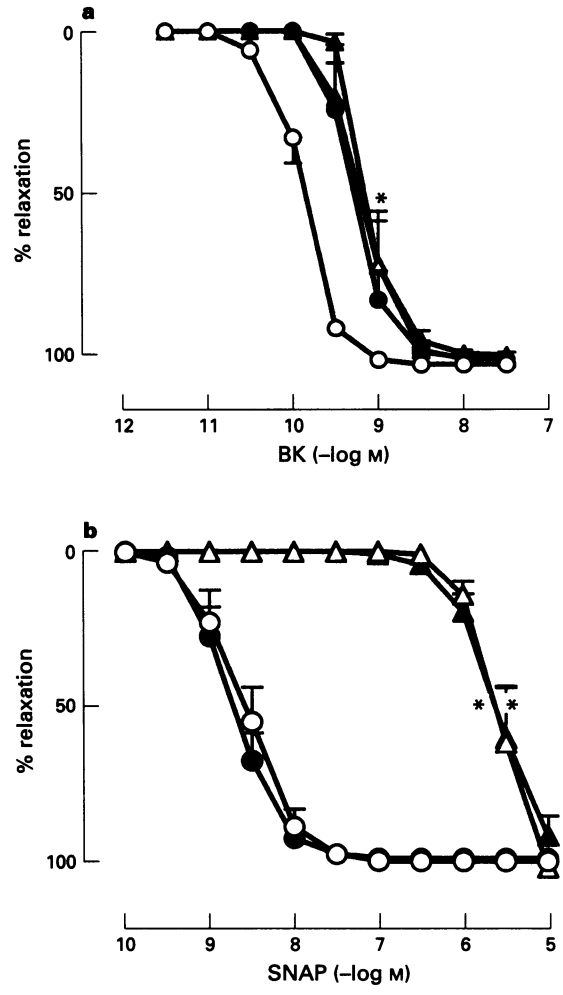

Figure 1 Effects of (a) bradykinin and (b) SNAP in rings of bovine epicardial coronary artery precontracted to approximately 40% of their respective $\%KPSS_{max}$: (○) control responses, (●), (△) and (▲) represent responses in the presence of L-NOARG (100 μM), oxyhaemoglobin (20 μM) and a combination of L-NOARG (100 M) and oxyhaemoglobin (20 μM), respectively. Initial levels of contraction to U46619 (expressed as $\%KPSS_{max}$) were (○) 37.2 ± 1.9, (●) 42.6 ± 1.2, (△) 43.6 ± 3.7 and (▲) 56.0 ± 4.1 in (a) and (○) 35.4 ± 1.4, (●) 36.9 ± 0.7, (△) 39.3 ± 5.8 and (●) 42.3 ± 4.5 in (b). Values [mean±s.e. mean from (a) 5 and (b) 3 to 5 experiments] are expressed as a percentage reversal of the initial U46619-induced force. *Indicates pEC_{50} values significantly different from controls ($P<0.05$, Tukey Kramer's modified t statistic after one-way ANOVA). Note, in (a) there was no significant differences between the pEC_{50} values for any of the treatment groups.

Effect of nifedipine

KCl Krebs solution (14 mM–68 mM) caused concentration-dependent contractions (F_{max}, $103.5\pm2.0\%$ $KPSS_{max}$; $n=4$) (Figure 2a). Nifedipine (0.1 μM) significantly reduced the F_{max} to 68 mM KCl Krebs solution by approximately 75% ($n=4$; $P<0.001$) compared to the control response (Figure 2a). The maximum inhibitory effect of nifedipine on KCl contractions (F_{max}, $13.0\pm1.6\%$) was observed at a concentration of 0.3 μM ($n=4$; $P<0.001$) (Figure 2a). No further significant reduction in F_{max} to KCl was observed at higher concentrations of nifedipine (1 μM, F_{max}, $15.8\pm2.8\%$ $KPSS_{max}$, $n=4$ and 3 μM, F_{max}, $9.9\pm1.2\%$ $KPSS_{max}$, $n=3$) (Figure 2a).

