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Contribution of K^+ channels and ouabain-sensitive mechanisms to the endothelium-dependent relaxations of horse penile small arteries

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1 Penile small arteries (effective internal lumen diameter of 300–600 μm) were isolated from the horse corpus cavernosum and mounted in microvascular myographs in order to investigate the mechanisms underlying the endothelium-dependent relaxations to acetylcholine (ACh) and bradykinin (BK).

2 In arteries preconstricted with the thromboxane analogue U46619 (3–30 nM), ACh and BK elicited concentration-dependent relaxations, pD_2 and maximal responses being 7.71 ± 0.09 and $91 \pm 1\%$ ($n=23$), and 8.80 ± 0.07 and $89 \pm 2\%$ ($n=24$) for ACh and BK, respectively. These relaxations were abolished by mechanical endothelial cell removal, attenuated by the nitric oxide (NO) synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NOARG, 100 μM) and unchanged by indomethacin (3 μM). However, raising extracellular K^+ to concentrations of 20–30 mM significantly inhibited the ACh and BK relaxant responses to $63 \pm 4\%$ ($P<0.01$, $n=7$) and to $59 \pm 4\%$ ($P<0.01$, $n=6$), respectively. ACh- and BK-elicited relaxations were abolished in arteries preconstricted with K^+ in the presence of 100 μM L-NOARG.

3 In contrast to the inhibitor of ATP-sensitive K^+ channels, the blockers of Ca^{2+}-activated K^+ (K_{Ca}) channels, charybdotoxin (30 nM) and apamin (0.3 μM), each induced slight but significant rightward shifts of the relaxations to ACh and BK without affecting the maximal responses. Combination of charybdotoxin and apamin did not cause further inhibition of the relaxations compared to either toxin alone. In the presence of L-NOARG (100 μM), combined application of the two toxins resulted in the most effective inhibition of the relaxations to both ACh and BK. Thus, pD_2 and maximal responses for ACh and BK were 7.65 ± 0.08 and $98 \pm 1\%$, and 9.17 ± 0.09 and $100 \pm 0\%$, respectively, in controls, and 5.87 ± 0.09 ($P<0.05$, $n=6$) and $38 \pm 11\%$ ($P<0.05$, $n=6$), and 8.09 ± 0.14 ($P<0.01$, $n=6$) and $98 \pm 1\%$ ($n=6$), respectively, after combined application of charybdotoxin plus apamin and L-NOARG.

4 The selective inhibitor of guanylate cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM) did not alter the maximal responses to either ACh or BK, but slightly decreased the sensitivity to both agonists, δpD_2 being 0.25 ± 0.07 ($P<0.05$, $n=6$) and 0.62 ± 0.12 ($P<0.01$, $n=6$) for ACh and BK, respectively. Combined application of ODQ and charybdotoxin plus apamin produced further inhibition of the sensitivity to both ACh ($\delta pD_2 = 1.39 \pm 0.09$, $P<0.01$, $n=6$) and BK (1.29 ± 0.11, $P<0.01$, $n=6$), compared to either ODQ or charybdotoxin plus apamin alone.

5 Exogenous nitric oxide (NO) present in acidified solutions of sodium nitrite ($NaNO_2$) and S-nitroso-cysteine (SNC) both concentration-dependently relaxed penile resistance arteries, pD_2 and maximal responses being 4.84 ± 0.06 and $82 \pm 3\%$ ($n=12$), and 6.72 ± 0.07 and $85 \pm 4\%$ ($n=19$), respectively. Charybdotoxin displaced to the right the dose-relaxation curves for both NO (δpD_2 0.38 ± 0.06, $P<0.01$, $n=6$) and SNC (δpD_2 0.50 ± 0.10, $P<0.01$, $n=5$), whereas apamin only reduced sensitivity ($\delta pD_2 = 0.35 \pm 0.12$, $P<0.05$, $n=5$) and maximum response ($65 \pm 9\%$, $P<0.05$, $n=6$) to SNC. ODQ shifted to the right the dose-relaxation curves to both NO and SNC. The relaxant responses to either NO or SNC were not further inhibited by a combination of ODQ and charybdotoxin or ODQ and charybdotoxin plus apamin, respectively, compared to either blocker alone.

