British Journal of Pharmacology (1996) 119, 1177-1186

Mechanisms of L-N^G nitroarginine/indomethacin-resistant relaxation in bovine and porcine coronary arteries

[1]Wolfgang F. Graier, *Sigrid Holzmann, Bernhard G. Hoebel, *Walter R. Kukovetz & Gert M. Kostner

Department of Medical Biochemistry, University of Graz, Harrachgasse 21/III, A-8010 Graz, Austria, and *Department of Pharmacology and Toxicology, University of Graz, Universitätsplatz 2, A-8010 Graz, Austria

1 Coronary arteries from bovines (BCA) and pigs (PCA) were used for measuring endothelium-dependent relaxation in the presence of L-N^G nitroarginine and indomethacin. As some compounds tested have been found to have an inhibitory effect on autacoid-activated endothelial Ca^{2+} signalling, endothelium-dependent relaxation was initiated with the Ca^{2+} ionophore A23187.

2 The common compounds for modulating arachidonic acid release/pathway, mepacrine and econazole only inhibited L-N^G nitroarginine-resistant relaxation in BCA not in PCA. In contrast, proadifen (SKF 525A) diminished relaxation in BCA and PCA. Mepacrine and proadifen inhibited Hoe-234-initiated relaxation in BCA and PCA, while econazole only inhibited Hoe 234-induced relaxation in PCA. Due to the multiple effects of these compounds, caution is necessary in the interpretation of results obtained with these compounds.

3 The inhibitor of Ca^{2+}-activated K^+ channels, apamin, strongly attenuated A23187-induced L-N^G nitroarginine-resistant relaxation in BCA while apamin did not affect L-N^G nitroarginine-resistant relaxation in PCA.

4 Pertussis toxin blunted L-N^G nitroarginine-resistant relaxation in BCA, while relaxation of PCA was not affected by pertussis toxin.

5 Thiopentone sodium inhibited endothelial cytochrome P450 epoxygenase (EPO) in PCA but not in BCA, while L-N^G nitroarginine-resistant relaxation of BCA and PCA were unchanged. Protoporphyrine IX inhibited EPO in BCA and PCA and abolished L-N^G nitroarginine-resistant relaxation of BCA not PCA.

6 An EPO-derived compound, 11,12-epoxy-eicosatrienoic acid (11,12-EET) yielded significant relaxation in BCA and PCA in three out of six experiments.

7 These findings suggest that L-N^G nitroarginine-resistant relaxation in BCA and PCA constitutes two distinct pathways. In BCA, activation of Ca^{2+}-activated K^+ channels via a pertussis-toxin-sensitive G protein and EPO-derived compounds might be involved. In PCA, no selective inhibition of L-N^G nitroarginine-resistant relaxation was found.

Keywords: G protein; bradykinin; A23187; endothelium-derived relaxing factor; ouabain; thiopentone sodium; protoporphyrine IX

Introduction

The vascular endothelium plays a dominant role in the local regulation of vascular tone. For this purpose endothelial cells produce, upon activation with autocoids like bradykinin, substance P or histamine, a variety of compounds with smooth muscle relaxing or contraction properties (for review, see Graier *et al.*, 1994). The three endothelium-derived compounds relax vascular smooth muscle cells via different mechanisms and have different chemical structures. The so called endothelium-derived relaxing factor, EDRF, is nitric oxide (NO) containing/releasing labile compound or NO itself which activates soluble guanylyl cyclase, resulting in a relaxation of vascular smooth muscle cells (Holzmann, 1982). Prostacyclin, another relaxing endothelium-derived factor, acts via activation of the adenylyl cyclase/adenosine 3′:5′-cyclic monophosphate (cyclic AMP) system (Holzmann *et al.*, 1980). The third relaxing compound, derived from the endothelium, relaxes smooth muscle cells by hyperpolarization (Feletou & Vanhotte, 1988). The chemical structure of this so called endothelium-derived hyperpolarizing factor (EDHF) is still unclear. There is evidence that some cytochrome P450-derived compounds might be involved in endothelium-derived smooth muscle hyperpolarization (Rubanyi & Vanhoutte, 1987). Very recently, this hypothesis has been confirmed by Hecker *et al.* (1995) who demonstrated EDHF-like activity from the cytochrome P450 mono-oxygenase-derived arachidonic acid epoxides (epoxyeicosatrienoic acids, EETs) in bovine and porcine coronary arteries. Furthermore, several groups (Gebremedhin *et al.*, 1992; Hu & Kim, 1993) clearly demonstrated that EETs stimulate K^+ channels, an effect suggested to be involved in the relaxation due to endothelium-derived hyperpolarizing factor (EDHF; for review see Harder *et al.*, 1995). However, most of the results representing EDHF as a cytochrome P450 mono-oxygenase-derived compound are due to the inhibitory properties on cytochrome P450. Inhibitors, like econazole, miconazole, SKF 525A, have been shown to possess a number of other effects, like inhibition of K^+ channels (Alvarez *et al.*, 1992a) and Ca^{2+}-ATPase inhibition (Mason *et al.*, 1993). On the other hand EDHF has been proposed to relax smooth muscle by activation of Na/K-ATPase in canine arteries (Feletou & Vanhoutte, 1988), suggested by the inhibition of EDHF-mediated relaxation with ouabain, a potent inhibitor of Na/K-ATPase. Thus, controversial results were obtained suggesting two principal mechanisms of L-N^G nitroarginine- and indomethacin-resistant relaxation: activation of smooth muscle K^+ channels and/or activation of Na/K-ATPase.

[1] Author for correspondence.

The aim of this study was first to clarify in isolated blood vessels whether the most common tools for modulating cytochrome P450 pathway(s) in the endothelium constitute other effects rather than specific inhibition of cytochrome P450 enzymes. Second, new pharmacological approaches were designed to verify whether L-N^G nitroarginine- and indomethacin-resistant relaxation constitutes a uniform mechanism in bovine and porcine coronary arteries.

Methods

Preparation of coronary artery strips

Left bovine (BCA) and right porcine coronary arteries (PCA) with intact endothelium were isolated from the hearts of adult animals obtained in the local slaughterhouse. The arteries were carefully freed from adherent tissue. Blood vessels were open longitudinally and cut alternatively from each side with a distance of 3–4 mm between each cut. To protect endothelium for damage during this procedure, touching or rubbing the intimal surface was avoided.

Organ bath studies

The strips were mounted in 5 ml organ baths containing 2 different physiological salt solutions, which were gassed with carbogen (95% O_2+5% CO_2) at 37°C. The PCA were put in Krebs Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2 and glucose 10.1 (i.e. PBS). All experiments with the BCA were performed in PBS and Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.68, $CaCl_2$ 1.36, $MgCl_2$ 1.16, $NaHCO_3$ 11.9 and glucose 10.1. The strips were allowed to equilibrate under 1 g tension for 1–1.5 h and then precontracted with 100 nM U 46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$). Changes in length were measured isotonically and calculated as % of maximum relaxation obtained at the end of each experiment by addition of 266 μM papaverine as described previously (Holzmann *et al.*, 1994).

All cumulative concentration-response curves of the Ca^{2+} ionophore A23187 and of Hoe 234 were performed in the presence of 10 μM indomethacin to exclude prostaglandin I_2 (PGI_2)-induced relaxations. Inhibition of the EDRF-mediated component of A23187-induced relaxation was achieved by pretreating the strips with 1 mM L-N^G nitroarginine for about 30 min. In the presence of L-N^G nitroarginine no increases in vessel guanosine 3′:5′-cyclic monophosphate (cyclic GMP) were measured (Holzmann *et al.*, 1994).

Determination of cyclic GMP in coronary strips

Blood vessel content of cyclic GMP was measured as described previously (Holzmann *et al.*, 1994). Briefly, vessels were freeze-clamped at maximal changes in length or during control periods, homogenized and cyclic GMP determined by use of radioimmunossay. Coronary artery cyclic GMP levels were expressed as pmol cyclic GMP g^{-1} wet wt.

