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Apamin-sensitive, non-nitric oxide (NO) endothelium-dependent relaxations to bradykinin in the bovine isolated coronary artery: no role for cytochrome P_{450} and K^+

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1 Since cytochrome P_{450}-derived metabolites of arachidonic acid and K^+ have been implicated in endothelium-derived hyperpolarizing factor (EDHF)-dependent responses, the aim of this study was to determine whether such factors contribute to non-nitric oxide (NO), endothelium-dependent relaxation to bradykinin (BK) in bovine isolated coronary artery.

2 In rings of artery contracted with U46619 and treated with indomethacin (3 μM) and N^G-nitro-L-arginine (L-NOARG; 100 μM), relaxation to BK (0.01 nM-0.3 μM) was blocked by $\sim$60% after inhibition of K^+ channels with either high extracellular K^+ (high $[K^+]_o$; 15–67 mM) or apamin (0.3 μM).

3 Ouabain (1 μM), an inhibitor of Na^+/K^+-ATPase, decreased the sensitivity to BK without affecting the maximum response. In L-NOARG-treated rings, ouabain had no further effect on the relaxation to BK. An inhibitor of inward-rectifying K^+ channels, Ba^{2+} (30 μM), had no effect on relaxations to BK in the absence or presence of either L-NOARG or ouabain.

4 KCl (2.5–10 mM) elicited small relaxations ($\sim$20%) that were abolished by nifedipine (0.3 μM) and ouabain.

5 Both the high $[K^+]_o$/apamin-sensitive relaxation to BK, and the relaxation to the K_{ATP} channel-opener, levcromakalim (0.6 μM), were unaffected by the cytochrome P_{450} inhibitor, 7-ethoxyresorufin (10 μM), or by co-treatment with a phospholipase A_2 inhibitor, arachidonyl trifluoromethyl ketone ($AACOCF_3$; 3 μM) and a diacylglycerol (DAG)-lipase inhibitor, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC 80267; 30 μM). The non-NO/high $[K^+]_o$-insensitive, $\sim$40% relaxation to BK was, however, abolished by these treatments.

6 Therefore, neither cytochrome P_{450}-derived metabolites of arachidonic acid nor K^+ appear to mediate the EDHF-like relaxation to BK (i.e the non-NO, high $[K^+]_o$/apamin-sensitive component) in bovine coronary arteries. Cytochrome P_{450}-derived metabolites may be released at higher BK concentrations to act in parallel with NO and the high $[K^+]_o$/apamin-sensitive mechanism.
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Keywords: Endothelium-dependent relaxation; EDHF; nitric oxide; bradykinin; bovine coronary artery; ouabain; cytochrome P_{450}; K^+; apamin; Ba^{2+}

Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; ACh, acetylcholine; Ba^{2+}, barium; BK, bradykinin; BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; DAG, diacylglycerol; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetranoic acid; high $[K^+]_o$, high extracellular K^+; K_{IR}, inward-rectifying K^+ channel; L-NOARG, N^G-nitro-L-arginine; NO, nitric oxide; ODQ, 1*H*-[1,2,4]-oxadiazolo[4,3-α]quinoxaline 1-one; PLA_2, phospholipase A_2; RHC 80267, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channel; SKF-525a, N,N-diethylaminoethyl-2,2-diphenylvalerate; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; U46619, 1,5,5-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid

Introduction

Non-prostanoid, endothelium-dependent relaxation in many blood vessels is mediated not only by nitric oxide (NO) but also by a non-NO, high K^+-sensitive mechanism which may be attributable to the release of an endothelium-derived hyperpolarizing factor (EDHF; Taylor & Weston, 1988; Kilpatrick & Cocks, 1994; Garland *et al*., 1995; Drummond & Cocks, 1996; Feletou & Vanhoutte, 1999). The mechanism of EDHF-mediated relaxation of vascular smooth muscle remains controversial. Two recent proposals are that EDHF is either K^+ (Edwards *et al*., 1998) or a cytochrome P_{450}-derived metabolite of arachidonic acid (Komori & Vanhoutte, 1990; Mombouli & Vanhoutte, 1997; Feletou & Vanhoutte, 1999; Fisslthaler *et al*., 1999).

In support of their claim that EDHF is K^+, Edwards *et al.* (1998) showed that non-prostanoid, non-NO, endothelium-dependent hyperpolarization to acetylcholine (ACh) in rat resistance arteries was blocked by a combination of the small (SK_{Ca}) and large (BK_{Ca}) conductance Ca^{2+}-activated K^+ channel inhibitors, apamin and charybdotoxin, respectively. The response was also blocked by a combination of ouabain and Ba^{2+}, inhibitors of Na^+/K^+-ATPase and inward-rectifying K^+ channels (K_{IR}), respectively. Edwards *et al.* (1998) found that low concentrations (<20 mM) of K^+ applied exogenously caused ouabain- and Ba^{2+}-sensitive smooth muscle hyperpolarization and relaxation, mimicking the non-NO, endothelium-dependent responses to ACh. Furthermore, using a K^+ measuring electrode strategically

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positioned between the endothelium and smooth muscle, they were able to show that in response to ACh, the K^+ concentration increased by approximately 5 mM in the myoendothelial space in an apamin- and charybdotoxin-sensitive manner. Thus, the authors concluded that EDHF is likely to be universally K^+, which after being released from endothelial cells *via* SK_{Ca} and BK_{Ca} channels activates smooth muscle Na^+/K^+-ATPase and K_{IR} to initiate hyperpolarization and relaxation (Edwards *et al*., 1998).