Concentration-dependent, tonic contractions to U46619 (pEC_{50}, 7.96 ± 0.07; F_{max}, $95.8\pm5.0\%$; $n=6$) (Figure 2b) were unaffected by nifedipine (0.3 μM) (pEC_{50}, 7.86 ± 0.10; F_{max}, $84.2\pm4.1\%$; $n=6$). A 10 fold higher concentration of nifedipine (3 μM) caused a small but significant decrease in both the sensitivity (pEC_{50}, 7.66 ± 0.02; $n=6$; $P<0.05$) and maximum contraction (F_{max}, $78.0\pm4.3\%$; $n=6$; $P<0.05$) to U46619 (Figure 2b). This high concentration of nifedipine (3 μM), however, had no effect on either the pEC_{50} (9.61 ± 0.13; $n=7$) or R_{max} ($101.0\pm0.5\%$; $n=7$) to BK in U46619-precontracted tissues (Figure 3). Furthermore, a combination of nifedipine (3 μM) and L-NOARG (100 μM) had no further inhibitory effect on relaxations to BK (pEC_{50}, 8.79 ± 0.10, R_{max}, $101.7\pm2.4\%$; $n=6$) than did treatment with L-NOARG (100 μM) alone (pEC_{50}, 9.05 ± 0.12; R_{max}, $99.6\pm1.2\%$; $n=6$) (Figure 3).

Effect of KCl Krebs solution

In the following group of experiments, all rings of artery were treated with nifedipine (0.3 μM) for at least 20 min to control smooth muscle contractions to 68 mM KCl and thus avoid any complications arising from functional antagonism. This concentration of nifedipine was chosen as it was shown to be the minimum concentration required for maximum inhibition of

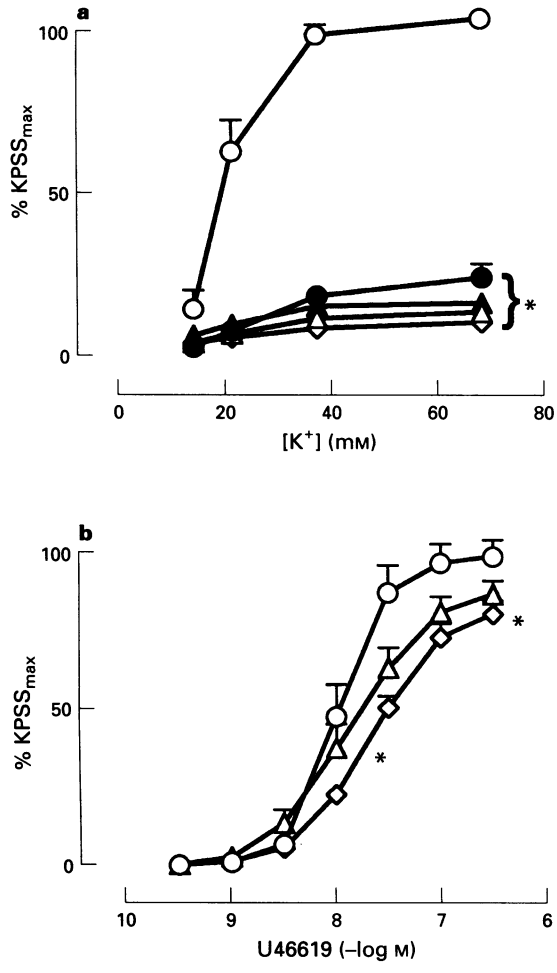

Figure 2 Effect of 0.1 (●), 0.3 (△), 1 (▲) and 3 μM (◇) nifedipine on contractions to increasing concentrations of (a) isotonic KCl Krebs solution and (b) cumulative half log molar additions of U46619 in rings of bovine epicardial coronary artery: (○) control responses. Values (mean±s.e. mean from 4 to 5 experiments) are expressed as percentages of the respective $KPSS_{max}$ for each tissue. *Indicates F_{max} or pEC_{50} values significantly different from controls ($P<0.05$, Tukey Kramer's modified t statistic after one-way ANOVA).