6 In the presence of 3 μM phentolamine, 5 μM ouabain contracted penile resistance arteries by $50 \pm 6\%$ ($n=17$) of K-PSS, but did not significantly change the relaxant responses to either ACh, BK or NO. However, in the presence of L-NOARG ouabain reduced the ACh- and BK-elicited relaxation from $94 \pm 3\%$ to $16 \pm 5\%$ ($P<0.0001$, $n=6$), and from $98 \pm 2\%$ to $13 \pm 3\%$ ($P<0.0001$, $n=5$), respectively. Combined application of ODQ and ouabain inhibited the relaxations to NO from $92 \pm 2\%$ to $26 \pm 3\%$ ($P<0.0001$, $n=6$).

7 The present results demonstrate that the endothelium-dependent relaxations of penile small arteries involve the release of NO and a non-NO non-prostanoid factor(s) which probably hyperpolarize(s) smooth muscle by two different mechanisms: an increased charybdotoxin and apamin-sensitive K^+ conductance and an activation of the Na^+-K^+ ATPase. These two mechanisms appear to be independent of guanylate cyclase stimulation, although NO itself can also activate charybdotoxin-sensitive K^+ channels and the Na^+-K^+ pump through both cyclic GMP-dependent and independent mechanisms, respectively.

Keywords: Endothelium; acetylcholine; bradykinin; nitric oxide; K^+-channels; charybdotoxin; apamin; cyclic GMP; ouabain; penile small arteries

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Introduction

Acetylcholine (ACh) and other neurohumoral substances produce relaxation of blood vessels via an endothelium-dependent mechanism in which, the endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO) or a closely related substance (Furchgott & Vanhoutte, 1989), plays a main role. However, in a variety of arteries, endothelium-dependent vasodilatations have been shown to be resistant to cyclo-oxygenase and NO synthase (NOS) blockades, both *in vivo* and *in vitro*, and there is a contribution of a diffussible substance the chemical identity of which remains unclear. This endothelial factor has been termed non-NO non-prostanoid endothelium-derived hyperpolarizing factor (EDHF), since *in vitro* studies have shown that upon release it causes hyperpolarization of the underlying smooth muscle by opening K^+ channels (Feletou & Vanhoutte, 1988; Cohen & Vanhoutte, 1995; Garland *et al*., 1995). The relative roles of NO and EDHF in arterial vasodilatation have been shown to be variable depending on the agonist, vessel type and animal species. In the systemic circulation, there seems to be a correlation between the vessel size and the differential contributions of NO and EDHF to the endothelium-dependent relaxations, and thus the smaller the artery the larger the component of the relaxation resistant to NOS blockade (Nagao *et al*., 1992; Hwa *et al*., 1994; Garland *et al*., 1995).

The main signal transduction mechanism accounting for the EDRF-NO-mediated vasodilatation is stimulation of guanylate cyclase and accumulation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Ignarro, 1990). In addition, in certain vascular beds NO can hyperpolarize the smooth muscle through both cyclic GMP-dependent and -independent mechanisms (Archer *et al*., 1994; Bolotina *et al*., 1994). The signal transduction pathway for EDHF in blood vessels is independent of cyclic GMP and is a hyperpolarization which has been attributed to an increased K^+ conductance of the smooth muscle cell membrane (Cohen & Vanhoutte, 1995; Garland *et al*., 1995).

Relaxation of the smooth muscle of penile arteries and cavernous trabeculae is a primary haemodynamic event in penile erection (Lue & Tanagho, 1987). This relaxation is under the control of vasodilator nerves and the vascular endothelium (Andersson & Wagner, 1995). It is well established that NO or a NO-related substance is involved in the non-adrenergic, non-cholinergic (NANC) neurogenic relaxation of corpus cavernosum (Kim *et al*., 1991; Andersson & Wagner, 1995), dorsal penile artery (Liu *et al*., 1991) and penile small arteries or helicine arteries (Simonsen *et al*., 1995; 1997b). However, discrepancies have been found concerning the relative contribution of NO to the endothelium-dependent relaxations of penile erectile tissues. Thus, whereas blockade of NO synthesis inhibited most of the relaxation to ACh in the corpus cavernosum (Azadzoi *et al*., 1992) and dorsal penile artery (Liu *et al*., 1991), NOS inhibitors did not affect the same responses in the circumflex veins of the penis (Kirkeby *et al*., 1993). Endothelial function of penile small arteries has not been clarified so far, although recent observations in our laboratory indicate that there is a differential effect of NOS blockade on the endothelium-dependent responses of human corpus cavernosum and helicine arteries (Simonsen *et al*., 1997b).