The conclusion by Edwards *et al*. (1998) that EDHF is K^+ may not hold true for all arteries. For example, it is well established that in large epicardial arteries of the pig, an EDHF-like mechanism compensates for up to 80% of the relaxation to BK after inhibition of NO synthesis (Kilpatrick & Cocks, 1994). This is despite the fact that these large arteries express few functional K_{IR} (Quayle *et al*., 1996). Also, Fisslthaler *et al*. (1999) have shown that while combined application of Ba^{2+} and ouabain had no effect on BK-induced, EDHF-mediated vascular responses in porcine large epicardial arteries, transfection with antisense oligonucleotides against cytochrome P_{450} 2C inhibited the response. Thus, these findings not only argue against a role for K_{IR} and Na^+/K^+-ATPase but also suggest that in porcine coronary arteries at least, EDHF represents a product of cytochrome P_{450} 2C metabolism.

Earlier observations also support a role for cytochrome P_{450} in EDHF-dependent vasorelaxation. For instance, a variety of inhibitors of arachidonic acid formation and cytochrome P_{450} have been shown to partially inhibit endothelium-dependent relaxations in many isolated arteries (Singer *et al*., 1984; Pinto *et al*., 1987; Hecker *et al*., 1994; Bauersachs *et al*., 1997; Satake *et al*., 1997) and perfused vascular beds (Fulton *et al*., 1992; 1996; Bauersachs *et al*., 1994; Adeagbo & Henzel, 1998). Also, products of cytochrome P_{450}-dependent metabolism of arachidonic acid including epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), have been shown to be released by cultured bovine coronary artery endothelial cells (Rosolowsky *et al*., 1996; Rosolowsky & Campbell, 1996; Gebremedhin *et al*., 1998) and to relax vascular smooth muscle (Proctor *et al*., 1987; Rosolowsky *et al*., 1990, 1996; Gebremedhin *et al*., 1992; Hecker *et al*., 1994; Campbell *et al*., 1996; Zou *et al*., 1996; Pratt *et al*., 1998). Also, EETs can activate smooth muscle calcium-dependent K^+ channels to initiate hyperpolarization (Gebremedhin *et al*., 1992; Hu & Kim, 1993; Campbell *et al*., 1996; Zou *et al*., 1996).

In the bovine coronary artery, the non-prostanoid relaxation to BK, which is sensitive to high extracellular K^+ and therefore likely attributable to an EDHF-like mechanism, compensates for up to 95% of the overall response after inhibition of NO (Drummond & Cocks, 1996). Also, a third mechanism independent of both NO and the EDHF-like mechanism is present in this tissue and acts as a second level backup behind NO and EDHF, accounting for ~40% of the overall response to BK. Therefore, the aim of this study was to determine whether endothelium-derived K^+ or cytochrome P_{450}-derived metabolites of arachidonic acid mediate non-NO, endothelium-dependent relaxation to BK in the bovine coronary artery. Our data show that while cytochrome P_{450}-derived metabolites of arachidonic acid may be involved, they do not mediate the EDHF-like response. Also, our results indicate that endothelium-derived K^+ does not appear to have any role in non-prostanoid, non-NO endothelium-dependent relaxation in the bovine coronary artery.

Methods

Tissue preparation

Sections of bovine myocardium containing the left anterior descending coronary artery were obtained from a local abattoir and transported to the laboratory in ice cold 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 and glucose 11.0). Ring segments (3 mm) of coronary artery were prepared as previously described (Drummond & Cocks, 1996) and 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 immersed in water-jacketed 30 ml organ baths containing warm (37°C), carbogen-bubbled (95% O_2, 5% CO_2) Krebs solution (pH 7.4). Changes in isometric force were amplified and displayed on dual-channel, flat-bed recorders (W & W Scientific Instruments, Basel, Switzerland).

Tissue equilibration

Rings of artery were allowed to equilibrate under zero tension for a period of 25 min, after which time they were passively stretched to a resting tension of 5 g. After a further 25 min, rings were stretched again to 5 g passive tension, allowed to equilibrate and then maximally contracted with an isotonic, high potassium physiological salt solution (KPSS; composition 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 and glucose 6.1). Once a stable plateau of active force to KPSS was obtained ($KPSS_{max}$), tissues were washed with normal Krebs solution and 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. Some tissues were also treated with the L-type voltage-operated Ca^{2+} channel (VOCC) inhibitor, nifedipine (0.3 μM), to prevent excessive levels of pre-contraction in experiments where an isotonic 67 mM KCl bathing solution (high $[K^+]_o$) was used to inhibit K^+ channels. Previously, we have shown that while nifedipine abolished contractions to a maximum depolarizing concentration of extracellular K^+, it had no effect on either the ability of U46619 to cause contraction or any component of the relaxation to BK (Drummond & Cocks, 1996).

Experimental protocol

Twenty-five minutes after the addition of indomethacin and nifedipine, rings were either left untreated (control) or treated with either the NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG; 100 μM), high $[K^+]_o$, the soluble guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxaline-1-one (ODQ; 10 μM), the SK_{Ca} inhibitor, apamin (0.3 μM), the Na^+/K^+-ATPase inhibitor, ouabain (1 μM or 1 mM) or the K_{IR} inhibitor, Ba^{2+} (30 μM). Some L-NOARG-treated tissues were further treated with either high $[K^+]_o$, ODQ, apamin, ouabain or Ba^{2+} or ouabain plus Ba^{2+}. Other L-NOARG-treated rings were treated with the combination of apamin (0.3 μM), charybdotoxin (0.1 μM) and the K_{ATP} channel inhibitor, glibenclamide (10 μM). Also, separate rings were treated with either inhibitors of arachidonic acid formation including the phospholipase A_2 inhibitors, quinacrine (30 μM; Bauersachs *et al*., 1994) and arachidonyl trifluoromethyl

ketone (AACOCF$_3$; 3 μM; Street *et al*., 1993; Riendeau *et al*., 1994) and the diacylglycerol (DAG) lipase inhibitor, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC 80267; 30 μM; Sutherland & Amin, 1982), or blockers of subsequent metabolism *via* the cytochrome P_{450} pathway such as N,N-diethylaminoethyl-2,2-diphenylvalerate (SKF-525a; 100 μM; Hecker *et al*., 1994), clotrimazole (100 μM; Hecker *et al*., 1994) or 7-ethoxyresorufin (10 μM; Tassaneeyakul *et al*., 1993). All tissues were then contracted with titrated concentrations of the thromboxane A_2-mimetic, U46619, to ~40% of their $KPSS_{max}$. Upon reaching a stable plateau, rings were exposed to cumulatively increasing half-log molar concentrations of either BK or NO-donors, S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP), or with either single concentrations of levcromakalim (0.6 μM) or KCl (2.5–10 mM).

