British Journal of Pharmacology (2003) 138, 39–46 © 2003 Nature Publishing Group All rights reserved 0007–1188/03 $25.00

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Acetylcholine-induced vasodilation may depend entirely upon NO in the femoral artery of young piglets

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1 To characterize agonist-induced relaxation in femoral artery rings from young piglets, we compared the effect of a NOS-inhibitor N^{ω}-nitro-L-arginine (L-NOARG), an NO-inactivator oxyhaemoglobin (HbO) and a soluble guanyl cyclase(sGC)-inhibitor 1*H*-[1,2,4]Oxadiazolo-[4,3,-α]quinoxalin-1-one (ODQ) on acetylcholine(ACh)-induced relaxation. The involvement of K^+ channel activation was studied on relaxations induced by ACh, the two NO donors sodium nitroprusside (SNP) and diethylamine (DEA) NONOate, and the cell membrane permeable guanosine 3′5′ cyclic monophosphate (cGMP) analogue 8-Br-cGMP.

2 Full reversal of phenylephrine-mediated precontraction was induced by ACh (1 nM – 1 μM) (pD_2 8.2 ± 0.01 and R_{max} $98.7 \pm 0.3\%$). L-NOARG (100 μM) partly inhibited relaxation (pD_2 7.4 ± 0.02 and R_{max} $49.6 \pm 0.8\%$). The L-NOARG/indomethacin(IM)-resistant response displayed characteristics typical for endothelium-derived hyperpolarizing factor (EDHF), being sensitive to a combination of the K^+ channel blockers charybdotoxin (CTX) (0.1 μM) and apamin (0.3 μM).

3 ODQ (10 μM) abolished relaxations induced by ACh and SNP. L-NOARG/IM-resistant relaxations to ACh were abolished by HbO (20 μM).

4 Ouabain (1 μM) significantly inhibited ACh-induced L-NOARG/IM-resistant relaxations and relaxations induced by SNP (10 μM) and 8-Br-cGMP (0.1 mM). A combination of ouabain and Ba^{2+} (30 μM) almost abolished L-NOARG/IM-resistant ACh-induced relaxation (R_{max} $7.7 \pm 2.5\%$ *vs* $23.4 \pm 6.4\%$, with and without Ba^{2+}, respectively, $P < 0.05$).

5 The present study demonstrates that in femoral artery rings from young piglets, despite an L-NOARG/IM-resistant component sensitive to K^+ channel blockade with CTX and apamin, ACh-induced relaxation is abolished by sGC-inhibition or a combination of L-NOARG and HbO. These findings suggest that relaxation can be fully explained by the NO/cGMP pathway.
British Journal of Pharmacology (2003) **138,** 39–46. doi:10.1038/sj.bjp.0705001

Keywords: Nitric oxide; EDRF; EDHF; ouabain; barium; potassium channels; sGC; cGMP; neonatal; femoral artery

Abbreviations: ACh, acetylcholine; cGMP, cyclic 3′,5′-guanosine monophosphate; CTX, charybdotoxin; DEA NONOate, N-ethylethanamine:1,1-diethyl-2hydroxy-2-nitrosohydrazine[1 : 1]; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; HbO, oxyhaemoglobin; IBX, iberiotoxin; K_{IR}, inward rectifier K^+ channel; L-NOARG, N^{ω}-nitro-L-arginine; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]Oxadiazolo-[4,3,-α]quinoxalin-1-one; PE, phenylephrine; sGC, soluble guanyl cyclase; SNP, sodium nitroprusside; TEA, tetraethylammonium chloride

Introduction

The existence of an endothelium-derived hyperpolarizing factor (EDHF) was suggested by several investigators (Brayden, 1990; Chen *et al*., 1988; Huang *et al*., 1988; Komori *et al*., 1988) shortly after the identification of nitric oxide (NO) as the endothelium-derived relaxing factor (Ignarro *et al*., 1987; Palmer *et al*., 1987). Hyperpolarization of vascular smooth muscle cells induced by acetylcholine (ACh) could not be reproduced by authentic NO or NO-donor drugs, and the characteristics of agonist-induced responses could not be fully explained by NO. These findings led to the conclusion that an endothelium-derived diffusible factor different from NO induced hyperpolarization and relaxation, possibly *via* closure of voltage-dependent calcium channels (Brayden, 1990). The identity of EDHF is, however, still a matter of debate, and the different results achieved from different vascular preparations suggest that a variety of mechanisms are involved in EDHF-mediated responses depending on vascular bed and species.

A cytochrome P_{450}-metabolite of arachidonic acid has been suggested as EDHF in coronary vasculature (for review: Fisslthaler *et al*., 2000; Fleming, 2000). In the rat hepatic artery, but also in the porcine coronary artery, it has been suggested that K^+ released from endothelial cells is EDHF (Beny & Schaad, 2000; Edwards *et al*., 1998). The released K^+ is believed to activate the Na^+/K^+-ATPase and/or K_{IR} channels situated on the vascular smooth muscle cells. The reactive oxygen species H_2O_2 is a candidate for EDHF in rabbit, murine and human mesenteric arteries (Fujimoto *et*

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al., 2001; Matoba, 2002; Matoba *et al*., 2000), and the transfer of electrical or chemical signals *via* myoendothelial gap junctions seems to play an important role in EDHF-like vasodilation in some vascular preparations (Edwards *et al*., 2000; Hutcheson *et al*., 1999b; Little *et al*., 1995; Sandow & Hill, 2000). Recently, an increase in cAMP was shown to be associated with endothelium-dependent relaxation of rabbit conduit arteries (Taylor, 2001). This increase seems to facilitate relaxation by enhancing electrotonic conductions *via* both myoendothelial and homocellular smooth muscle gap junction (Griffith, 2002).