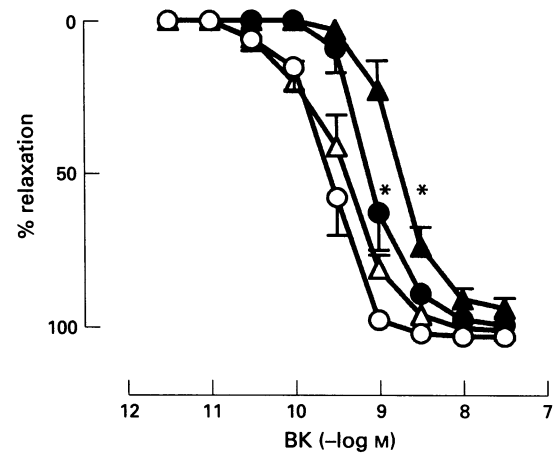

Figure 3 Relaxation responses to BK in rings of artery treated with L-NOARG (100 μM; ●), nifedipine (3 μM; △) or a combination of L-NOARG (100 μM) and nifedipine (3 μM; ▲); (○) control responses. Initial levels of contraction to U46619 (expressed as % $KPSS_{max}$) were (○) 38.8±3.5, (●) 42.7±1.9, (△) 33.8±1.3 and (▲) 35.2±1.1. Values (mean±s.e. mean from 6 experiments) are expressed as a percentage reversal of the initial precontraction level. *Indicates pEC_{50} values significantly different from controls ($P<0.05$, Tukey Kramer's modified t statistic after one-way ANOVA). Note, there was no significant difference between the response to BK in the presence of L-NOARG alone and in the presence of a combination of L-NOARG and nifedipine.

KCl-induced contractions (from Figure 2a above). The response to BK in 68 mM KCl Krebs solution (pEC_{50}, 9.42±0.10; R_{max}, 93.9±1.8% $n=5$) was not significantly different from that in normal Krebs (pEC_{50}, 9.58±0.09; R_{max}, 98.4±0.8%; $n=5$) (Figure 4a). In the presence of high KCl Krebs solution, however, L-NOARG (100 μM) markedly reduced the sensitivity (pEC_{50}, 8.53±0.20; $n=5$; $P<0.05$) and R_{max} (31.0±11.3%; $n=5$; $P<0.001$) of relaxations to BK to values that were significantly less than those observed following L-NOARG (100 μM) treatment in the presence of normal Krebs solution (pEC_{50}, 9.12±0.08; R_{max}, 91.5±2.0%; $n=5$). None of these treatments had any effect on relaxations induced by SNAP ($n=3$ to 4) (Figure 4b).

Effect of nifedipine and KCl Krebs solution on response to levcromakalim

Nifedipine (0.3 μM and 3 μM) had no effect on the maximum relaxation to levcromakalim (0.3 μM) (97.3±3.1%; $n=6$; Figure 5). However, in rings incubated with nifedipine (0.3 μM) and 68 mM KCl Krebs solution, levcromakalim (0.3 μM) failed to elicit any response ($n=6$) (Figure 5).

Discussion

This study provides strong evidence that BK causes bovine coronary artery endothelial cells to release not only NO, but a second factor, EDHF, which can initiate vascular smooth muscle relaxation via a mechanism which is independent of the closure of voltage-operated Ca^{2+} channels. Thus, in the presence of L-NOARG to inhibit NO synthesis and nifedipine to inhibit vascular L-type Ca^{2+} channel activity, BK still elicited maximum relaxations in artery rings contracted with U46619.