Statistics

All cumulative concentration-relaxation curves were normalized as percentages of relaxation from the initial U46619-induced contraction level. Each normalized curve was then computer-fitted (Graphpad Prism, version 1.00) with a sigmoidal regression curve of the following equation,

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

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 HILLSLOPE controls the slope of the curve. Mean sensitivity (pEC_{50} values), maximum relaxations (R_{max}) and their standard errors (s.e.) were then calculated for each response curve. U46619-induced levels of contraction were expressed as percentages of the respective $KPSS_{max}$ values for each tissue. Values of n represent number of rings of artery, each from different animals. Differences in mean pEC_{50} and R_{max} values were tested for significance by means of one way analysis of

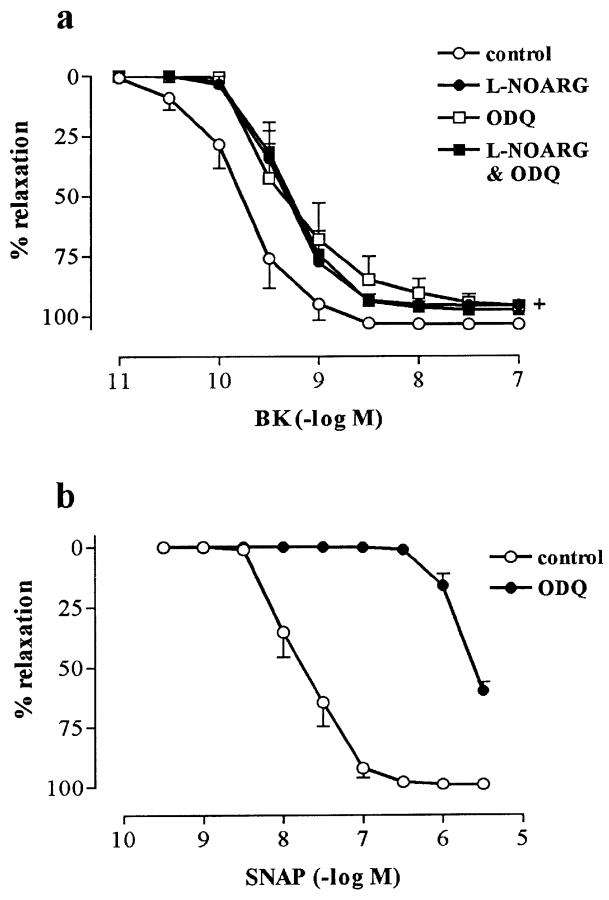

Figure 1 Effect of (a) the NO synthase inhibitor, L-NOARG (100 μM) and guanylate cyclase inhibitor, ODQ (10 μM), on endothelium-dependent relaxations to BK in rings of bovine coronary artery contracted with U46619. (b) Effect of ODQ (10 μM) on endothelium-independent relaxations to the NO-donor, SNAP in similarly contracted rings of bovine coronary artery. Values (mean ± s.e.mean from $n=6$ experiments) are expressed as a percentage reversal of the initial U46619-induced contraction. (+) indicates that R_{max} values for all treatments significantly different from those obtained in control tissues ($P<0.05$ for Tukey-Kramer's t-statistic after one way ANOVA).