Measurement of microsomal cytochrome P450 epoxygenase in endothelial cells

Endothelial cytochrome P450 epoxygenase activity was measured as described previously (Graier *et al.*, 1996). Primary cultured endothelial cells isolated from BCA and PCA were harvested by enzymatic digestion (0.025% trypsin and 0.02% EDTA in PBS), centrifuged and resuspended in intracellular-like buffer containing (mM): KCl 140, NaCl 1, $MgCl_2$ 1 and HEPES acid 10, pH adjusted at 7.1. In the presence of the NADPH-generating system (D,L-isocitric acid, NADP and isocitric-dehydrogenase) and 25 μM 1-ethoxypyrene-3,6,8-tris-(dimethyl-sulphonamide) (EPSA) cells were permeabilized by saponin (1 mg ml^{-1}). Cytochrome P450 epoxygenase-catalyzed dealkylation of EPSA to 1-hydro-xypyrene-3,6,8-tris-(dimethylsulphonamide) (HPSA) was measured at 495 nm excitation and 550 nm emission. By use of an *in situ* calibration curve of HPSA in the presence of 25 μM EPSA, absolute amounts of HPSA produced were calculated. Results are expressed in pmol HPSA $min^{-1} \times 10^6$ cells.

Statistics

Analysis of variance (ANOVA) including a *post hoc* analysis (Scheffe's F test) was used to test differences in the absence and presence of the compound to be tested. Each point represents the mean value ± s.e.mean. $P<0.05$ was considered significant and indicated with an asterisk.

Materials

A23187 (calcimycin), L-N^G nitroarginine, pertussis toxin, proadifen, econazole, mepacrine, apamin, ouabain, U 46619, were purchased from Sigma, Vienna, Austria. Indomethacin-Na-trihydrate was from MSD, Munich, Germany. (35,4R)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulfonylchroman hemihydrate (Hoe 234) was a gift of Dr B Schölkens, Hoechst AG, Frankfurt/Main, Germany. 11,12-epoxy-eicosatrienoic acid (11,12-EET) was obtained from Biomol, Hamburg, Germany; Cayman Chemicals, Vienna, Austria; Paesel & Lorei, Frankfurt/Main, Germany and Cascade Biochem LTD, Reading, U.K. All other chemicals were purchased from Merck, Darmstadt, Germany.

Results

Effects of inhibitors of the arachidonic acid/cytochrome P450 pathway on L-N^G nitroarginine-resistant relaxation

To exclude any modulating effect on endothelial Ca^{2+} signalling of the compounds used (Graier *et al.*, 1995), endothelium-dependent relaxation was initiated with the Ca^{2+} ionophore A23187 throughout the study. In the presence of indomethacin, A23187 relaxed BCA and PCA in a concentration-dependent manner (Figure 1). Maximal relaxation upon addition of 300 nM A23187 in porcine blood vessels (Figure 1b; 80.5±2.8%) was significantly greater than that obtained in bovine tissues (Figure 1a; 60.8±6.9%). However, in the presence of 1 mM L-N^G nitroarginine relaxation responses to 300 nM A23187 in both tissues were comparable (39.3±1.4 in PCA and 27.4±6.5% in BCA, respectively). While the cytochrome P450 inhibitor econazole (10 μM) failed to affect A23187-induced relaxation in PCA (Figure 1b), econazole (10 μM) diminished relaxation of BCA in the absence and presence of L-N^G nitroarginine by 43±4.4 and 42±4.1%, respectively (Figure 1a). Identical results were obtained with BCA in Tyrode solution, where econazole (10 μM) attenuated relaxation due to A23187 in the absence and presence of L-N^G nitroarginine by 49±5.0 and 47±3.5% (data not shown).

Similar to econazole, the phospholipase A_2 (PLA_2) inhibitor, mepacrine, inhibited BCA relaxation induced by A23187 in the absence (not shown) and presence of L-N^G nitroarginine in Tyrode solution (Figure 2a) and PBS (by 26±3.7 in the absence and 32±4.5% in the presence of L-N^G nitroarginine), while mepacrine failed to affect A23187-induced relaxation in PCA (Figure 2b). In contrast to econazole and mepacrine which did not affect PCA relaxation to A23187, proadifen diminished L-N^G nitroarginine-resistant relaxation in PCA and BCA (Table 1).

In view of the proposed involvement of K^+ channels in the L-N^G nitroarginine-resistant relaxation (see Introduction) the effects of the compounds used were tested on K^+ channels in intact blood vessels. Therefore, ATP-sensitive K^+ channels were activated by Hoe 234 (Busse *et al.*, 1991), which yielded a concentration-dependent relaxation of BCA and PCA. While econazole (up to 100 μM) weakly affected

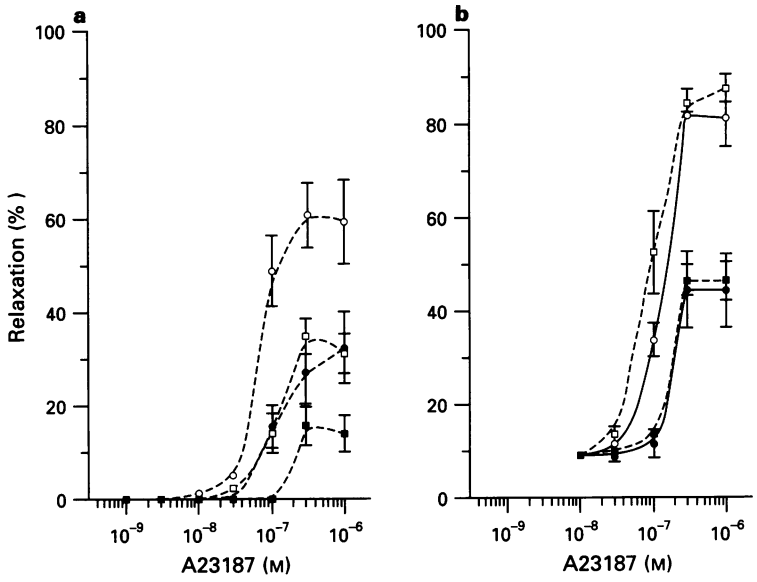

Figure 1 Concentration-relaxation relationship for A23187 in BCA (a) and PCA (b) under control conditions (○), in the presence of L-N^G nitroarginine (1 mM; ●), in the presence of econazole (10 μM; □), and in the presence of L-N^G nitroarginine (1 mM) plus econazole (10 μM) (■). All experiments were performed in PBS solution containing 10 μM indomethacin. Relaxation is expressed as % of the papaverine-induced relaxation (obtained after the experiments). Each point represents the mean and vertical lines show s.e.mean ($n=4-8$).

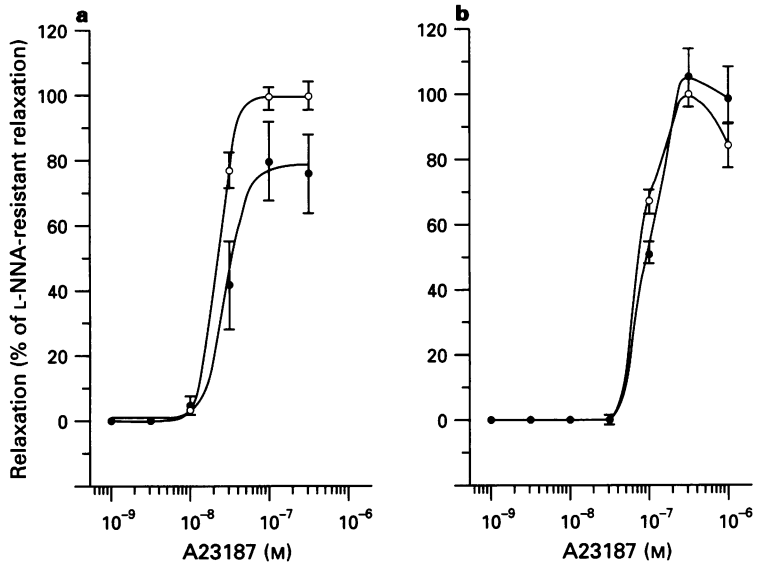

Figure 2 Effect of mepacrine (10 μM; ●) vs. control (○) on concentration-relaxation relationship for A23187 in BCA (a) and PCA (b) in the presence of L-N^G nitroarginine (1 mM) and indomethacin (10 μM). Relaxation is expressed as % of the L-N^G nitroarginine-resistant relaxation obtained in the vessels. Each point represents the mean and vertical lines show s.e.mean ($n=4$).