It has previously been shown that relaxations to BK in the bovine coronary artery are endothelium-dependent and partially inhibited by L-NOARG (Holzmann *et al.*, 1994; Drummond & Cocks, 1995). In the present study, we confirmed the finding that L-NOARG only partially blocked the response to BK. L-Arginine analogues such as L-NOARG are potent inhibitors of NO synthase (Moore *et al.*, 1990). Their ability to abolish NO production by the endothelium in *in situ* vascular preparations where only smooth muscle relaxation is measured must, however, be independently established, due to possible tissue specializations in their transport, metabolism and potency. Thus, in the present study the effects of oxyhaemoglobin, a potent NO scavenger (Martin *et al.*, 1986), were examined against the relaxation response remaining to BK after treatment with L-NOARG. Oxyhaemoglobin alone blocked the response to BK to a similar degree to L-NOARG and more importantly had no further inhibitory effect on the L-NOARG-resistant response to BK. The same concentration of oxyhaemoglobin caused an approximate 600 fold decrease in sensitivity to the NO donor, SNAP. It can therefore be concluded from these experiments that L-NOARG abolished NOS activity and that the L-NOARG-resistant response to BK was not mediated by NO formed by any other pathway. Furthermore, the L-NOARG-resistant response to BK was not mediated by prostanoids since all experiments were carried out in the presence of the cyclo-oxygenase inhibitor, indomethacin. The L-NOARG-resistant response was, however, significantly blocked by approximately 70% by high extracellular KCl, a treatment both known to inhibit K^+ channel activity (Chen & Suzuki, 1989) and used widely to inhibit the effects of EDHF (Nagao & Vanhoutte, 1992; Adeagbo & Triggle, 1993; Cowan *et al.*, 1993; Kilpatrick & Cocks, 1994; Waldron & Garland,