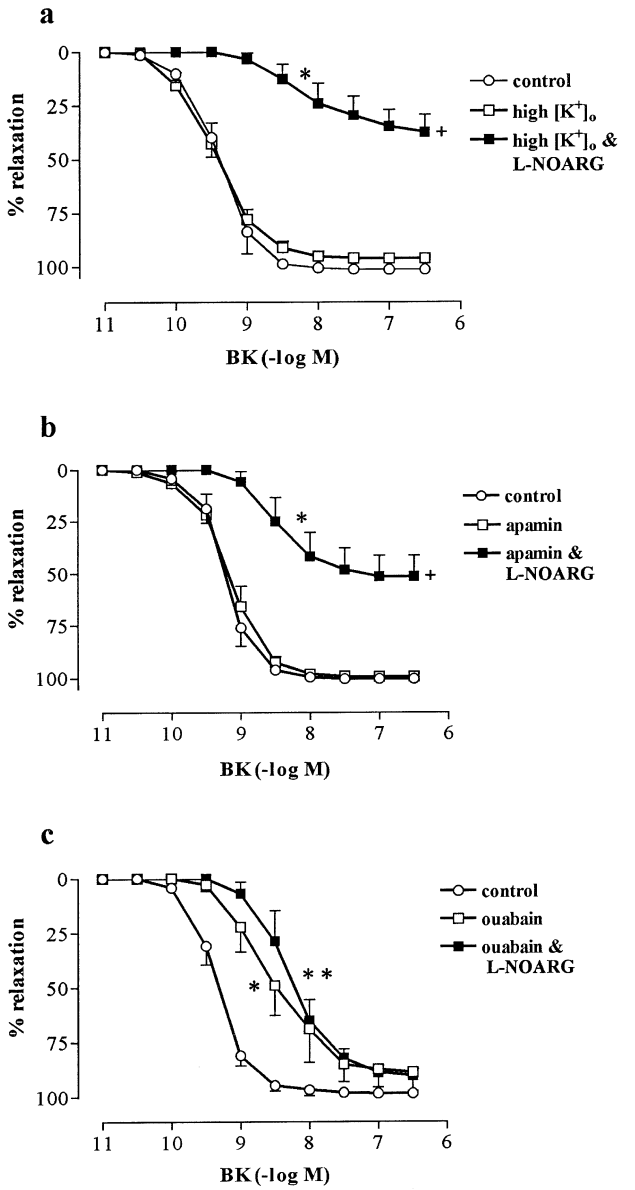

Figure 2 Effect of (a) 67 mM extracellular K^+ (high $[K^+]_o$), (b) apamin (0.3 μM) and (c) ouabain (1 μM) on endothelium-dependent relaxations to BK in the absence and presence of L-NOARG (100 μM) in rings of bovine coronary artery contracted with U46619. Values (mean ± s.e.mean from $n=5-6$ experiments) are expressed as a percentage reversal of the initial U46619-induced contraction. (*$P<0.05$ and **$P<0.001$) and ($^+P<0.001$) indicate that pEC_{50} and R_{max} values, respectively were significantly different from those obtained in control tissues (for Tukey-Kramer's t-statistic after one way ANOVA).

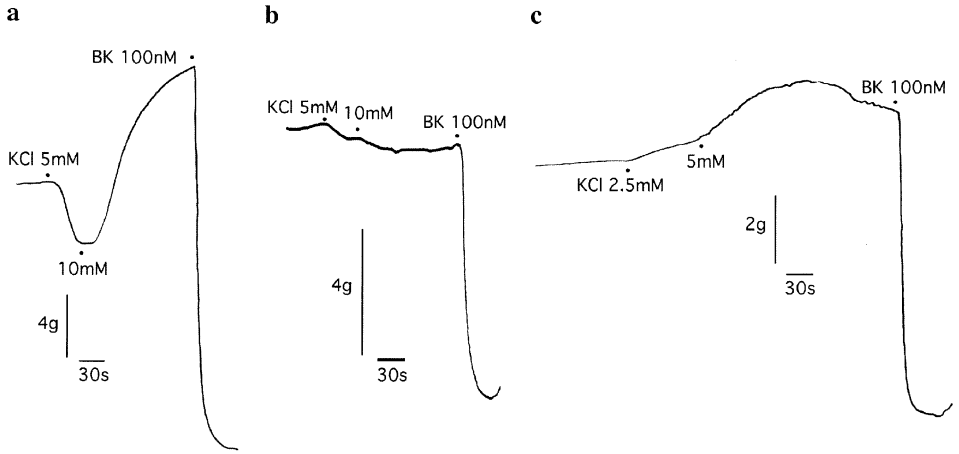

Figure 3 Digitized recordings showing responses to exogenous administration of 2.5–10 mM KCl in rings of bovine coronary artery contracted with U46619 in the (a) absence or presence of either (b) nifedipine (0.3 μM) or (c) ouabain (1 μM). Also shown are maximum relaxations to bradykinin (BK; 100 nM).

variance (ANOVA) with multiple comparisons *via* Dunnett's test (when treatment groups were being compared with controls only) or Tukey-Kramer's test (when treatment groups were being compared amongst themselves as well as with controls). All differences were accepted as significant at the $P<0.05$ level.

Drugs and their sources

Ba^{2+} chloride, bradykinin triacetate, clotrimazole, indomethacin, N^G-nitro-L-arginine (L-NOARG), quinacrine (Sigma, MO, U.S.A.); apamin, arachidonyl trifluoromethyl ketone ($AACOCF_3$), 7-ethoxyresorufin, nifedipine, 1*H*-[1,2,4]oxadizolo[4,3-α]quinoxaline-1-one (ODQ), N,N-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525a), 1,5,5-hydroxy-11,9-(epoxymethano) prosta-5Z,13E-dienoic acid (U46619), S-nitroso-N-acetylpenicillamine (SNAP), 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC 80267; Sapphire Bioscience, N.S.W., Australia); ouabain (Calbiochem); sodium nitroprusside (SNP; David Bull Laboratories, Australia) and levcromakalim (kind gift from Dr Grant McPherson).

Stock solutions of nifedipine (10 mM), ouabain (100 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 $AACOCF_3$ (1 mM), clotrimazole (100 mM), 7-ethoxyresorufin (10 mM), ODQ (10 mM), RHC 80267 (10 mM) and levcromakalim (10 mM) were made up in dimethyl sulphoxide. All subsequent dilutions of these drugs were in distilled water. All other drug stocks were made up in distilled water. Note that none of the vehicles used in this study had an effect on any component of the response to BK or on the responses to SNAP, SNP, levcromakalim and KCl.

Results

Confirmation that an EDHF-like mechanism acts as a backup relaxation mechanism for NO

BK caused concentration-dependent (pEC_{50}, 9.74 ± 0.16) and maximum (R_{max}, $103.6\pm0.9\%$) relaxation in endothelium-intact rings of bovine isolated coronary artery contracted to $\sim$40% $KPSS_{max}$ with U46619 (Figure 1). L-NOARG (100 μM) caused a small, significant reduction in R_{max} to BK ($95.8\pm1.6\%$) and although there was a trend for L-NOARG to cause a reduction in sensitivity (pEC_{50}, 9.37 ± 0.13), it did not reach significance (Figure 1). Also, a near-identical pattern of inhibition of responsiveness to BK was observed with ODQ (10 μM; pEC_{50}, 9.25 ± 0.23; R_{max}, $96.2\pm2.1\%$; Figure 1). The combination of L-NOARG and ODQ was no more effective at inhibiting the response to BK than was either compound alone (pEC_{50}, 9.29 ± 0.12; R_{max}, $97.8\pm2.3\%$; Figure 1). ODQ caused an $\sim$100-fold increase in the concentration of the NO donor, SNAP, required to elicit a threshold response (Figure 1).