Hoe 234-induced relaxation in BCA in Tyrode solution (Figure 3a), econazole markedly attenuated relaxation in PCA in PBS induced by direct activation of ATP-sensitive K^+ channels (Figure 3b). Mepacrine and proadifen concentration-dependently diminished Hoe 234-induced relaxation in BCA in Tyrode solution (Figures 4a and 5a) and PCA in PBS (Figures 4b and 5b, respectively). Relaxations to Hoe 234 of BCA in PBS were similarly sensitive to mepacrine and proadifen, while the inhibitory potency of econazole was slightly increased compared with relaxations in Tyrode solution (data not shown).

In contrast to the inhibitory properties of the cytochrome P450 inhibitors, econazole and proadifen, on Ca^{2+} activated K^+ channels (Alvarez *et al.*, 1992a) and ATP-sensitive K^+ channels (this study), ouabain enhanced Hoe 234-induced relaxation in BCA (Tyrode solution) and PCA (PBS) (Table 2).

Table 1 Proadifen inhibits L-N^G nitroarginine-resistant relaxation in BCA and PCA in a concentration-dependent manner

Proadifen (μM)	*BCA* *Relaxation to A23187*	*PCA*
none	100±2.5	100±7.0
10	66.8±5.2*	73.3±2.9*
30	55.3±3.0*	27.1±2.1*
100	19.0±1.5*	10.3±0.9*

Blood vessels were precontracted with U46619 (100 nM) in PBS (PCA) and Tyrode solution (BCA) and relaxation was initiated by 100 nM A23187 in the presence of 1 mM L-N^G nitroarginine and 10 μM indomethacin. The response is expressed as % of that obtained in the absence of proadifen. *$P<0.05$ vs. relaxation in the absence of proadifen ($n=6-8$).

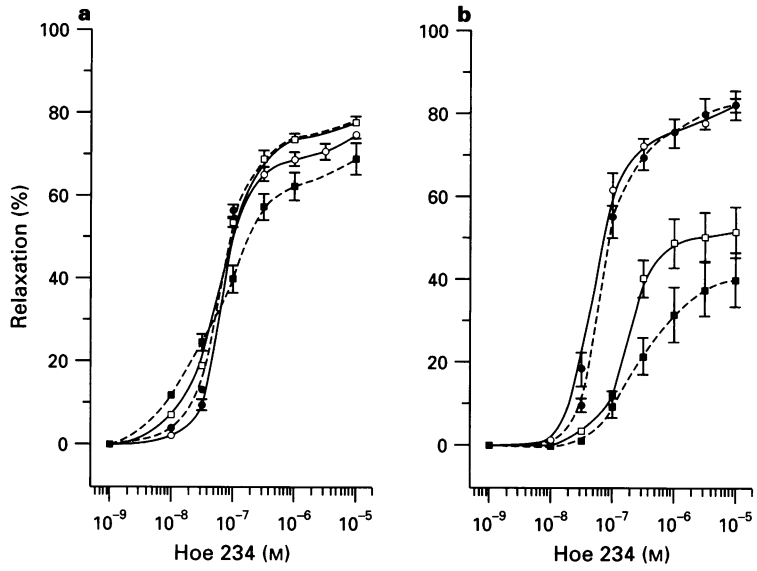

Figure 3 Concentration-relaxation for Hoe 234 in BCA (a) and PCA (b) under control conditions (○), in the presence of 1 μM (●), 10 μM (□), and 100 μM (■) econazole. Relaxation is expressed as % of the papaverine-induced relaxation (obtained after the experiments). Each point represents the mean and vertical lines show s.e.mean ($n=6$).

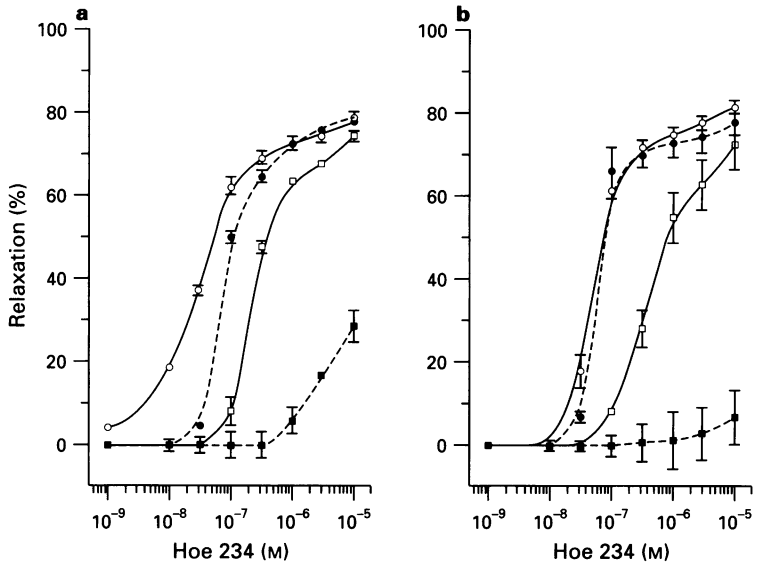

Figure 4 Effect of mepacrine on the concentration-relaxation relationship for Hoe 234 in BCA (a) and PCA (b). Relaxation was measured in the absence of mepacrine (○) and in the presence of 1 μM (●), 10 μM (□) and 100 μM (■) mepacrine. Relaxation is expressed as % of the papaverine-induced relaxation (obtained after the experiments). Each point represents the mean and vertical lines show s.e.mean ($n=6$).

Effects of apamin and ouabain on L-N^G nitroarginine-resistant relaxation of BCA and PCA

Apamin, an inhibitor of small conductance Ca^{2+}-activated K^+ channels (Hecker *et al.*, 1995) prevented L-N^G nitroarginine-resistant BCA relaxation to A23187 in PBS by about 87% (Figure 6a), while it failed to prevent L-N^G nitroarginine-resistant PCA relaxation to A23187 in PBS (Figure 6b). In Tyrode solution, apamin reduced maximal BCA relaxation to A23187 by 53% (data not shown).

Ouabain (10 μM), an inhibitor of Na/K-ATPase slightly decreased L-N^G nitroarginine-resistant relaxation to A23187 of BCA by about 19% in Tyrode solution (NS; Figure 7a). In contrast, ouabain (again 10 μM) significantly diminished BCA relaxation to A23187 in PBS by 79% (Figure 7c) and attenuated A23187-induced L-N^G nitroarginine-resistant PCA relaxation to A23187 in PBS by about 55% (Figure 7b). Therefore, the effects turned out to be significant at all concentrations of A23187 inducing a relaxation of the blood vessel.

In agreement with the differences in ouabain-mediated inhibition of A23187-induced BCA relaxation depending upon the solution used, L-N^G nitroarginine-resistant BCA relaxation initiated with A23187 was more resistant to a decrease in extracellular Na^+ concentration in Tyrode solution (by half, 38%) than in PBS (79%; data not shown). In PCA, decreasing extracellular Na^+ by half resulted in an attenuation of A23187-initiated relaxation by 82% in the presence L-N^G nitroarginine.

Effect of pertussis toxin on L-N^G nitroarginine-resistant relaxation of BCA and PCA

Pretreatment with pertussis toxin diminished A23187-induced L-N^G nitroarginine-resistant relaxation of BCA in a

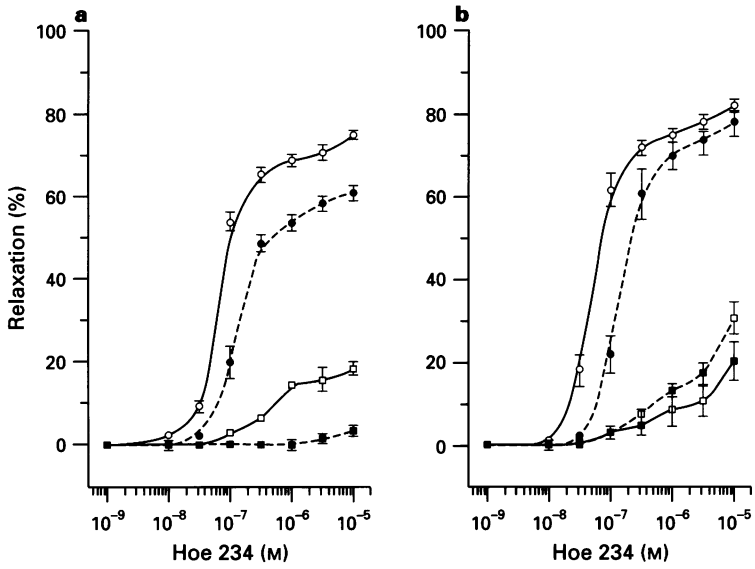

Figure 5 Concentration-relaxation relationship for Hoe 234 in BCA (a) and PCA (b) under control conditions (○), in the presence of 1 μM (●), 10 μM (□), and 100 μM (■) proadifen. Relaxation is expressed as % of the papaverine-induced relaxation (obtained after the experiments). Each point represents the mean and vertical lines show s.e.mean ($n=6$).

concentration-dependent manner (Figure 8, solid columns; Tyrode solution), while it failed to affect L-N^G nitroarginine-resistant relaxation in PCA (Figure 8, open columns; PBS). The inhibitory properties of pertussis toxin on A23187-initiated relaxation of BCA remained unchanged when PBS was used (reduction by 87% at 400 μg ml^{-1} pertussis toxin).