Bioassay experiments with sandwich preparation of rabbit conduit arteries have failed to demonstrate release of a freely diffusible EDHF after addition of ACh (Hutcheson *et al*., 1999a; Plane *et al*., 1995; Vanheel, 2000). In addition, there are indications that responses induced by NO and a putative EDHF are not easily distinguished. Tare *et al*. (1990) showed that NOS inhibition inhibited both ACh-induced relaxation and hyperpolarization, suggesting a role for a NOS product also in the hyperpolarizing response. Furthermore, Bolotina *et al*. (1994) demonstrated that NO could directly activate voltage-dependent calcium (K_{Ca}) channels in cell-free membrane patches. An additional problem is that evidence of an EDHF, either of an electrical or chemical origin, has to a large extent been indirect; i.e. the apparent inability of NOS- and cyclo-oxygenase (COX)-inhibitors to abolish relaxant responses. However, the commonly used L-arginine analogues may not necessarily inhibit NO production completely (Cohen *et al*., 1997), and it can therefore not be excluded that commonly used 'EDHF-inhibitors' block responses mediated by a rest production of NO. A universal characteristic of EDHF-mediated responses is the sensitivity to K^+ channel blockade with a combination of charybdotoxin (CTX) and apamin. These compounds typically have little or no effect in the absence of NOS inhibitors. It has been suggested that CTX and apamin may exert their effects mainly *via* K^+ channels located on the endothelial cells rather than on smooth muscle cells (Beny & Schaad, 2000; Edwards *et al*., 1998). It can therefore not be excluded that CTX/apamin directly affects NO production/release or gap junction activity.

The aim of the present study was to characterize agonist-induced vasodilation in femoral artery rings from young piglets, which have previously been demonstrated to exhibit L-NOARG/indomethacin (IM)-resistant relaxations to ACh (Stoen *et al*., 1997). The effect of L-NOARG was compared to that of the soluble guanyl cyclase (sGC) inhibitor ODQ and the NO-inactivator oxyhaemoglobin (HbO). We also examined the effect of the two NO donors SNP and DEA NONOate, and the cell membrane permeable cGMP analogue 8-Br-cGMP. Based on the observations that the Na^+/K^+-ATPase and/or inward rectifying K^+ channels (K_{IR}) channels may be responsible for EDHF-like responses, inhibitors of the respective mechanisms, ouabain and Ba^{2+}, were also included in the study.

Methods

Tissue preparation and mechanical measurements

The study was approved by the National Committee for Animal Studies. Male piglets 2 weeks (14–18 days) old were killed by a blow to the head followed by rapid exsanguination. The femoral arteries were immediately removed and mounted in organ chambers for measuring isometric tension. Cylindrical segments were mounted on L-shaped holders and immersed in organ baths containing Krebs' buffer solution ($38 \pm 0.5^{\circ}C$) bubbled with 5% CO_2 and 95% O_2 to maintain a pH of 7.4. The rings were stretched in 200 mg increments to a resting tension of 500 mg and allowed to equilibrate for 1 h. Segments were subsequently exposed to 60 mM K^+ buffer solution (with an equimolar reduction in the Na^+ content) until a stable contraction was achieved (reference contraction). A short equilibration period was allowed after washout and relaxation experiments were started. Indomethacin (10 μM) was present in the buffer solution in all experiments to inhibit cyclo-oxygenase. Cumulative concentration-response curves to either of the vasorelaxants ACh (1 nM–1 μM), SNP (1 nM–10 μM), DEA NONOate (1 nM–1 μM) or 8-Br-cGMP (1 μM–0.3 mM) were obtained in the presence or absence of the various inhibitors. Incubation time for the inhibitors was 10 (L-NOARG, ouabain), 15 (ODQ, Ba^{2+}) and 30 min (apamin, CTX, IBX, TEA). To ensure similar precontraction in all experimental groups inhibitors were added first, followed by phenylephrine (PE) at a concentration necessary to induce 60–65% of the reference contraction (see above).

When a stable precontraction was reached, the vasorelaxant was added in cumulative doses. A new dose of ACh was added when a plateau in relaxant response was reached, usually less than a minute after the preceding dose. A new dose of SNP/DEA NONOate and 8-Br-cGMP was added 3–5 and 30 min, respectively, after the previous one. The time intervals between doses were based on the time needed to reach a full response (in pilot experiments). Each arterial ring was exposed to only one combination of inhibitor and vasorelaxant.

Because ouabain has time-dependent effects on vascular tone, the ouabain experiments with one dose of vasorelaxant were conducted with a limited and set exposure time. To ensure sufficient incubation time for ouabain to inhibit the Na^+/K^+-ATPase, the effect of ouabain on K^+-induced relaxation was tested prior to relaxation experiments. Small increases in extracellular K^+ may dilate arteries *via* activation of K_{IR} (Knot *et al*., 1996; Chrissobolis *et al*., 2000) and/or the Na^+/K^+-ATPase (Doughty *et al*., 2000). After incubation in K^+-free buffer solution, addition of 7.5 mM K^+ induced a relaxation corresponding to approximately 90% of PE-induced precontraction (Figure 1). Incubation for 10 min with ouabain (1 μM) abolished this relaxant response whereas Ba^{2+} (30 μM) had no effect, suggesting that K^+-induced relaxation was mediated *via* the Na^+/K^+-ATPase in our preparation. Based on this, we also concluded that 10 min incubation time was sufficient to inhibit the Na^+/K^+-ATPase. The relaxation results in these experiments were corrected for the increase in tension observed in control rings in the presence of ouabain.