In the presence of L-NOARG, the response to BK was markedly inhibited by high $[K^+]_o$ (67 mM; pEC_{50}, 8.07 ± 0.23; R_{max}, $38.6\pm8.6\%$; Figure 2). By contrast, high $[K^+]_o$ had no effect on the response to BK in the absence of L-NOARG (Figure 2). A similar degree of inhibition of L-NOARG-resistant relaxation to BK was observed with a lower concentration of $[K^+]_o$ (15 mM) ($n=5$; data not shown). Like high $[K^+]_o$, apamin (0.3 μM) inhibited the L-NOARG-resistant response to BK (pEC_{50}, 8.35 ± 0.13; R_{max}, $51.5\pm10.0\%$) and had no effect on the control response (Figure 2). A combination of apamin (0.3 μM), glibenclamide (10 μM) and charybdotoxin (0.1 μM) was no more effective at inhibiting the L-NOARG-resistant response to BK than was apamin on its own ($n=4$; data not shown).

Effect of ouabain and Ba^{2+}

The pEC_{50} (8.4 ± 0.15; $n=6$) and R_{max} ($92.3\pm1.7\%$) to BK in tissues treated with a combination of ouabain (1 mM) and Ba^{2+} (30 μM) were significantly ($P<0.05$) reduced compared to controls. The relaxation to BK was further reduced by ouabain and Ba^{2+} (30 μM) in L-NOARG-treated tissues (pEC_{50}, 7.9 ± 0.15; R_{max}, $62.1\pm5.5\%$; $n=6$). Ouabain alone caused the same degree of inhibition of BK-induced relaxations in the absence and presence of L-NOARG as that observed with the combination of ouabain and Ba^{2+} ($n=6$; data not shown). Ba^{2+} (30 μM) had no effect on control or L-NOARG-resistant responses to BK ($n=6$; data not shown). Ouabain (1 μM) caused a significant inhibition of the pEC_{50} (8.58 ± 0.22) but unlike the higher concentration, had no effect on R_{max} ($88.9\pm8.3\%$) to BK (Figure 2). The subsequent addition of L-NOARG to tissues treated with 1 μM ouabain,

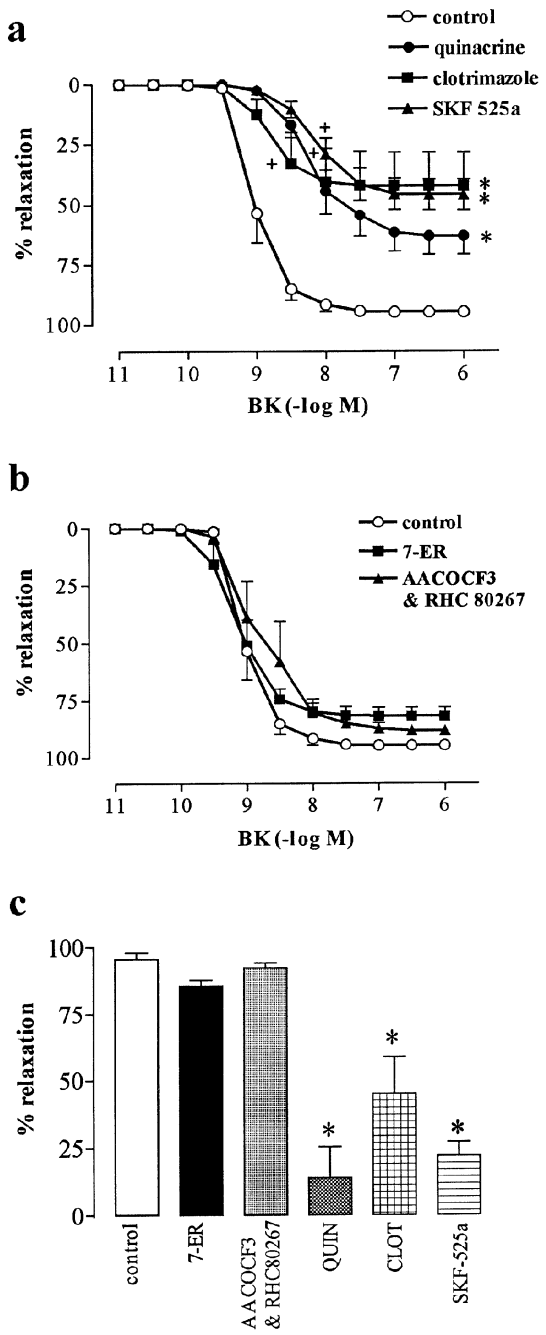

Figure 4 Effect of inhibitors of free arachidonic acid formation [quinacrine (QUIN; 30 μM); $AACOCF_3$ (3 μM); RHC 80267 (30 μM)] and cytochrome P_{450} activity [7-ethoxyresorufin (7-ER; 10 μM); clotrimazole (CLOT; 100 μM); SKF 525a (100 μM)] on L-NOARG-resistant responses to BK (a and b) and on relaxations to levcromakalim (0.6 μM; c) in rings of bovine coronary artery contracted with U46619. All values (mean$\pm$s.e.mean from $n=4-6$ experiments) are expressed as a percentage reversal of the initial U46619-induced contraction. * ($P<0.01$) and (+) ($P<0.01$) indicate R_{max} and pEC_{50} values significantly different from control (Dunnett's modified *t*-statistic after a single one-way ANOVA).

caused no further inhibition of relaxations to BK (pEC_{50}, 8.30 ± 0.21; R_{max}, $88.46\pm2.5\%$; Figure 2).