Effect of the cytochrome P450 epoxygenase-derived compound, 11,12-epoxy-eicosatrienoic acid (11,12-EET) on blood vessel tone

The effect of 11,12-EET, a candidate for EDHF (Harder *et al.*, 1995; Hecker *et al.*, 1995), was tested on BCA and PCA with endothelium intact or removed. When the stock solution (50 μg/ml^{-1} in ethanol) was used, 11,12-EET-induced relaxation responses (312 nM and 3.12 μM) in BCA and PCA were indistinguishable from those measured with the solvent alone. When the 11,12-EET solution was concentrated 10 fold, the relaxation induced in three out of six experiments was significantly greater than that induced by the corresponding solvent control (ethanol; Table 3). In addition, the kinetics of the 11,12-EET-initiated relaxation were compared with those of an equipotent concentration of A23187 (i.e. 100 nM) in the presence of L-N^G nitroarginine (1 mM); the time needed to obtain 50% of the effect (τ) with 11,12-EET was significantly slower in BCA and PCA than that of A23187 (Table 4).

Effect of thiopentone sodium on endothelial epoxygenase and L-N^G nitroarginine-resistant relaxation

Very recently, thiopentone sodium was shown to prevent L-N^G nitroarginine-resistant relaxation in rabbit carotid artery (Lischke *et al.*, 1995). By use of our fluorescence enzyme assay for microsomal cytochrome P450 epoxygenase in endothelial cells (Graier *et al.*, 1996), the inhibitory effect of thiopentone sodium on this enzyme was tested in BCA and PCA (Figure 9). While thiopentone sodium (100 μM) weakly attenuated microsomal cytochrome P450 epoxygenase in endothelial cells isolated from BCA by 14.7% (NS vs. enzyme activity in the absence of thiopentone sodium), enzyme activity in endothelial cells derived from PCA was diminished by 50.4% ($P<0.05$ vs. enzyme activity in the absence of thiopentone sodium).

The effect of thiopentone sodium on blood vessel relaxation was also studied. Thiopentone sodium (100 μM) failed to change the relaxation of BCA (Figure 10a) and PCA (Figure 10b) induced by A23187 in PBS in the absence and presence of L-N^G nitroarginine.

Table 2 Ouabain enhanced relaxation of BCA and PCA by activation of ATP-sensitive K^+ channels with Hoe 234

Ouabain (μM)	*BCA* *Relaxation to Hoe 234 (%)*	*PCA*
None	35.5±1.3	58.7±3.6
1	59.3±10.8*	73.5±4.3*
10	78.3±5.2*†	84.3±1.9*†

Blood vessel relaxation induced by 30 nM Hoe 234 in the presence or absence of ouabain. Relaxation is expressed as % of the maximal relaxation obtained to papaverine. *$P<0.05$ vs. relaxation in the absence of oaubain and †$P<0.05$ vs. relaxation in the presence of 1 μM ouabain ($n=4-8$).

Effect of protoporphyrine IX on L-N^G nitroarginine-resistant relaxation

In the presence of protoporphyrine IX maximal BCA relaxation to A23187 (1 μM) was strongly diminished; protoporphyrine IX reduced BCA relaxation in PBS by 59% under control conditions (Figure 11), which was similar to the inhibition induced by L-N^G nitroarginine (1 mM). In the presence of L-N^G nitroarginine (1 mM) and protoporphyrine (1 μM), BCA relaxation in PBS to A23187 (3 nM to 1 μM) was attenuated (Figure 11). Protoporphyrine IX slightly decreased A23187-initiated increases in vessel cyclic GMP content from 46.6±6.0 to 28.0±10.4 pmol cyclic GMP g^{-1} wet wt. (NS, $n=5$). In contrast, in the PCA propoporphyrine IX either alone or in combination with L-N^G nitroarginine had no effect on maximal relaxation and cyclic GMP increase due to A23187 (1 μM; Figure 11). In PCA, A23187-induced increases in vessel cyclic GMP content remained unchanged by protoporphyrine IX (control: 220.0±48.0 and protoporphyrine IX: 224.3±50.3; NS, $n=5$).

Discussion

The data presented in this study clearly indicate that A23187-initiated L-N^G nitroarginine-resistant relaxation in bovine and

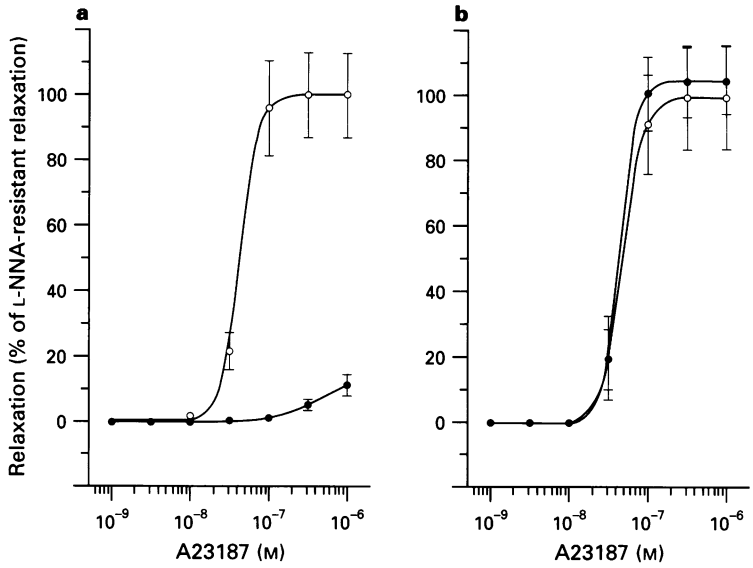

Figure 6 Effect of apamin on L-N^G nitroarginine-resistant relaxation induced by A23187 in BCA (a) and PCA (b) in PBS. In all experiments L-N^G nitroarginine (1 mM) and indomethacin (10 μM) were present and the concentration-relaxation relationship for A23187 is shown in the absence (○) and presence (●) of 1 μM apamin. Relaxation is expressed as % of the maximal relaxation obtained in the presence of L-N^G nitroarginine and indomethacin. Points represent the mean and vertical lines show s.e.mean ($n=4-8$).