Drugs

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indomethacin was dissolved in a few drops of NaOH and diluted in demineralized water to a stock

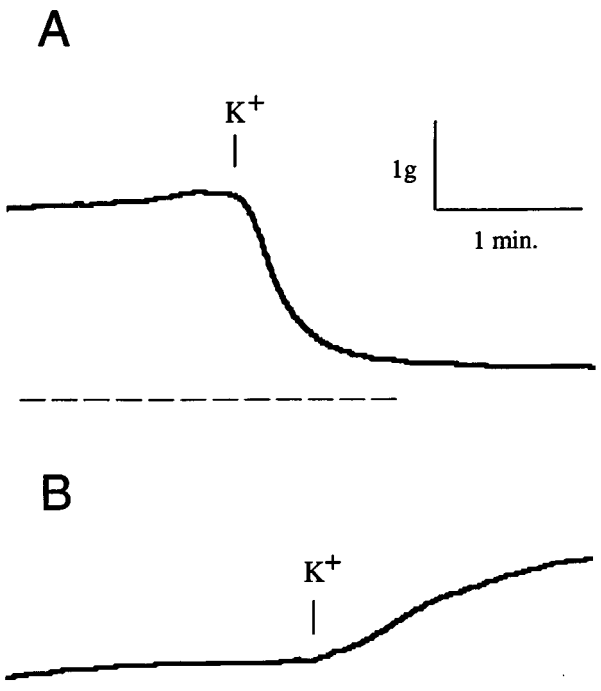

Figure 1 (a,b) Representative tracings of three experiments showing K^+ induced relaxation in the (a) absence and (b) presence of 1 μM ouabain. Relaxation was induced in PE-precontracted femoral artery rings by addition of 7.5 mM K^+ after incubation in K^+ free solution. Broken line indicates baseline tension before addition of PE.

solution of 10 mM which was made fresh weekly. L-NOARG was dissolved in 0.1 M HCl to a stock solution of 100 mM daily. DEA NONOate was dissolved in 10 mM NaOH, stored on ice and protected from light until it was used. Haemoglobin was dissolved in 0.9% NaCl to make up a 1 mM stock solution. The stock solution was subsequently reduced to HbO by the addition of a small amount of sodium dithionite. Excess sodium dithionite was extracted by running the solution through a sephadex (PD-10) column equilibrated with 0.9% NaCl. All other substances were prepared in demineralized water as stock solutions and stored at 4°C (ouabain, Ba^{2+}, TEA, PE) or frozen at −70°C, thawed and diluted further at the day of the experiment (IBX, CTX, apamin, ACh). SNP was prepared immediately before the experiment and protected from light.

Calculations and analysis of results

All values are given as mean±s.e.mean of n experiments, n being equal to number of animals. Responses to relaxing agents were expressed as a percentage reversal of the PE-induced precontraction. The individual relaxation curves were fitted to the sigmoidal four parameter logistic equation (Graphpad Prism, version 2.01). From this analysis the pD_2 (negative log of the concentration of a drug that produces half its maximal response, $-\log EC_{50}$) and R_{max} (maximum relaxation) values of the test substances were calculated. pD_2 and R_{max} values were compared using unpaired t-test (two-tail) or ANOVA with Bonferroni post tests as appropriate. P-values <0.05 were considered statistically significant.

Results

Effect of L-NOARG, ODQ and HbO on ACh-induced relaxation

ACh (1 nM–1 μM) induced a concentration-dependent relaxation in piglet femoral artery rings precontracted with PE (pD_2 8.2±0.01 and R_{max} 98.7±0.3%). In the presence of the NOS inhibitor L-NOARG (100 μM), the relaxant response was significantly reduced with a decrease in both pD_2 value and maximal relaxation (pD_2 7.4±0.02 and R_{max} 49.6±0.8%, $P<0.001$ *vs* controls). The sGC inhibitor ODQ (10 μM) almost abolished the relaxant response to ACh (R_{max} 4.4±0.06%) (Figure 2a).

In a separate series of experiments relaxation was studied in the presence of the NO inactivator HbO (2 μM). HbO alone attenuated the sensitivity as well as the maximal response to ACh (pD_2 7.6±0.02 *vs* 7.8±0.03, $P<0.05$, and R_{max} 72.3±1.2% *vs* 98.5±1.3%, $P<0.001$, with and without