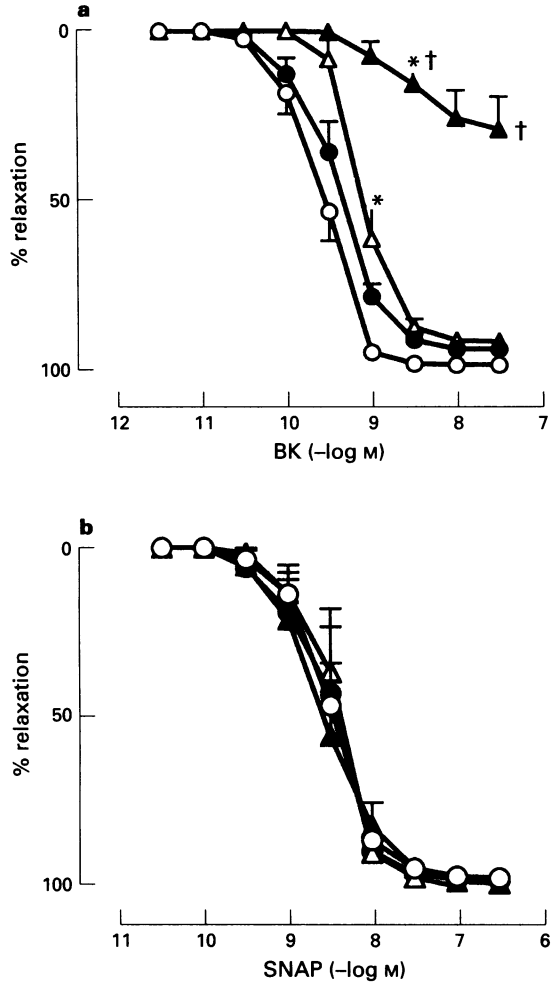

Figure 4 Relaxation responses of bovine epicardial coronary arteries to (a) BK and (b) SNAP in the presence of normal Krebs (○), normal Krebs and L-NOARG (100 μM; △), 68 mM KCl Krebs (●) and a combination of 68 mM KCl Krebs and L-NOARG (100 μM; ▲). All rings of artery were treated with nifedipine (0.3 μM) for at least 20 min prior to precontraction with 68 mM KCl and/or U46619. Initial levels of contraction to U46619 (expressed as % $KPSS_{max}$) were (○) 38.6±1.5, (△) 37.6±1.0, (●) 41.2±3.7 and (▲) 44.7±3.3 in (a) and (○) 34.0±1.4, (△) 35.2±0.3, (●) 36.2±0.5 and (▲) 37.2±0.5 in (b). Values [mean±s.e. mean from (a) 5 and (b) 3 to 4 experiments] are expressed as a percentage reversal of the initial level of precontraction. *Represents pEC_{50} values significantly different from those values obtained in tissues exposed to normal Krebs in the absence of L-NOARG. †Represents pEC_{50} or R_{max} values significantly different from those obtained in tissues exposed to normal Krebs in the presence of L-NOARG ($P<0.05$, Tukey Kramer's modified t statistic after one-way ANOVA).

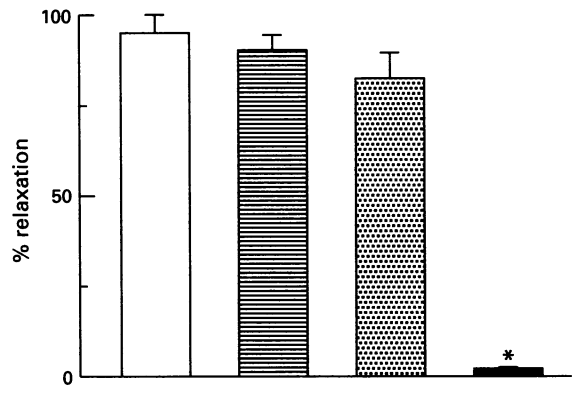

Figure 5 Maximum relaxation responses to levcromakalim (0.3 μM) in the absence (open column) or presence of nifedipine (0.3 μM; striped column), nifedipine (3 μM; stippled column) or a combination of nifedipine (0.3 μM) and 68 mM KCl Krebs solution (solid column). Initial levels of contraction to U46619 (expressed as % $KPSS_{max}$) were (open column) 39.1±1.7, (striped column) 36.8±1.7, (stippled column) 32.2±1.3 and (solid column) 43.5±5.0. Values (mean±s.e. mean from 6 experiments) are expressed as a percentage reversal of the initial level of precontraction. *Indicates R_{max} values significantly different from those obtained in control tissues ($P<0.05$, Tukey Kramer's modified t statistic after one-way ANOVA).

1994; Kemp *et al.*, 1995; Kühberger *et al.*, 1995). Thus, it is likely that L-NOARG-resistant relaxations in the bovine coronary artery are at least partially mediated by EDHF (see Holzmann *et al.*, 1994).

The finding from this study that relaxations mediated by EDHF were observed in tissues contracted independently of Ca^{2+} entry through voltage-operated Ca^{2+} channels is surprising since it is generally assumed that hyperpolarization-mediated relaxations are brought about by closure of such channels (Beny & von der Weid, 1991; Garland *et al.*, 1995; Godfraind & Govoni, 1995). As in most arterial smooth muscle preparations, the predominant voltage-operated Ca^{2+} channel in the bovine coronary artery was of the L-type since the L-type Ca^{2+} channel blocker, nifedipine, virtually abolished contractions to maximum depolarization with KCl. As previously reported for the dog coronary artery (Angus & Brazenor, 1983), contractions to the thromboxane A_2 agonist, U46619, were only marginally dependent on the entry of Ca^{2+} through voltage-operated channels since nifedipine had only a small effect on the response to U46619. Thus, relaxations by EDHF of tissues contracted with U46619 were probably due to reduction of either Ca^{2+} entry through a non- L-type Ca^{2+} channel or release from an unidentified internal store.