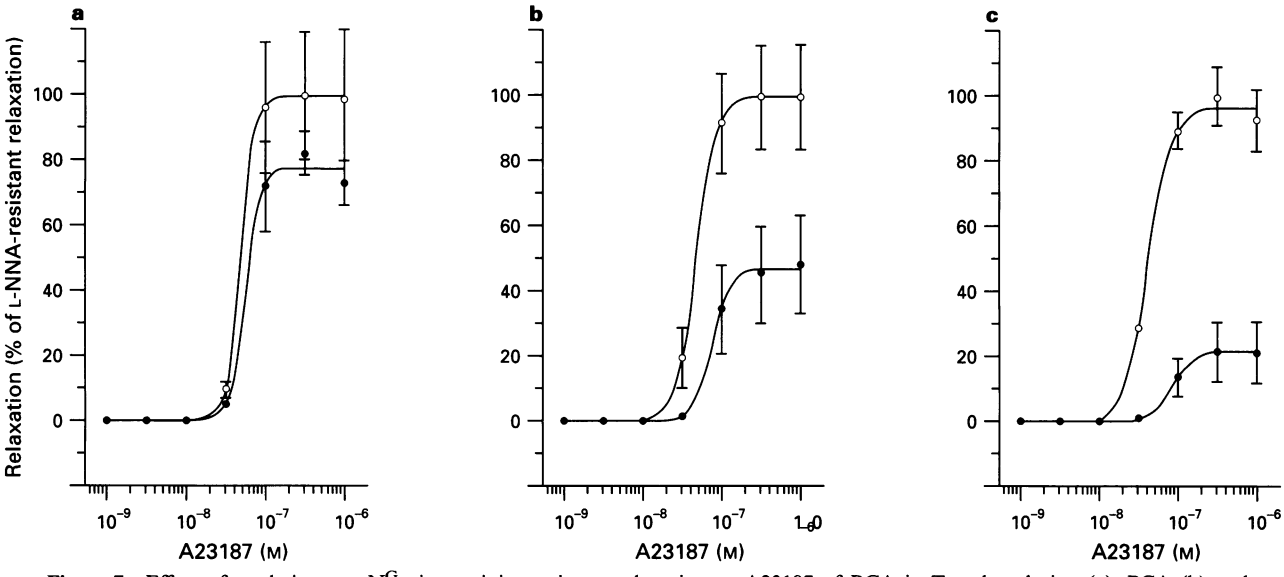

Figure 7 Effect of ouabain on L-N^G nitroarginine-resistant relaxation to A23187 of BCA in Tyrode solution (a), PCA (b) and BCA (c) in PBS. In all experiments L-N^G nitroarginine (1 mM) and indomethacin (10 μM) were present and the concentration-relaxation relationship for A23187 is shown in the absence (○) and presence (●) of ouabain (10 μM). Relaxation is expressed as % of the maximal relaxation obtained in the presence of L-N^G nitroarginine and indomethacin. Points represent the mean and vertical lines show s.e.mean ($n=4-8$).

porcine coronary arteries does not represent a uniform mechanism. In BCA, the remaining relaxation in the presence of L-N^G nitroarginine and indomethacin depends on the activation of Ca^{2+}-activated K^+ channels (K_{Ca} channels) via a pertussis toxin-sensitive pathway (G_i family G protein) in which possibly cytochrome P450 epoxygenase-derived compound(s) is (are) involved. In contrast, L-N^G nitroarginine/indomethacin-resistant relaxation to A23187 of PCA was resistant to pertussis toxin, K_{Ca} channel blockade and inhibition of cytochrome P450 epoxygenase, while it was sensitive to proadifen and a reduction in extracellular Na^+. An overview of our data presented in this paper is shown in Table 5.

Formation of EDHF essentially depends on increases in endothelial free Ca^{2+} concentration due to stimulation with autacoids like bradykinin or the Ca^{2+} ionophore, A23187 (Suzuki *et al.*, 1992). To investigate a possible involvement of cytochrome P450 enzyme in the effect of L-N^G nitroarginine-resistant relaxation we used well known inhibitors for this type of enzyme, like econazole, mepacrine and proadifen. However, several groups have shown that these cytochrome P450-inhibitors inhibit Ca^{2+} entry evoked by Ca^{2+} store depletion in various non-excitable cells, such as platelets (Sargeant *et al.*, 1992), rat thymocytes (Alvarez *et al.*, 1991), neutrophils (Montero *et al.*, 1991; 1992) and endothelial cells (Graier *et al.*, 1992). In addition, the involvement of cytochrome P450 enzyme(s) in the generation of the cellular signal cascade for Ca^{2+} entry activation in non-excitable cells has very recently been suggested (Alvarez *et al.*, 1991; 1992b; Graier *et al.*, 1995). Finally, IC_{50} values of these compounds for preventing autacoid-stimulated Ca^{2+} entry in endothelial cells are ten times lower than those obtained for inhibition of cytochrome P450 enzymes. With

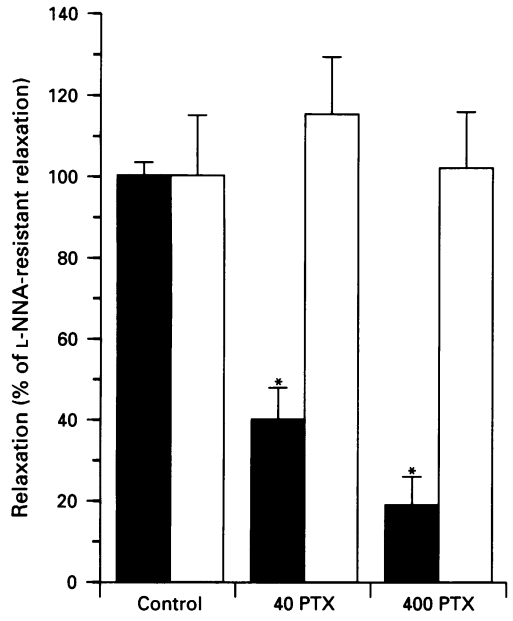

Figure 8 Effect of various concentrations (ng ml^{-1}) of pertussis toxin (PTX) on endothelium-dependent relaxation of BCA in Tyrode solution (solid columns) and PCA in PBS (open columns) to A23187 (100 nM) in the presence of L-N^G nitroarginine (1 mM) and indomethacin (10 μM). Relaxation is expressed as % of the maximal obtained in these vessels in the presence of L-N^G nitroarginine and indomethacin. Each column represents the mean and vertical lines show s.e.mean ($n=6-14$). *$P<0.05$ vs. relaxation in the absence of pertussis toxin.

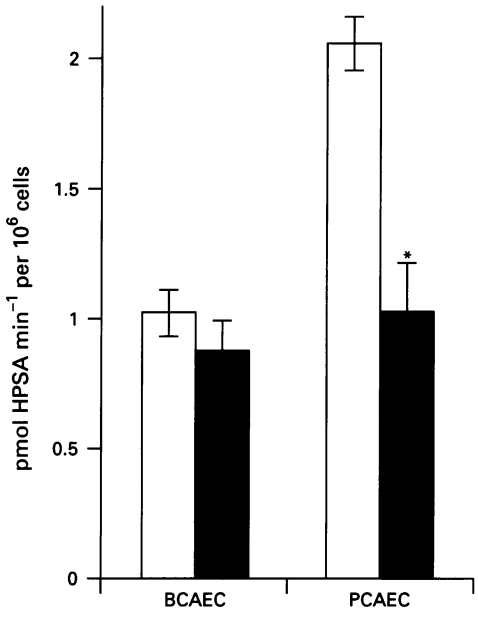

Figure 9 Effect of thiopentone sodium on microsomal cytochrome P450 epoxygenase activity in endothelial cells derived from BCA (BCAEC) and PCA (PCAEC). Enzyme activity was determined by the conversion of 1-ethoxypyrene-3,6,8-tris-(dimethylsulphonamide) (EPSA) to 1-hydroxypyrene-3,6,8-tris-(dimethylsulphonamide) (HPSA) in the absence of (open columns) and presence (solid columns) of 100 μM thiopentone sodium and is expressed as pmol HPSA detected per min and 10^6 cells. Each column represents the mean ± s.e.mean ($n=3-16$). *$P<0.05$ vs. enzyme activity in the absence of thiopentone sodium.

Table 3 Comparison of the relaxing effect of 11, 12-epoxy-eicosatrienoic acid (11,12-EET) with the effect of the corresponding solvent (ethanol) concentrations

	Concentration (μM)	*BCA Relaxation (%)*	*PCA*
11,12-EET	3.12	3.5±1.5*	5.5±1.5
Ethanol	corr. to 3.12 μM 11,12-EET	0.0±0.0	1.5±1.0
11,12-EET	6.24	16.5±1.5*	26.0±8.7
Ethanol	corr. to 6.24 μM 11,12-EET	5.3±1.8	5.7±1.3

Relaxation of pre-contracted BCA and PCA upon addition of 11,12-EET (in ethanol solution) or the corresponding ethanol quantity was determined in PBS. Ethanolic solutions of 11,12-EET (50 μg ml^{-1}) were concentrated 10 fold by lyophilization immediately before the experiments. There was no difference between the effect of 11,12-EET or ethanol in strips with and without endothelium. Relaxation response is expressed as % of the maximal relaxation induced by papaverine (see Methods). *$P<0.05$ vs. solvent control ($n=3-8$).