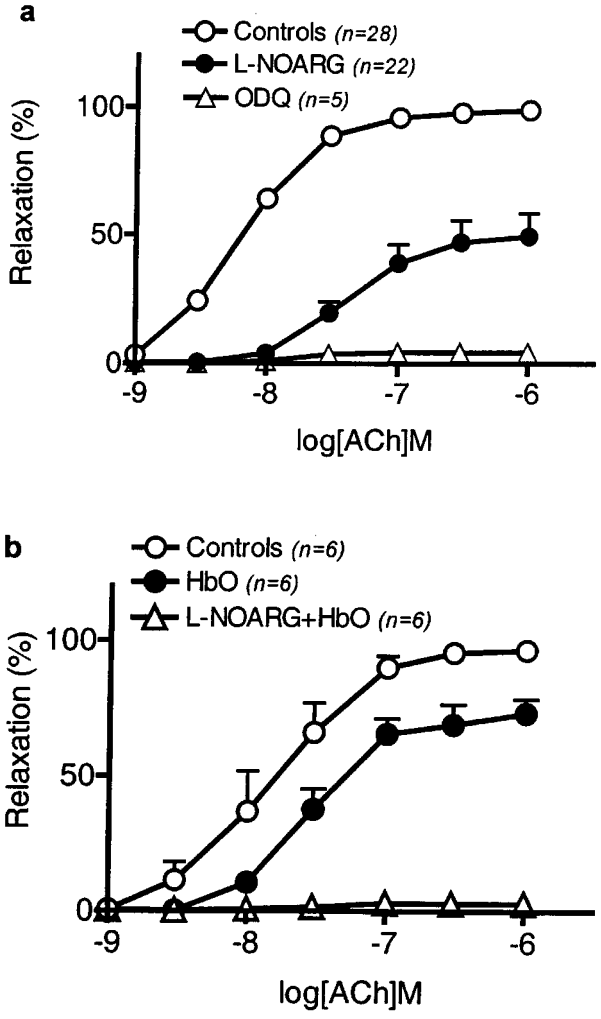

Figure 2 (a,b) Concentration–response curves to ACh (1 nM–1 μM) in the presence of (a) L-NOARG (100 μM) or ODQ (10 μM) and (b) HbO (2 μM) alone or in combination with L-NOARG (100 μM). Relaxations are expressed in % reversal of PE-induced contraction. All experiments were performed in the presence of indomethacin (10 μM). Data are presented as mean±s.e.mean.

HbO, respectively). A combination of L-NOARG and HbO abolished the relaxant response (Figure 2b).

Effect of K^+ channel inhibition on ACh-induced relaxation

To address the contribution of K^+ channels to ACh-induced relaxation, several selective K^+ channel blockers were tested alone, in combination with each other or in combination with L-NOARG (Figure 3a,b). CTX (0.1 μM), a blocker of K_{Ca} and some K_V, inhibited the vasorelaxant response to ACh with a small decrease in pD_2 value (pD_2 8.0 ± 0.03 *vs* 8.2 ± 0.01, $P<0.01$) and R_{max} ($95.2 \pm 1.3\%$ *vs* $98.7 \pm 0.34\%$, $P<0.05$). The selective blocker of BK_{Ca}, IBX (0.1 μM), and apamin (0.3 μM), a selective blocker of SK_{Ca}, did not inhibit the ACh response. There were no synergistic effects when combining apamin with either of the other toxins in the absence of NOS inhibition. The L-NOARG/IM-resistant part of the relaxant response was completely abolished by a combination of CTX and apamin. IBX could not replace CTX in this combination as $34.3 \pm 0.5\%$ relaxation persisted in the presence of L-NOARG, IBX and apamin.

Relaxation induced by the NO donors SNP or DEA NONOate

SNP (1 nM – 10 μM) and DEA NONOate (1 nM – 1 μM) induced concentration-dependent relaxations in PE-precontracted femoral artery rings (Figure 4a,b). The pD_2 and R_{max} values for SNP-induced relaxation were 6.8 ± 0.04 and $102.8 \pm 2.3\%$, respectively, whereas the corresponding values for DEA NONOate were 7.6 ± 0.02 and $103.6 \pm 1.1\%$. The sGC inhibitor ODQ (10 μM) completely abolished relaxation induced by SNP ($n=3$, data not shown).

A high K^+ (20 mM) solution gave a rightward shift in the concentration – response curves to SNP and DEA NONOate

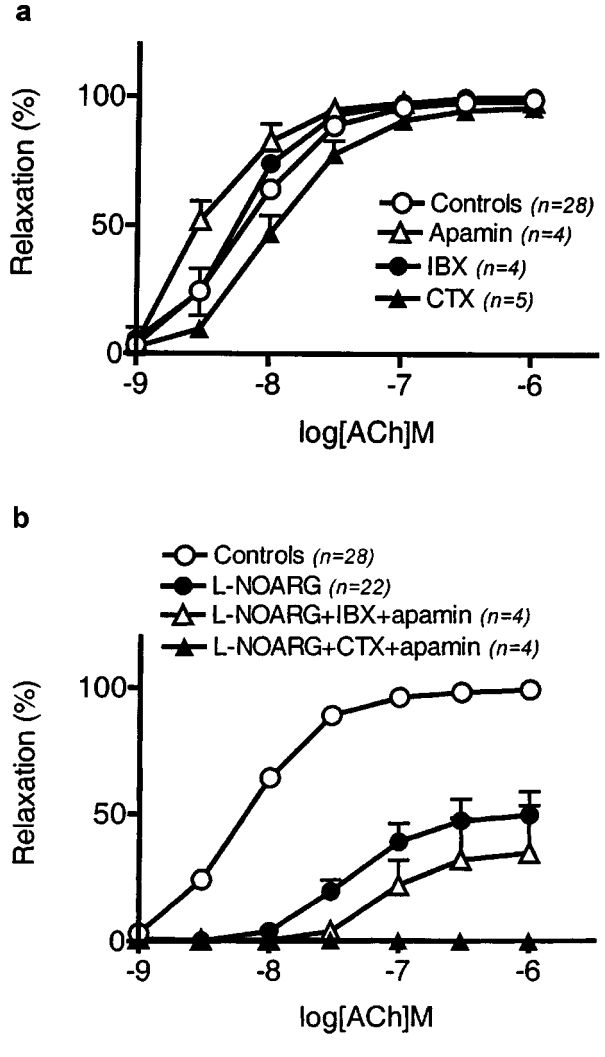

Figure 3 (a,b) Concentration – response curves to ACh (1 nM – 1 μM) in the presence of (a) K^+ blockade with IBX (0.1 μM), CTX (0.1 μM) or apamin (0.3 μM) alone and (b) in combination with L-NOARG (100 μM). Relaxations are expressed in % reversal of PE-induced contraction. All experiments were performed in the presence of indomethacin (10 μM). Data are presented as mean ± s.e.mean.