Combined treatment with ouabain (1 mM) and Ba^{2+} (30 μM) had no effect on relaxation to the NO donor, SNP (control: pEC_{50}, 7.65 ± 0.2; R_{max}, 91.5 ± 4.6; ouabain and Ba^{2+}: pEC_{50}, 7.28 ± 0.12; R_{max}, $76.3\pm9.3\%$; $n=6$).

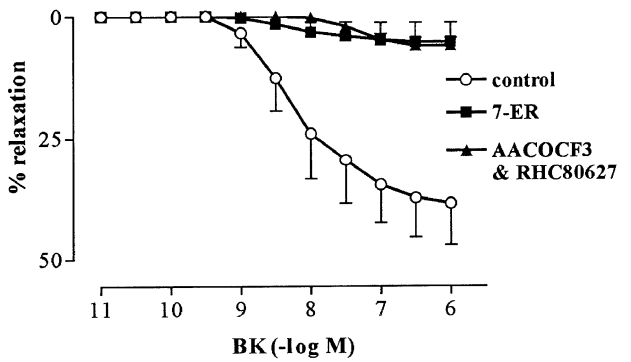

Figure 5 Effect of 7-ethoxyresorufin (7-ER; 10 μM) and a combination of $AACOCF_3$ (3 μM) and RHC 80267 (30 μM) on L-NOARG/high $[K^+]_o$-resistant responses to BK in rings of bovine coronary artery contracted with U46619. All responses were obtained in the presence of L-NOARG (100 μM) and 67 mM $[K^+]_o$. Values (mean$\pm$s.e.mean from $n=6$ experiments) are expressed as a percentage reversal of the initial U46619-induced contraction.

Response to KCl

Increasing the extracellular concentration of K^+ from 5.3 to 10.3 mM with KCl caused relaxation of $18.0\pm3.7\%$ ($n=5$) followed by contraction (Figure 3). No further relaxation to K^+ was observed when the concentration was raised to 15.3 mM (data not shown). Both relaxation (R_{max}, $4.8\pm1.2\%$) and contraction to K^+ were inhibited by nifedipine (0.3 μM; Figure 3). Also, the relaxation to K^+ was abolished by ouabain (1 μM; Figure 3).

Effect of inhibitors of arachidonic acid formation and cytochrome P_{450} activity on NO-independent relaxations to BK and responses to levcromakalim

Quinacrine (30 μM), SKF 525a (100 μM) and clotrimazole (100 μM), each significantly reduced both the R_{max} ($63.0\pm8.6\%$, $45.8\pm7.0\%$, $42.1\pm15.2\%$, respectively) and sensitivity (pEC_{50}, 8.21 ± 0.18, 8.09 ± 0.14, 8.64 ± 0.17, respectively) of the L-NOARG-resistant response to BK (pEC_{50}, 8.98 ± 0.08; R_{max}, $94.7\pm2.1\%$; Figure 4). By contrast, $AACOCF_3$ (3 μM) and RHC 80267 (30 μM), either alone ($n=6$; data not shown) or in combination (pEC_{50}, 8.77 ± 0.23; R_{max}, $88.9\pm2.2\%$) and 7-ethoxyresorufin (10 μM; pEC_{50}, 9.15 ± 0.17; R_{max}, $81.8\pm4.2\%$) had no significant effect on the L-NOARG-resistant response to BK (Figure 4).

The ATP-sensitive K^+ channel opener, levcromakalim (0.6 μM), caused a near-maximum relaxation (R_{max}, $95.3\pm2.5\%$) which was unaffected by either 7-ethoxyresorufin (10 μM) or the combination of RHC 80267 (30 μM) and $AACOCF_3$ (3 μM; Figure 4). By contrast, quinacrine (30 μM), clotrimazole (100 μM) and SKF-525a (100 μM) significantly reduced the R_{max} to levcromakalim to $13.8\pm11.5\%$ $45.0\pm13.7\%$ and $21.8\pm5.1\%$, respectively (Figure 4).

Effect of inhibitors of arachidonic acid formation and cytochrome P_{450} activity on non-NO, high K^+-resistant relaxations to BK

While both $AACOCF_3$ ($n=5$) and RHC 80267 ($n=6$) alone had no effect on non-NO, K^+-resistant relaxation to BK, in combination they virtually abolished this response (R_{max}, $6.1\pm1.7\%$; Figure 5). Similarly, 7-ethoxyresorufin (R_{max}, $5.4\pm4.0\%$) abolished the L-NOARG/high $[K^+]_o$-resistant

relaxations to BK (Figure 5), despite the fact that the latter of these compounds had no effect on the L-NOARG-resistant responses to BK.

Effect of the combination of $AACOCF_3$ and RHC 80267 and 7-ethoxyresorufin on control and high $[K^+]_o$-resistant relaxation to BK

In the absence of any treatments, the combination of $AACOCF_3$ (3 μM) and RHC 80267 (30 μM) had no effect on the control response to BK (pEC_{50}, 9.3 ± 0.2; R_{max}, $99.9 \pm 2.0\%$; $n=6$). Under the same conditions, however, 7-ethoxyresorufin (10 μM) caused a significant ($P<0.01$) reduction in R_{max} to BK ($82.8 \pm 3.9\%$; $n=5$) without affecting the pEC_{50}.