Table 4 Comparison of the time needed to obtain 50% of the effect of a single dose (τ) with equipotent concentrations of 11,12-epoxy-eicosatrienoic acid (11,12-EET) and A23187

Compound	*Conc. (μM)*	τ in BCA (min)	τ in PCA (min)
11,12-EET	6.24	2.75±1.25	3.25±0.25
A23187	0.10	1.20±0.12*	1.57±0.27*

*$P<0.05$ vs. τ measured for 11,12-EET-initiated relaxation.

regard to the effects of the cytochrome P450 inhibitors on autacoid-initiated Ca^{2+} signalling in endothelial cells, the Ca^{2+} ionophore A23187 was used throughout the study to elevate endothelial Ca^{2+} levels by a non-cytochrome P450-dependent mechanism (Graier *et al.*, 1995).

The most common compounds used for preventing cytochrome P450 enzyme activity (i.e. econazole, proadifen) and interfering with arachidonic acid release (mepacrine) also modulate blood vessel relaxation by the ATP-sensitive K^+ channel activator, Hoe 234 (Busse *et al.*, 1991). Mepacrine and proadifen were equally potent in BCA and PCA as inhibitors of Hoe 234-induced relaxation. In contrast, econazole weakly affected Hoe 234-induced relaxation in BCA, while it was more potent in preventing relaxation due to Hoe 234 in PCA. These data indicate for the first time, that beside Ca^{2+}-activated K^+ channels (Alvarez *et al.*, 1992a), these so-called cytochrome P450 inhibitors strongly inhibit ATP-sensitive K^+ channel activity in smooth muscle cells of BCA and PCA. Thus, results obtained with these compounds may be used to exclude the involvement of cytochrome P450 enzymes, Ca^{2+}-activated or ATP-sensitive and Ca^{2+} activated K^+ channels if no inhibition is found. However, caution is necessary to speculate over an involvement of cytochrome P450 enzyme(s) simply because the compounds mentioned above showed some inhibitory effect.

With respect to these findings, our results that econazole and mepacrine did not affect A23187-initiated L-N^G nitroarginine-resistant relaxation of PCA suggest that this relaxation is not due to activation of these types of K^+ channels or cytochrome P450 enzyme(s). The lack of an involvement of cytochrome P450 enzymes and/or K^+ channels is further confirmed by our findings that the inhibitor of Ca^{2+} activated K^+ channels apamin and the cytochrome P450 epoxygenase inhibitors thiopentone sodium and protoporphyrine IX had no effect on A23187-initiated relaxation in PCA. Hence, proadifen which also affected A23187-induced L-N^G nitroarginine-resistant relaxation of PCA may have additional inhibitory properties on L-N^G nitroarginine-resistant relaxation in PCA.

In contrast to PCA, L-N^G nitroarginine-resistant relaxation of BCA was highly sensitive to econazole, which has been shown to inhibit Ca^{2+}-activated K^+ channels (Alvarez

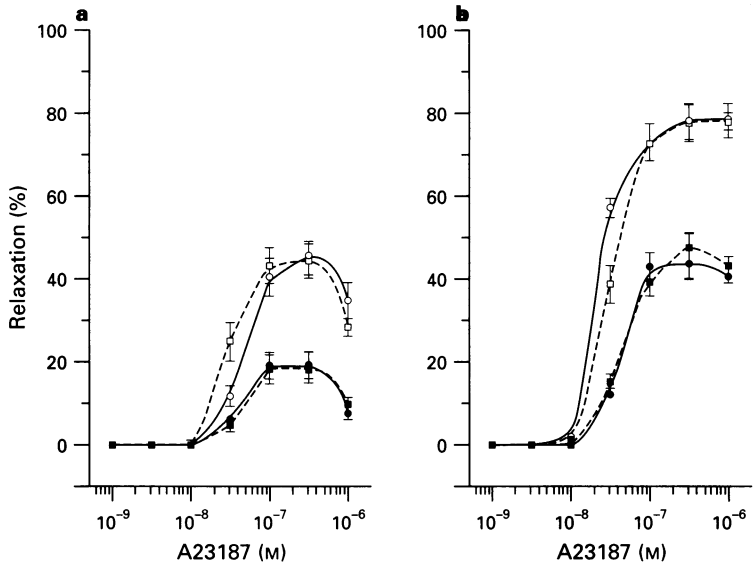

Figure 10 Concentration-relaxation relationship for A23187 in BCA (a) and PCA (b) under control conditions (○), in the presence of L-N^G nitroarginine (1 mM; ●), in the presence of thiopentone sodium (100 μM; □), and in the presence of L-N^G nitroarginine (1 mM) plus thiopentone sodium (100 μM; ■). In all experiments indomethacin (10 μM) was present. Relaxation is expressed as % of the papaverine-induced relaxation (obtained after the experiments). Points represent the mean and vertical lines show s.e.mean ($n=17-18$).

et al., 1992a). In agreement with these results, the inhibitor of Ca^{2+}-activated K^+ channels, apamin (Hecker *et al.*, 1995), inhibited L-N^G nitroarginine-resistant relaxation of BCA, while it was without effect on the relaxation of PCA resistant to L-N^G nitroarginine. Convincingly, the inhibitory potency of these compounds on Ca^{2+}-activated K^+ channels (Alvarez *et al.*, 1992a) and ATP-sensitive K^+ channels (this study) are almost identical to that obtained on A23187-induced L-N^G nitroarginine-resistant relaxation of BCA. These data are in agreement with our previous findings demonstrating the efficiency of other K^+ channel inhibitors in preventing EDHF in BCA (Holzman *et al.*, 1994) and suggest, that in A23187-induced L-N^G nitroarginine-resistant relaxation of BCA, activation of Ca^{2+}-activated K^+ channels is involved.

A further possibility is that direct inhibition of the EDHF forming cytochrome 450 epoxygenase by these compounds might explain the inhibitory properties of econazole and proadifen on BCA relaxation (Harder *et al.*, 1995; Hecker *et al.*, 1995). However, the potencies of econazole and proadifen for inhibition of endothelial cytochrome P450 epoxygenase are about 3 to 5 times lower than for inhibition of A23187-initiated L-N^G nitroarginine-resistant relaxation of BCA (Graier *et al.*, 1996). Although the inhibitory properties of econazole and proadifen on A23187-induced L-N^G nitroarginine-resistant relaxation of BCA are more likely due to inhibition of EDHF-activated K^+ channels than to the inhibition of endothelial cytochrome P450 epoxygenase, at least at the lower concentrations, an involvement of cytochrome P450 epoxygenase cannot be ruled out completely. While the low sensitivity of imidazole-antimycotics, proadifen and mepacrine does not allow us to speculate about the involvement of cytochrome P450 epoxygenase in L-N^G nitroarginine-resistant relaxation of BCA, convincing results were obtained with protoporphyrine IX. This compound inhibited cytochrome P450 epoxygenase in endothelial cells isolated from BCA and thereby abolished L-N^G nitroarginine-resistant relaxation, without having a strong effect on nitric oxide synthase and K^+ channels activated by Hoe-234 (data not shown). Thiopentone sodium, a compound recently shown to prevent EDHF-mediated relaxation in rabbit carotid artery (Lischke *et al.*, 1995), failed to affect L-N^G nitroarginine-resistant relaxation in BCA. This might be related to the inability of thiopentone sodium to prevent cytochrome P450 epoxygenase activity in endothelial cells