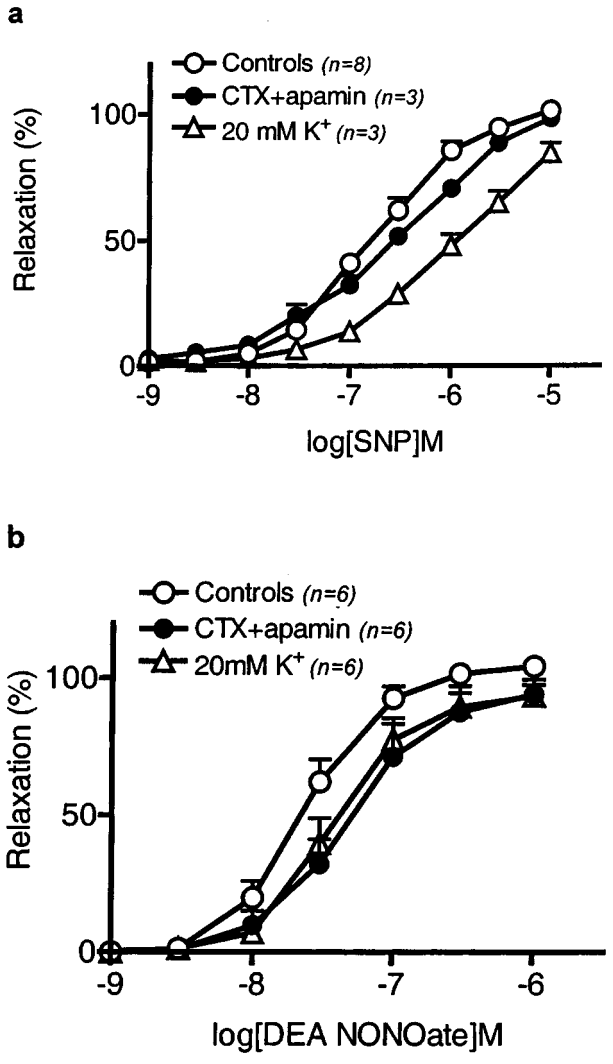

Figure 4 (a,b) Concentration – response curves to (a) SNP (1 nM – 10 μM) and (b) DEA NONOate (1 nM – 1 μM) in the absence and presence of 20 mM K^+ or a combination of CTX (0.1 μM) and apamin (0.3 μM). All experiments were performed in the presence of indomethacin (10 μM). Data are presented as mean ± s.e.mean.

(Figure 4a,b). Maximal relaxation was not reached within the tested concentration range for SNP, but for DEA NONOate R_{max} was significantly reduced in the presence of high K^+ ($92.8 \pm 1.3\%$, $P<0.001$ *vs* controls). Inhibition of K^+ channels with CTX and apamin in combination reduced the sensitivity to both SNP and NONOate (pD_2 6.4 ± 0.05 and 7.3 ± 0.01, respectively, $P<0.001$ *vs* controls for both). Maximal relaxation to NONOate was also reduced by CTX and apamin ($94.9 \pm 1.0\%$, $P<0.001$ *vs* controls).

Effect of L-NOARG, ODQ and K^+ channel blockade on relaxation induced by 8-Br-cGMP

The cell permeable cyclic GMP analogue 8-Br-cGMP (1 μM – 0.3 mM) induced concentration-dependent relaxation (pD_2 4.5 ± 0.04 and R_{max} $89.1 \pm 3.5\%$) in femoral artery rings (Figure 5). Inhibition of NOS and sGC with L-NOARG (100 μM) and ODQ (10 μM), respectively, amplified to some extent the relaxation induced by 8-Br-cGMP although the differences were not statistically significant (Figure 5). K^+ channel blockade with 20 mM K^+, TEA (1 mM), Ba^{2+} (30 μM) or CTX/apamin did not have any inhibitory effect on 8-Br-cGMP-induced relaxation (data not shown).

Role of the Na^+/K^+-ATPase and K_{IR}

Ouabain (1 μM) and Ba^{2+} (30 μM) were used to inhibit the Na^+/K^+-ATPase and K_{IR}, respectively. Ouabain had time-dependent effects on the vascular tone, and in resting artery rings ouabain induced a slow, monophasic increase in tension ($36 \pm 6\%$ of the 60 mM K^+-induced reference contraction after 1 h, $n=8$). Within the time frame of experiments with one dose of vasorelaxant (10 – 15 min) ouabain caused only about 3% increase of the reference contraction. Ba^{2+} had no effect on resting tension.