Similar to its effects on the control response, the combination of $AACOCF_3$ (3 μM) and RHC 80267 (30 μM) also had no effect on the high $[K^+]_o$-resistant response to BK (pEC_{50}, 9.2 ± 0.09; R_{max}, $95.7 \pm 2.3\%$; $n=6$). By contrast, 7-ethoxyresorufin (10 μM) abolished this response (R_{max}, $4.8 \pm 3.7\%$; $n=5$; $P<0.01$). Concentration-dependent relaxations to SNAP (pEC_{50}, 7.80 ± 0.11; R_{max}, $98.4 \pm 0.7\%$; $n=12$) were unaffected by the combination of $AACOCF_3$ and RHC 80267 ($n=5$) but were markedly inhibited by 7-ethoxyresorufin (pEC_{50}, 5.65 ± 0.64; R_{max}, 20.3 ± 6.6; $n=6$, $P<0.01$ for both values).

Discussion

The results of this study suggest that a cytochrome P_{450}-derived metabolite of arachidonic acid is involved in endothelium-dependent relaxation in the bovine coronary artery, but that it does not mediate the component of the response attributable to an EDHF-like mechanism. The cytochrome P_{450}-dependent mechanism appeared to be activated only by high concentrations of BK, to function as a backup with the EDHF-like mechanism for NO, the predominant non-prostanoid, endothelium-derived relaxing factor (EDRF) in this tissue. Also, our findings do not support a role for K^+ as an EDRF in the bovine coronary artery.

Recently, Fisslthaler *et al.* (1999) reported that cytochrome P450 2C is an EDHF synthase in the porcine coronary artery. Our findings that both the PLA_2 inhibitor, quinacrine, and the cytochrome P_{450} inhibitors, SKF-525a and clotrimazole, all attenuated the L-NOARG-resistant response to BK in the bovine coronary artery appear to support the notion that a cytochrome P_{450}-derived metabolite of arachidonic acid is involved in a smooth muscle relaxation mechanism attributable to EDHF (Komori & Vanhoutte, 1990; Fulton *et al.*, 1992; Hecker *et al.*, 1994; Bauersachs *et al.*, 1994; Mombouli & Vanhoutte, 1997; Feletou & Vanhoutte, 1999). However, the inhibitory effects of these compounds most likely resulted from non-specific actions on smooth muscle K^+ conductance since each compound also blocked relaxation to the K^+ channel opener, levcromakalim. Similar inhibition of relaxations to the K_{ATP} channel openers, levcromakalim and pinacidil, has been reported in rat hepatic (Zygmunt *et al.*, 1996) and mesenteric (Fukao *et al.*, 1997) arteries by quinacrine and SKF-525a. By contrast, neither $AACOCF_3$, an inhibitor of PLA_2 which is structurally and mechanistically distinct from quinacrine (Gelb *et al.*, 1994), nor 7-ethoxyresorufin, a xenobiotic cytochrome P_{450} inhibitor (Tassaneeyakul *et al.*, 1993) which inhibits endothelium-dependent relaxations to arachidonic acid in the perfused rat kidney (Oyeken *et al.*, 1991), had any effect on relaxations to either levcromakalim or BK after NO inhibition. Also, combined PLA_2 and DAG lipase inhibition with $AACOCF_3$ and RHC 80267, respectively, failed to have any effect on the response to BK after NO inhibition. Therefore, relaxations to the EDHF-like mediator do not appear to involve cytochrome P_{450}-derived metabolites of arachidonic acid, at least in bovine coronary arteries.

A cytochrome P_{450}-derived metabolite of arachidonic acid may, however, have mediated the component of the response to BK not attributable to either NO or EDHF, since inhibition of the two main sources of arachidonic acid with $AACOCF_3$ and RHC 80267, as well as inhibition of cytochrome P_{450} with 7-ethoxyresorufin abolished this L-NOARG/high $[K^+]_o$-resistant response. 7-ethoxyresorufin is a relatively specific competitive substrate inhibitor of 1A isoforms of cytochrome P_{450} (Tassaneeyakul *et al.*, 1993). Also, cytochrome P_{450}1A isozymes have been immunohistochemically localized in both mammalian and non-mammalian endothelial cells. Thus, the block by 7-ethoxyresorufin of L-NOARG/high $[K^+]_o$-resistant relaxations to BK may implicate a role for members of the cytochrome P_{450}1A family in this low efficacy component of the response.

While 7-ethoxyresorufin inhibited relaxations to BK in the presence of L-NOARG and high K^+, it also blocked the NO-dependent component of the response. This effect most likely occurred downstream of NO synthase since relaxations to the NO-donor, SNAP, were also markedly attenuated by 7-ethoxyresorufin. These findings are consistent with previous studies which have shown 7-ethoxyresorufin inhibits endothelium-dependent relaxation known to be due solely to NO (Rees *et al.*, 1990) as well as relaxation to exogenous NO in the rat isolated aorta (Bennett *et al.*, 1992; Oyeken *et al.*, 1994; Li & Rand, 1996) and guinea-pig taenia coli (Selemidis *et al.*, 1997). One possible mechanism underlying these inhibitory actions of 7-ethoxyresorufin involves the generation of superoxide anions which are known to inactivate NO (Ignarro *et al.*, 1988). Irrespective of the mechanism involved, however, the findings here that 7-ethoxyresorufin abolished all the response to BK in the presence of high K^+ indicates that it may prove to be a useful compound to study responses due to EDHF-like mechanisms in isolation from other non-prostanoid relaxing factors.