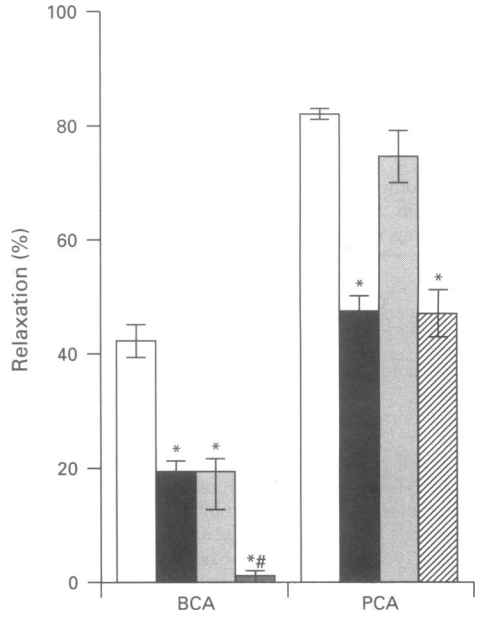

Figure 11 Endothelium-dependent relaxation of BCA and PCA in PBS to A23187 (1 μM) under control conditions (open columns), in the presence of L-N^G nitroarginine (1 mM; solid columns), in the presence of protoporphyrine IX (1 μM; stippled columns) and in the presence of 1 mM L-N^G nitroarginine and 1 μM protoporphyrine IX (1 μM; hatched columns). In all experiments indomethacin (10 μM) was present. Relaxation is expressed as % of the maximal relaxation obtained to papaverine. Each column represents the mean and vertical lines show s.e.mean ($n=10-18$). *$P<0.05$ vs. relaxation under control conditions and $^{\#}P<0.05$ vs. relaxation in the presence of L-N^G nitroarginine.

derived from BCA, which suggests that different isoforms of these enzymes exist in BCA and PCA. It has been shown that heme oxygenase, another protoporphyrine-IX-sensitive enzyme, generates carbon monoxide, resulting in an increase in smooth muscle cyclic GMP associated with vessel relaxation (Verma *et al.*, 1993). Since we did not find an increase in cyclic GMP in the presence of L-N^G nitroarginine in BCA and PCA, the possible involvement of heme-oxy-

Table 5 Comparisons between the pharmacological modulation of the L-N^G nitroarginine-resistant relaxation of BCA and PCA in PBS

	Concentration	*Inhibitory action on:*	*BCA*	*PCA*
Ecanazole	$>1\,\mu M$	K_{Ca} > CaATPase, cytP450	+	−
Mepacrine	$>1\,\mu M$	K_{Ca} > PLA_2	+	−
Proadifen	$>1\,\mu M$	? > cytP450	+	+
Ouabain	$10\,\mu M$	Na^+/k^+-ATPase, Depol.	+	+
Low $[Na^+]_e$	<70 mM	Na^+ gradient ↓, Depol.	+	+
Apamin	$1\,\mu M$	K_{Ca}	+	−
Pertussis toxin	>40 ng ml^{-1}	G_i	+	−
Thiopentone-Na	$100\,\mu M$	cytP450 epoxygenase	−	−
Protoporphyrine	$1\,\mu M$	cytP450 epoxygenase	+	−

+: strongly inhibited; +/−: weakly inhibited; −: not affected.

genase-derived carbon monoxide as EDHF can be excluded. Overall, these data suggest that in BCA, L-N^G nitroarginine-resistant relaxation is related to the activation of cytochrome P450 epoxygenase.

Our finding that ouabain strongly inhibited the L-N^G nitroarginine-resistant relaxation induced by A23187 in PCA and BCA in PBS is in agreement with previous data demonstrating that acetylcholine-induced L-N^G nitroarginine-resistant relaxation of canine arteries is prevented by ouabain (Feletou & Vanhoutte, 1988; Hoeffner *et al.*, 1989). Convincingly, A23187-induced L-N^G nitroarginine-resistant relaxation of PCA and BCA in PBS strictly depends on extracellular Na^+ ($[Na^+]_e$). However, the effect of ouabain and decreased $[Na^+]_e$ on L-N^G nitroarginine-resistant relaxation strongly depends on the experimental solutions used. In Tyrode solution, ouabain and a decrease in $[Na^+]_e$ had much less effect on L-N^G nitroarginine-resistant relaxation of BCA; this has been also shown in guinea-pig coronary arteries (Chen & Cheung, 1992), rat mesenteric arteries (Ayotunde *et al.*, 1991) and human coronary arteries (Nakashima *et al.*, 1993). Since the inhibitory properties of ouabain and a reduced $[Na^+]_e$ critically depend on the solutions used, the effect observed with PBS is more likely due to some depolarizing effect of ouabain and reduced $[Na^+]_e$ rather than interference with EDHF directly. Thus, caution is needed when these pharmacological tools are used for studying L-N^G nitroarginine-resistant relaxation.

Very recently, the cytochrome P450-derived arachidonic acid epoxides (EETs) have been suggested to be released from the endothelial cells as EDHF (Harder *et al.*, 1995; Hecker *et al.*, 1995). This study provides evidence for an involvement of cytochrome P450 epoxygenase in L-N^G nitroarginine-sensitive relaxation in BCA but not in PCA (see discussion above). In our experiments, the cytochrome P450 epoxygenase-derived compound 11,12-EET yielded significant relaxation in BCA and PCA. By comparing the time needed to evoke 50% of the relaxation of a given dose (τ), we demonstrated that the relaxant response to 11,12-EET was induced at a slightly slower rate than that to an equipotent concentration of A23187 in the presence of L-N^G nitroarginine. This might be due to the fact that extracellularly applied 11,12-EET has to diffuse to the smooth muscle cells, while EDHF is vectorially released from the endothelial cells towards the smooth muscle layer.

In agreement with our results, indicating two distinct pathways of A23187-induced L-N^G nitroarginine-resistant relaxation in BCA and PCA, the effect of pertussis toxin differed between BCA and PCA. In PCA, A23187-induced L-N^G nitroarginine-resistant relaxation was not affected by pertussis toxin, which excludes a major G_i proteins in this type of relaxation. Similar results were presented by Lüscher & Noll (1994) who found that pertussis toxin did not affect bradykinin-induced relaxation of PCA in the presence and absence of L-N^G nitroarginine. In contrast, pertussis toxin markedly attenuated L-N^G nitroarginine-resistant relaxation of BCA. The (bromo)-A23187-induced Ca^{2+} increase in endothelial cells freshly isolated from BCA measured by a microfluorometric fura-2 method (Graier *et al.*, 1995) was not affected by pertussis toxin (data not shown). Thus, pertussis toxin either interferes with the formation of EDHF directly or, more likely, prevents the effect of EDHF on smooth muscle K^+ channels. The involvement of pertussis toxin-sensitive G protein in muscarinic receptor-mediated inhibition of Ca^{2+}-activated K^+ channels has been described in vascular smooth muscle cells (Kume & Kotlikoff, 1991), airway smooth muscle cells (Kume *et al.*, 1995) and colonic smooth muscle (Cole *et al.*, 1989). Furthermore G protein-regulated K^+ channels have been shown in the epithelial-cell line (Hemada *et al.*, 1993), cortical collecting duct cells (Suzuki *et al.*, 1994), adrenal chromaffin cells (Cannon *et al.*, 1994) and rabbit atrai (Ray & Macheod, 1994). Since the nature of L-N^G nitroarginine-resistant muscle cell relaxation remains unclear, we cannot speculate whether this compound or pathway relaxes smooth muscle cells via a receptor-G protein-K^+ channel cascade or via direct activation of G protein within the smooth muscle membrane. As far as we know this is the first study to describe a pertussis toxin-sensitive mechanism in L-N^G nitroarginine-resistant relaxation of BCA.

Our data (summarized in Table 5) revealed that A23187-initiated L-N^G nitroarginine-resistant relaxation of BCA and PCA differ markedly in terms of pharmacological modulation by econazole, mepacrine, apamin, protoporphyrine IX and pertussis toxin. In PCA, vessel relaxation due to A23187 in the presence of L-N^G nitroarginine is not mediated by apamin-sensitive, Ca^{2+} activated K^+ channels and cytochrome P450 epoxygenase, although cytochrome P450 epoxygenase is present in PCA and 11,12-EET evokes relaxation. Based on the inhibitory properties of pertussis toxin, econazole, apamin and protoporphyrine IX, the involvement of G protein-regulated Ca^{2+}-activated K^+ channels in A23187-initiated L-N^G nitroarginine-resistant relaxation of BCA seems likely. Thus, L-N^G nitroarginine-resistant relaxation does not constitute a uniform mechanism and includes at least two distinct pathways in BCA and PCA.

The authors wish to thank Mrs Betty Oberer for her excellent technical assistance and Prof. Dr Gerald Pöch for his critical review of the manuscript. This work was supported by grants of the Austrian Science Fund (P-10029-Med.), the Austrian National Bank (Jubiläumsfondsprojekt 5406 to W.F.G.), and Franz Lanyar Stiftung as an associate member of a project of the European Union (EureCa:No. BMH1-CT94-1375).