Ouabain (1 μM) inhibited the ACh-induced L-NOARG-resistant relaxation (R_{max} $23.4 \pm 6.4\%$ *vs* $59.1 \pm 4.8\%$, with and without ouabain, respectively, $P<0.05$) (Figure 6). Ouabain also had a significant inhibitory effect on the relaxation induced by SNP (10 μM) (R_{max} $79.0 \pm 3.9\%$ *vs* $93.4 \pm 3.6\%$, $P<0.05$) and 8-Br-cGMP (0.1 mM) (R_{max} $39.1 \pm 4.7\%$ *vs* $65.4 \pm 7.1\%$, $P<0.05$) (Figure 7A). Relaxations induced by a lower dose of SNP (0.3 μM) or DEA NONOate (30 nM) were not significantly altered by ouabain, although there was a trend towards inhibition in the presence of ouabain (R_{max} 15.5 ± 7.1 *vs* $33.8 \pm 9.3\%$ and 31.0 ± 5.3 *vs* $54.5 \pm 13.4\%$, SNP and DEA NONOate, respectively) (Figure 7B). When a full concentration – response curve to SNP (1 nM – 10 μM) was performed in the presence of ouabain, relaxation was abolished ($n=6$; data not shown).

Ba^{2+} did not inhibit relaxations induced by ACh (1 nM – 1 μM). In the presence of L-NOARG, Ba^{2+} amplified the relaxant response (R_{max} $75.6 \pm 3.4\%$ *vs* $59.1 \pm 4.8\%$, with and without Ba^{2+}, respectively, $P<0.05$) (Figure 6).

SNP-induced relaxation was significantly inhibited in the presence of Ba^{2+} with decreased sensitivity and R_{max} (pD_2 6.3 ± 0.05 *vs* 6.8 ± 0.04 and R_{max} 60.9 ± 1.9 *vs* $102.8 \pm 2.3\%$, $P<0.001$ for both). Relaxant responses to DEA NONOate and 8-Br-cGMP were unaltered by Ba^{2+} (data not shown).

In combination with ouabain, Ba^{2+} had a significant inhibitory effect on ACh-induced L-NOARG-resistant relaxation (R_{max} $7.7 \pm 2.5\%$ *vs* $23.4 \pm 6.4\%$, with and without Ba^{2+}, respectively, $P<0.05$) (Figure 6). A combination of ouabain and Ba^{2+} almost abolished relaxation to 0.3 μM SNP (R_{max} 3.5 ± 1.9 *vs* $33.8 \pm 9.3\%$, $P<0.01$) and significantly inhibited relaxation induced by 30 nM DEA NONOate (R_{max} $23 \pm 4.1\%$ *vs* 54.5 ± 13.4, $P<0.05$) (Figure 7B).

Discussion

The main finding of this study is that despite the presence of an L-NOARG-resistant component, the relaxant response to ACh was abolished by an inhibitor of sGC, ODQ, in isolated

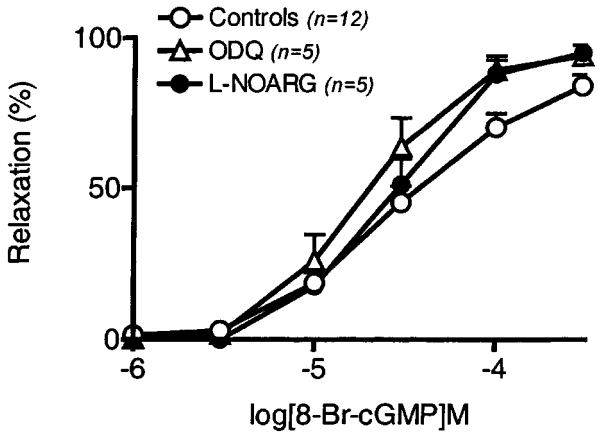

Figure 5 Concentration – response curves to 8-Br-cGMP (1 μM – 0.3 mM) in the presence of L-NOARG (100 μM) or ODQ (10 μM). All experiments were performed in the presence of indomethacin (10 μM). Data are presented as mean $\pm$ s.e.mean.

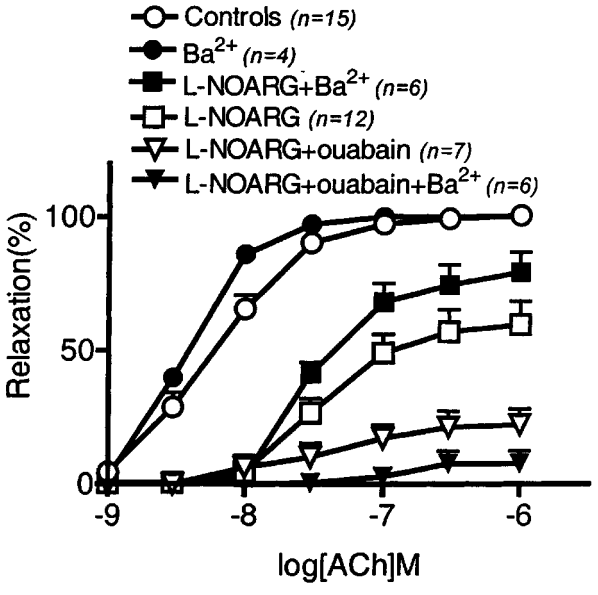

Figure 6 Concentration – response curves to ACh (1 nM – 1 μM) in the presence of Ba^{2+} (30 μM), L-NOARG (100 μM), or L-NOARG in the presence of Ba^{2+}, ouabain (1 μM) or a combination of ouabain and Ba^{2+}. Relaxations are expressed in % reversal of PE-induced contraction. All experiments were performed in the presence of indomethacin (10 μM). Data are presented as mean $\pm$ s.e.mean.