It has previously been shown in porcine (Yamagishi *et al.*, 1992a) and canine (Yamagishi *et al.*, 1992b) coronary arteries that the ATP-dependent K^+ channel opener, levcromakalim (Edwards & Weston, 1993), may mediate relaxation by preventing U46619-induced inositol trisphosphate (IP_3) production, thereby reducing the amount of Ca^{2+} released from internal stores (see also Kühberger *et al.*, 1993). The precise mechanism by which this occurs, however, is unknown, although it may be associated with smooth muscle hyperpolarization since the effects of levcromakalim were blocked by raising extracellular K^+ and by the K^+ channel inhibitor, tetrabutylammonium (Yamagishi *et al.*, 1992a,b). It is tempting to speculate that a similar mechanism is also involved in the EDHF-mediated relaxation to BK in U46619-contracted cow coronary arteries reported here. As in the previous studies (Yamagishi *et al.*, 1992a,b) we too found that relaxations to levcromakalim, like EDHF, were not necessarily the result of closure of voltage-operated Ca^{2+} channels since they were not affected by nifedipine. Relaxations to levcromakalim were, however, blocked by high extracellular KCl suggesting that they were mediated by smooth muscle hyperpolarization. Therefore, hyperpolarization-induced decreases in IP_3 accumulation offers an attractive explanation as to how EDHF may mediate relaxation independently of the closure of L-type Ca^{2+} channels.

This study also confirmed previous findings that in large arteries such as pig coronary arteries (Kilpatrick & Cocks, 1994), rabbit aorta and carotid arteries (Cowan *et al.*, 1993) and pulmonary arteries from pulmonary hypertensive sheep (Kemp *et al.*, 1995) the relaxant action of EDHF was generally silent but acted as a reserve or 'backup' mechanism of relaxation when NO synthesis was compromised. Thus, in the present study, treatment with high extracellular KCl alone had no effect on the response to BK, whilst treatment with L-NOARG alone caused a small but significant rightward shift in the BK concentration-response curve. A combination of high

extracellular KCl and L-NOARG, however, abolished all but 30% of the relaxation to BK. The means by which the relaxant action of EDHF remains silent is at present unknown. One possibility is that the formation and/or release of EDHF requires higher concentrations of BK and thus intracellular Ca^{2+} than does the formation of NO. Thus, under normal conditions, NO release would be expected to account for 100% of the total relaxation before the concentration of BK was high enough to generate sufficient Ca^{2+} to cause EDHF release. In support of the idea that EDHF requires a greater stimulus strength for formation and/or release was the observation in porcine coronary arteries that the ratio of EDHF:NO contributing to endothelium-dependent relaxation increases with the ability of an agonist to raise intracellular endothelial cell Ca^{2+} (Nagao & Vanhoutte, 1992).

Finally, even after treatment with both high extracellular KCl and L-NOARG, approximately 30% of the total relaxation response to BK still remained. This response was probably not due to the inability of 68 mM KCl to inhibit totally the effect of EDHF, since previous studies have demonstrated that concentrations of extracellular KCl above 20–30 mM are sufficient to abolish all K^+ channel activity (Chen & Suzuki, 1989). Thus, the remaining response may be mediated by yet another endothelium-derived relaxing factor. One candidate for such a factor is an epoxyeicosatrienoic acid formed by the action of cytochrome P450 on arachidonic acid since these substances are produced by endothelial cells (Pritchard *et al.*, 1990; Rosolowsky *et al.*, 1990) and have been shown to cause relaxation in bovine coronary arteries (Rosolowsky *et al.*, 1990).

In conclusion, this study clearly suggests that relaxations to BK after treatment with L-NOARG are not due to NO but to a K^+-sensitive mechanism which may represent EDHF and which is able to mediate smooth muscle relaxation in tissues contracted independently of voltage-operated Ca^{2+} channel activity. Future studies will determine if the endogenous hyperpolarizing mechanism (i.e. EDHF), like levcromakalim, inhibits the IP_3/Ca^{2+} signalling pathway in vascular smooth muscle.

This work was supported by a grant from the National Health and Medical Research Council of Australia. The authors wish to thank Dr D. Pruneau and Mr R. Benaud for their valuable comments.

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(Received July 20, 1995
Revised October 9, 1995
Accepted November 7, 1995)