References

ALVAREZ, J., MONTERO, M. & GARCÍA-SANCHO, J. (1991). Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. *Biochem. J.*, **274**, 193–197.

ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992a). High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *J. Biol. Chem.*, **267**, 11789–11793.

ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992b). Cytochrome P450 may regulate plasma membrane Ca^{2+} permeability according to the filling state of the intracellular Ca^{2+} stores. *FASEB J.*. **6,** 786–792.

AYOTUNDE, S., AGEAGBO, O. & MALIK, K.U. (1991). Contribution of K^+ channels to arachidonic acid-induced endothelium-dependent vasodilation in rat isolated perfused mesenteric arteries. *J. Pharmacol. Exp. Ther.*, **258,** 452–458.

BUSSE, R., LÜCKHOFF, A. & MÜLSCH, A. (1991). Cellular mechanisms controlling EDRF/NO formation in endothelial cells. *Basic Res. Cardiol.*, **86** (Suppl. 2), 7–16.

CANNON, S.D., WILSON, S.P. & WALSH, K.B. (1994). A G-protein-activated K^+ current in bovine adrenal chromaffin cells – Possible regulatory role in exocytosis. *Molec. Pharmacol.*, **45,** 109–116.

CHEN, G. & CHEUNG, D.W. (1992). Characterization of acetylcholine-induced membrane hyperpolarization in endothelial cells. *Circ. Res.*, **70,** 257–263.

COLE, W.C., CARL, A. & SANDERS, K.M. (1989). Muscarinic suppression of the Ca^{2+}-dependent K current in colonic smooth muscle. *Am. J. Physiol.*, **257,** C481–C487.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93,** 515–524.

GEBREMEDHIN, D., MA, Y.-H., FALCK, J.R., ROMAN, R.J., VANROLLINS, M. & HARDER, D.R. (1992). Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am. J. Physiol.*, **263,** H519–H525.

GRAIER, W.F., GROSCHNER, K., SCHMIDT, K. & KUKOVETZ, W.R. (1992). SK&F 96365 inhibits histamine-induced formation of endothelium-derived relaxing factor in human endothelial cells. *Biochem. Biophys. Res. Commun.*, **186,** 1539–1545.

GRAIER, W.F., SIMECEK, S., HOEBEL, B.G., STUREK, M., KUKOVETZ, W.R. & KOSTNER, G.M. (1996). Cytochrome P450 monooxygenase regulates endothelial Ca^{2+} entry and formation of endothelium-derived relaxing factors. In *Endothelium-Derived Hyperpolarizing Factor*, ed Vanhoutte P.M. London: Peters Press. (in press).

GRAIER, W.F., SIMECEK, S. & STUREK, M. (1995). Cytochrome P450 mono-oxygenase-regulated signaling of endothelial Ca^{2+} entry. *J. Physiol.*, **482,** 259–274.

GRAIER, W.F., STUREK, M. & KUKOVETZ, W.R. (1994). Ca^{2+} regulation and endothelial vascular function. *Endothelium*, **1,** 223–236.

HARDER, D.R., CAMPBELL, W.B. & ROMAN, R.J. (1995). Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. *J. Vasc. Res.*, **5,** 79–92.

HECKER, M., BARA, A.T., BAUERNSACHS, J. & BUSSE, R. (1995). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481,** 407–414.

HEMADA, E., NAKAJIMA, T., OTA, S., TERANO, A., OMATA, M., NAKADE, S., MIKOSHIBA, K. & KURACHI, Y. (1993). Activation of a Ca^{2+}-dependent K^+ current by acetylcholine and histamine in a human epithelial-cell line. *J. Gen. Physiol.*, **102,** 667–692.

HOEFFNER, U., FELETOU, M., FLAVAHAN, N.A. & VANHOUTTE, P.M. (1989). Canine arteries release two different endothelium-derived relaxing factors. *Am. J. Physiol.*, **257,** H330–H333.

HOLZMANN, S. (1982). Endothelium-induced relaxation by acetylcholine is associated with larger rises in cyclic GMP in coronary artery strips. *J. Cyclic Nucleotide Protein Res.*, **8,** 409–419.

HOLZMANN, S., KUKOVETZ, W.R. & SCHMIDT, K. (1980). Mode of action of coronary arterial relaxation by prostacyclin. *J. Cyclic Nucleotide Protein Res.*, **6,** 451–460.

HOLZMANN, S., KUKOVETZ, W.R., WINDISCHHOFER, W., PASCHKE, E. & GRAIER, W.F. (1994). Pharmacologic differentiation between endothelium-dependent relaxations sensitive and resistant to nitro-L-arginine in coronary arteries. *J. Cardiovasc. Pharmacol.*, **23,** 747–756.

HU, S. & KIM, H.S. (1993). Activation of K^+ channels in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acids. *Eur. J. Pharmacol.*, **230,** 215–221.

KUME, H. & KOTLIKOFF, M.I. (1991). Muscarinic inhibition of single K_{Ca} channels in smooth muscle cells by a pertussis toxin-sensitive G protein. *Am. J. Physiol.*, **261,** 1204–1209.

KUME, H., MIKAWA, K., TAKAGI, K. & KOTLIKOFF, M.I. (1995). Role of G-proteins and K^+-Ca^{2+}-channels in the muscarinic and beta adrenergic regulation of airway smooth muscle. *Am. J. Physiol.*, **12,** L221–L229.

LISCHKE, V., BUSSE, R. & HECKER, M. (1995). Selective inhibition by barbiturates of the synthesis of endothelium-derived hyperpolarizing factor in the rabbit carotid artery.*Br. J. Pharmacol.*, **115,** 969–974.

LÜSCHER, T.F. & NOLL, G. (1994). Endothelium dysfunction in the coronary circulation. *J. Cardiovasc. Pharmacol.*, **24** (Suppl. 3), S16–S26.

MASON, J.M., MAYER, B. & HYMEL, L.J. (1993). Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole and SKF 96365. *Am. J. Physiol.*, **264,** C654–C662.

MONTERO, M., ALVAREZ, J. & GARCÍA-SANCHO, J. (1991). Agonist-induced Ca^{2+} influx into human neutrophils is secondary to the emptying of intracellular Ca^{2+} stores. *Biochem. J.*, **277,** 73–79.

MONTERO, M., ALVAREZ, J. & GARCÍA-SANCHO, J. (1992). Control of plasma-membrane Ca^{2+} entry by the intracellular Ca^{2+} stores – Kinetic evidence for a short-lived mediator. *Biochem. J.*, **288,** 519–525.

NAKASHIMA, M., MOMBOULI, J.-V., TAYLOR, A.A. & VANHOUTTE, P.M. (1993). Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J. Clin. Invest.*, **92,** 2867–2871.

RAY, A. & MACHEOD, K.M. (1994). Effect of carbachol in the absence and presence of phenylephrine on Rb^+ efflux and tension in rabbit left atria. *Eur. J. Pharmacol.*, **256,** 311–319.

RUBANYI, G.M. & VANHOUTTE, P.M. (1987). Nature of endothelium-derived relaxing factor: are there two relaxing mediators? *Circ. Res.*. **61,** I161–I167.

SARGEANT, P., CLARKSON, W.D., SAGE, S.O. & HEEMSKERK, J.W.M. (1992). Calcium influx evoked by Ca^{2+} store depletion in human platelets is more susceptible to cytochrome P-450 inhibitors than receptor-mediated calcium entry. *Cell Calcium*, **13,** 553–564.

SUZUKI, H., CHENG, G. & YAMAMOTO, Y. (1992). Endothelium-derived hyperpolarizing factor (EDHF). *Jap. Circ. J.*, **56,** 170–174.

SUZUKI, M., TAKAHASHI, K. & SAKAI, O. (1994). Regulation by GTP of Ca^{2+}-activated K^+ channels in the apical membrane of rabbit cortical collecting duct cells. *J. Membrane Biol.*, **141,** 43–50.

VERMA, A., HIRSCH, D.J., GLATT, C.E., RONNETT, G.V. & SNYDER, S.H. (1993). Carbon monoxide: a putative neural messenger. *Science*, **259,** 381–384.

(Received July 18, 1996
Revised August 12, 1996
Accepted August 16, 1996)