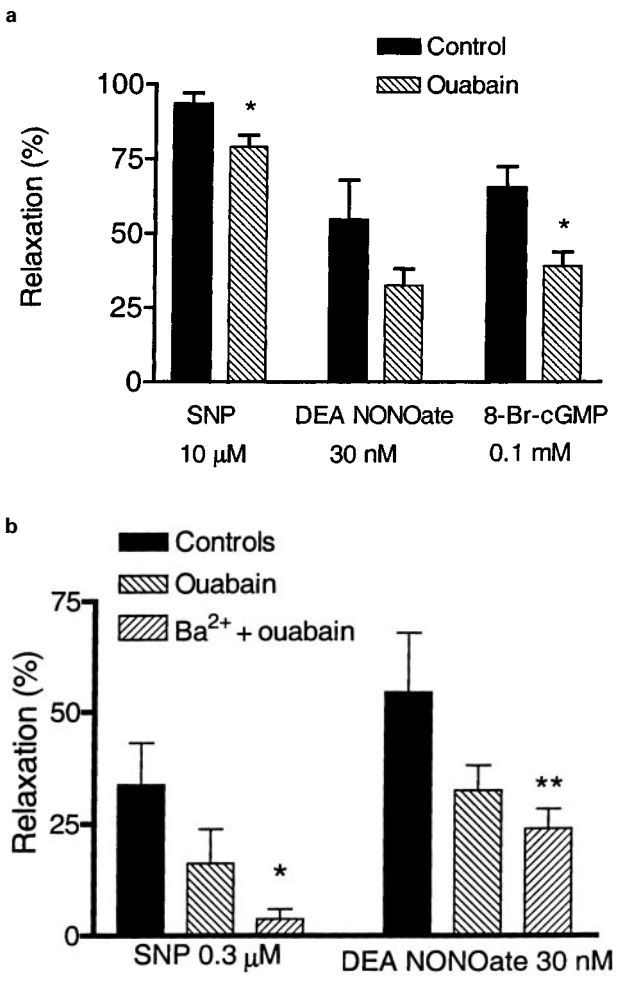

Figure 7 (a) Relaxation induced by SNP (10 μM), DEA NONOate (30 nM) and 8-Br-cGMP (0.1 mM) in the absence and presence of ouabain (1 μM). (b) Relaxation induced by SNP (0.3 μM) or DEA NONOate (30 nM) in the absence and presence of ouabain (1 μM) or a combination of ouabain and Ba^{2+} (30 μM). Relaxations are expressed as % reversal of PE-induced contraction. All experiments were performed in the presence of indomethacin (10 μM). Data are expressed as mean$\pm$s.e.mean of (a) 4–8 experiments or (b) 4 experiments. *$P<0.05$ *vs* control. **$P<0.08$ *vs* control.

femoral artery rings from newborn piglets. The involvement of the NO/cGMP pathway was further supported by the finding that HbO abolished the L-NOARG-resistant relaxation, suggesting that NO mediated the entire relaxant response to ACh. However, L-NOARG-resistant relaxation was sensitive to a combination of the K^+ channel inhibitors CTX and apamin. Such L-NOARG/IM-resistant and CTX/apamin-sensitive responses are commonly interpreted as being mediated by a factor different from NO, namely a yet unidentified EDHF.

ODQ abolished relaxations induced by SNP and ACh, suggesting that the relaxant responses were mediated *via* activation of sGC and an increase in cGMP. ODQ is a potent and selective inhibitor of sGC which, in contrast to the other commonly used sGC inhibitors, methylene blue and LY 58383, do not generate superoxide anions or affect NOS activity in concentrations up to 10 μM (Garthwaite *et al*., 1995). Although species differences in sensitivity to ODQ can not be excluded, present knowledge of ODQ and its effects do not suggest non-specific effects at the concentrations used in the present study. Effects distal to its effects on sGC have been suggested based on an inhibitory effect of ODQ on 8-Br-cGMP-induced relaxation in rat aorta (Feelisch *et al*., 1999). The present study did not show any inhibitory effect on relaxation induced by 8-Br-cGMP. Contrary, like L-NOARG, ODQ tended to amplify the 8-Br-cGMP-induced relaxation.

One possible explanation for the discrepancy between the effects of NOS inhibition and sGC inhibition on ACh-induced relaxation is that a relaxing factor different from NO mediates relaxation *via* stimulation of sGC. H_2O_2, which have been suggested as an EDHF in rabbit, mice and human mesenteric arteries (Fujimoto *et al*., 2001; Matoba, 2002; Matoba *et al*., 2000) has been shown to stimulate sGC (Fujimoto *et al*., 2001). However, in the present study the NO-inactivator HbO abolished the L-NOARG-resistant relaxation. To the best of our knowledge there are no studies available demonstrating inhibitory effects of HbO on H_2O_2-induced responses, and in the present study it is therefore assumed that NO is responsible for the HbO-sensitive relaxation which occurred despite NOS inhibition with L-NOARG. Cohen *et al*. (1997) demonstrated that even a combination of L-NOARG 300 μM and L-NAME 30 μM did not abolish NO release in the rabbit carotid artery. In the same study highly correlated relaxant and hyperpolarizing responses was demonstrated in the rabbit carotid artery after application of NO gas or an NO-donor, and it was suggested that the ability of endothelium-dependent vasodilation to persist in the presence of NOS inhibitors may be related more to the amount of NO produced than to the existence of other factors.