Although the nature of the cytochrome P_{450}-derived metabolite of arachidonic acid which appeared to be involved in the response to BK is unknown, two possible classes of compounds are EETs and HETEs. Such compounds are not only released from bovine endothelial cells in culture, but also have been shown to relax isolated segments of bovine coronary arteries (Rosolowsky *et al.*, 1996; Rosolowsky & Campbell, 1996; Gebremedhin *et al.*, 1998; Pratt *et al.*, 1998). If these metabolites did play a role in mediating the non-NO/$[K^+]$-resistant component, they need not have acted in a paracrine manner. For example, inhibitors of cytochrome P_{450} are known to prevent the influx of Ca^{2+} that follows agonist-induced depletion of intracellular Ca^{2+} stores in many cell types including bovine coronary artery endothelial cells (Alvarez *et al.*, 1992; Graier *et al.*, 1995). Furthermore, the exogenous application of 5,6-EET to bovine coronary and human umbilical artery endothelial cells was shown to cause a sustained increase in intracellular Ca^{2+} which was similar in time course and magnitude to the second phase increase in Ca^{2+} observed in response to BK (Graier *et al.*, 1995). Taken together, these observations suggest that EETs can act as intracellular second messengers in endothelial cells, signalling transmembrane Ca^{2+} influx in response to agonist-induced depletion of internal Ca^{2+} stores (Baron *et al.*, 1997). In the bovine coronary artery, non-NO/high $[K^+]_o$-resistant relaxa-

tions occurred over a higher concentration range of BK than that which stimulated NO and the EDHF-like mechanism. This may indicate that activation of the remaining non-prostanoid mechanism requires higher levels of intracellular endothelial cell Ca^{2+}. Thus, cytochrome P_{450}-derived metabolites of arachidonic acid like EETs and HETEs may act intracellularly to raise endothelial cell Ca^{2+} levels high enough to activate an as yet unidentified smooth muscle relaxing mechanism.

The present study extends our earlier findings in the same tissue (Drummond & Cocks, 1996) that NO is the predominant mediator of endothelium-dependent relaxation with the EDHF-like and the low-efficacy, cytochrome-P_{450}-dependent mechanisms, both acting as backup vasodilator systems for NO. Thus, the NO synthase inhibitor, L-NOARG, and the guanylate cyclase inhibitor, ODQ (Garthwaite *et al*., 1995), when used alone or in combination with each other, inhibited relaxations to BK to the same small but significant degree. Also, the NO-independent relaxation to BK was blocked by $\sim$60% by either high $[K^+]_o$ (Drummond & Cocks, 1996) or the SK_{Ca} channel inhibitor, apamin, neither of which had any effect in the absence of L-NOARG. Furthermore, the observation that combined treatment with $AACOCF_3$ and RHC 80267, as well as 7-ethoxyresorufin, all had no effect in L-NOARG-treated tissues when the EDHF-like mechanism was active (i.e. in the absence of high $[K^+]_o$) indicates that the cytochrome P_{450}-dependent mechanism also acts as backup for the EDHF-like mechanism.

This study also shows that EDHF-like relaxation to BK in the bovine coronary artery is unlikely to be mediated by endothelial cell K^+ (Edwards *et al*., 1998). Thus, while the EDHF-like mechanism mediated near maximum relaxation ($\sim$95%), 5–10 mM extracellular K^+ caused only small relaxations ($\sim$20%). Also, relaxations to K^+ appeared to be mediated by activation of Na^+/K^+-ATPase since they were abolished by low concentrations of ouabain. By contrast, ouabain had no effect on the EDHF-like component of the response to BK. This finding supports those of Quignard *et al*. (1999) who showed that endothelium-dependent hyperpolarization to BK in the pig coronary artery was unaffected by ouabain. Finally, we have previously demonstrated that EDHF-like relaxations to BK in bovine and pig coronary arteries are unaffected by nifedipine in concentrations (>0.3 μM) that abolished contractions to KCl (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). Thus, EDHF-like relaxations to BK in these tissues can occur independently of the need to close L-type VOCCs. This was not the case for the small relaxations to K^+ since they were abolished by nifedipine.

Since our studies appear to exclude a role for cytochrome P_{450} and K^+, it remains a possibility that direct communication between endothelial cells and smooth muscles cells, *via* heterocellular gap junctions, was involved in the EDHF-like relaxations to BK (Kúhberger *et al*., 1994; Chaytor *et al*., 1998; Dora *et al*., 1999; Yamamoto *et al*., 1999). As described for similar EDHF-like responses in the guinea-pig mesenteric artery (Yamamoto *et al*., 1999), we have preliminary data which shows that the proposed gap junctional uncoupling agent, 18-β-glycyrrhetinic acid (Goldberg *et al*., 1996), appears to inhibit EDHF-like relaxation in the bovine isolated coronary artery (Selemidis & Cocks, unpublished observations). The pattern of inhibition we observed with 18-β-glycyrrhetinic acid was similar to that with the high (1 mM) concentration of ouabain used here. That is, 18-β-glycyrrhetinic acid not only inhibited the response to BK, but it also significantly improved the degree of block by L-NOARG. High concentrations of ouabain (0.1–1 mM) are known to prevent the formation of gap junctions and conversely 18-β-glycyrrhetinic acid is known to inhibit Na^+/K-ATPase (Rabito *et al*., 1987; Watsky *et al*., 1990). Since our findings with lower concentrations of ouabain suggest that Na^+/K^+-ATPase is not involved in the EDHF-like response in the bovine coronary artery, the shared inhibitory effects of 18-β-glycyrrhetinic and high concentrations of ouabain may implicate a role for gap junctions. It remains to be determined whether such gap junctions are involved in transfer of the EDHF-like response from endothelium to smooth muscle (i.e. myoendothelial gap junctions) or if they co-ordinate the spread of hyperpolarization within a single layer of the vessel wall (i.e. endothelial-endothelial or muscle-muscle).

In conclusion, our studies show that three non-prostanoid factors operate in parallel to mediate endothelium-dependent relaxations to BK in the bovine coronary artery. NO is the dominant factor and is responsible for most of the response. After block of NO, an EDHF-like mechanism which does not involve either endothelial K^+ or a cytochrome P_{450}-derived metabolite of arachidonic acid, compensates for nearly all of the maximum relaxation, whilst inhibition of both NO and the EDHF-like mechanism reveals a second level of backup perhaps mediated by a cytochrome P_{450}-dependent mechanism.

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