Another finding of the present study was that the L-NOARG/IM-resistant part of ACh-induced relaxation possessed typical characteristics of an EDHF-mediated response, i.e. it was sensitive to the combination of CTX and apamin and could only be demonstrated in the presence of NOS inhibition. In addition, this particular relaxation was abolished by high potassium in a previous study (Stoen *et al*., 1997). An effect of CTX/apamin *per se* does not, however, prove a role for a non-prostanoid non-NO EDHF in ACh-induced relaxation. The mechanisms of action of CTX/apamin are not clear, and to exclude an effect of CTX/apamin on NO-mediated relaxation, the inhibition of NO production must be complete. In the present study, results with L-NOARG in the presence and absence of HbO suggest that this was not the case. If CTX/apamin decreases the opening probability of K_{Ca} channels on endothelial cells (Beny & Schaad, 2000; Edwards *et al*., 1998), and thereby inhibits the hyperpolarization which provides the driving force for sustained Ca^{2+}-influx into the endothelial cell, an effect on the production and/or release of NO can not be excluded. As the CTX/apamin combination also had a small, but significant inhibitory effect on relaxations induced by SNP or DEA NONOate, this combination of K^+ blockers may in addition inhibit steps downstream from the production/release of NO.

Ouabain has been used to characterize the involvement of the Na^+/K^+-ATPase in EDHF-induced responses (Doughty *et al*., 2000; Edwards *et al*., 1998; Zygmunt & Hogestatt, 1996; Zygmunt *et al*., 2000), but the results are contradictory

as to whether the Na^+/K^+-ATPase is involved in these responses or not. On the other hand, ouabain may also inhibit increases in cGMP induced by the NO pathway (Rapoport & Murad, 1983; Rapoport *et al*., 1985). A role for the Na^+/K^+-ATPase has gained renewed interest after K^+ was suggested as an EDHF in the rat hepatic artery, and this response could be inhibited by a combination of Ba^{2+} and ouabain (Beny & Schaad, 2000; Edwards *et al*., 1998).

In the present study, ouabain inhibited relaxation induced by SNP (10 μM) and 8-Br-cGMP (0.1 mM) by 15 and 40%, respectively, suggesting a role for the Na^+/K^+-ATPase in NO/cGMP-mediated responses in this preparation. The inhibitory effect of ouabain on ACh-induced L-NOARG/IM-resistant relaxation may also indicate an effect on EDHF-like responses, but these results must be interpreted with caution. Inhibition of the sodium pump will depolarize the cell membrane with a concomitant increase in calcium influx into vascular smooth muscle cells. This may counteract a relaxing effect of EDRF/EDHF. The effect of ouabain on resting membrane potential varies between species and vascular beds and is probably also dependent on age (Hayashi & Park, 1987). Whether changes in resting membrane potential may have an effect on conduction *via* gap junctions is at present not clear, but the importance of the Na^+/K^+-ATPase in endothelium-dependent relaxation requires further investigations.

Results obtained in the presence of Ba^{2+} were somewhat contradictory in the present study. As K_{IR}-dependent vasodilation seems to become more important as arterial size decreases (Quayle *et al*., 1996), one may not expect K_{IR}-activation to play a major role in agonist-induced vasodilation in conduit arteries. In accordance with this, relaxation to DEA NONOate and 8-Br-cGMP were unaffected by Ba^{2+}. The strong inhibitory effect of Ba^{2+} on SNP-induced relaxation was in contrast to this, and is also in contrast to what has been reported in coronary porcine arterioles (Rivers, 2001) and canine middle cerebral arteries (Onoue, 1997). This finding is at present unexplained, as is the unexpected finding of an augmented relaxant response to ACh in the presence of Ba^{2+}.

In the presence of ouabain, Ba^{2+} had an inhibitory effect on L-NOARG-resistant relaxation to ACh. This is in accordance with the hypothesis that ouabain/Ba^{2+}-sensitive mechanisms may be responsible for the EDHF-like part of agonist-induced vasodilation (Beny & Schaad, 2000; Edwards *et al*., 1998). An inhibitory effect of ouabain/Ba^{2+} was found also on SNP-induced relaxations, and considering the strong inhibitory effect of Ba^{2+} alone on SNP-induced relaxation this could be a simple additive effect of the two inhibitors. The results with DEA NONOate, however, may indicate a combined ouabain/Ba^{2+} effect also on NO-mediated relaxation. This will have to be confirmed in future studies.

In conclusion, the present study demonstrated that L-NOARG/IM-resistant ACh-induced relaxations in isolated artery rings from newborn piglets display typical characteristics of an EDHF-like response. The relaxant response to ACh was, however, abolished by inhibition of sGC with ODQ and by a combination of L-NOARG and HbO, suggesting that NO/cGMP mediated the entire relaxation. Relaxations induced by the two NO donors SNP and DEA NONOate were inhibited by the CTX/apamin combination commonly used to characterize EDHF-like responses, suggesting that activation of K^+ channels are involved in NO/cGMP-mediated responses. This was supported by the finding that inhibition of the Na^+/K^+-ATPase with ouabain reduced the relaxation to both SNP and 8-Br-cGMP. We suggest that EDHF is NO in this vessel preparation, although the existence of another mechanism independent of NO, but sensitive to HbO can not be excluded.

This study was supported by the Norwegian Council of Cardiovascular Diseases.

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(Received July 22, 2002
Revised August 28, 2002
Accepted September 23, 2002)