The purpose of the present study was to evaluate the effects of ACh and bradykinin (BK) on penile small arteries or helicine arteries, terminal branches of the deep penile arteries which control the blood flow between the systemic circulation and the cavernous sinusoids. Moreover, the mechanisms underlying these relaxant responses, as well as the relative contributions of EDRF-NO and other factor(s) resistant to inhibition of the L-arginine/NO pathway have been clarified, in order to determine whether the heterogeneity of the endothelial function found for other erectile tissues can be extended to the penile small arteries.

Methods

Dissection and mounting

Penises from normal horses were obtained once a week at the local slaughterhouse immediately after death and placed in cold physiological salt solution (PSS). Throughout the subsequent dissection the penis was bathed in cold PSS, 4°C, of the following composition (mmol l^{-1}): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.5, ethylenediaminetetraacetic acid (EDTA) 0.027 and glucose 11. The solution was gassed with 5% CO_2 in 95% O_2 to maintain pH at 7.4. The corpus cavernosum of the penis was open and penile small arteries, which are second- or third-order branches of the deep penile artery having a normalized internal lumen diameter of 200–600 μm, were dissected carefully removing the adhering trabecular tissue, as previously described (Simonsen *et al*., 1997b,c). Segments (ca 2 mm long) of the small vessels were subsequently mounted as ring preparations on two 40 μm wires in microvascular double myographs by fixing one of the wires to a force transducer for isometric tension recording and the second wire to a length displacement device (Mulvany & Nyborg, 1980).

The vessels were allowed to equilibrate in PSS, 37°C, pH 7.4 for about 30 min. The relation between resting wall tension and internal circumference was determined, and from this the internal circumference L_{100}, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ*, was calculated. The vessels were set to the internal circumference L_1, given by $L_1 = 0.9 \times L_{100}$. Preliminary experiments showed that the force development is close to maximal at this internal circumference (Simonsen *et al*., 1997c). The effective internal lumen diameter was determined as $l_1 = L_1\pi^{-1}$.

Experimental procedure

After normalization, the contractile ability of the vessels was tested by stimulating with K-PSS (equivalent to PSS but NaCl exchanged with KCl on an equimolar basis giving a final concentration of 123.7 mM K^+) until reproducible responses were obtained, normally after 3 stimulations. The relaxant responses to ACh, bradykinin (BK), S-nitroso-cysteine (SNC) and exogenous NO (present in acidified solutions of $NaNO_2$) were tested by cumulative addition of the agonists in arteries precontracted with the thromboxane analogue U46619 (3–30 nM) or a 20–25 mM K^+ solution, giving a contraction averaging 40–60% of the response to K-PSS in each preparation. Since the concentration-relaxation response curves to the different agonists could be repeated at least twice in the same preparation, when the effect of the different blockers was tested, a first control curve was constructed, and after washing for 30 min, a second concentration-response curve was repeated in the presence of the blocker. In the first set of experiments, the role of endothelial cells in the ACh- and BK-induced relaxations was tested by removing mechanically the vascular endothelium. After the first control curve to the agonist, the arteries were stretched to an internal lumen

diameter lower than l_1, and a horse hair was guided through the vessel lumen and gently moved forth and back several times. After this procedure, the artery was challenged once with K-PSS to check its viability and a second concentration-response curve to the agonist was obtained. When the effects of inhibition of NO synthase (NOS), cyclo-oxygenase, guanylate cyclase, K^+ channels or the Na^+-K^+ ATPase were tested, a first concentration-response curve which served as control was constructed and then the arteries were repeatedly washed and subsequently incubated for 20–30 min with either N^G-nitro-L-arginine (L-NOARG, 100 μM), indomethacin (3 μM), ODQ (5 μM), glibenclamide (3 μM), charybdotoxin (30 nM) or apamin (0.3 μM), or 10–15 min with ouabain (5 μM), before a second concentration-response curve was obtained.

Drugs

Acetylcholine chloride, apamin, bradykinin acetate salt, charybdotoxin, L-cysteine, glibenclamide, indomethacin, methylene blue, N^G-nitro-L-arginine (L-NOARG), ouabain, 9,11-dideoxy-11 α 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) and sodium nitrite were purchased from Sigma Chemical Co (St Louis, MO U.S.A.). 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson

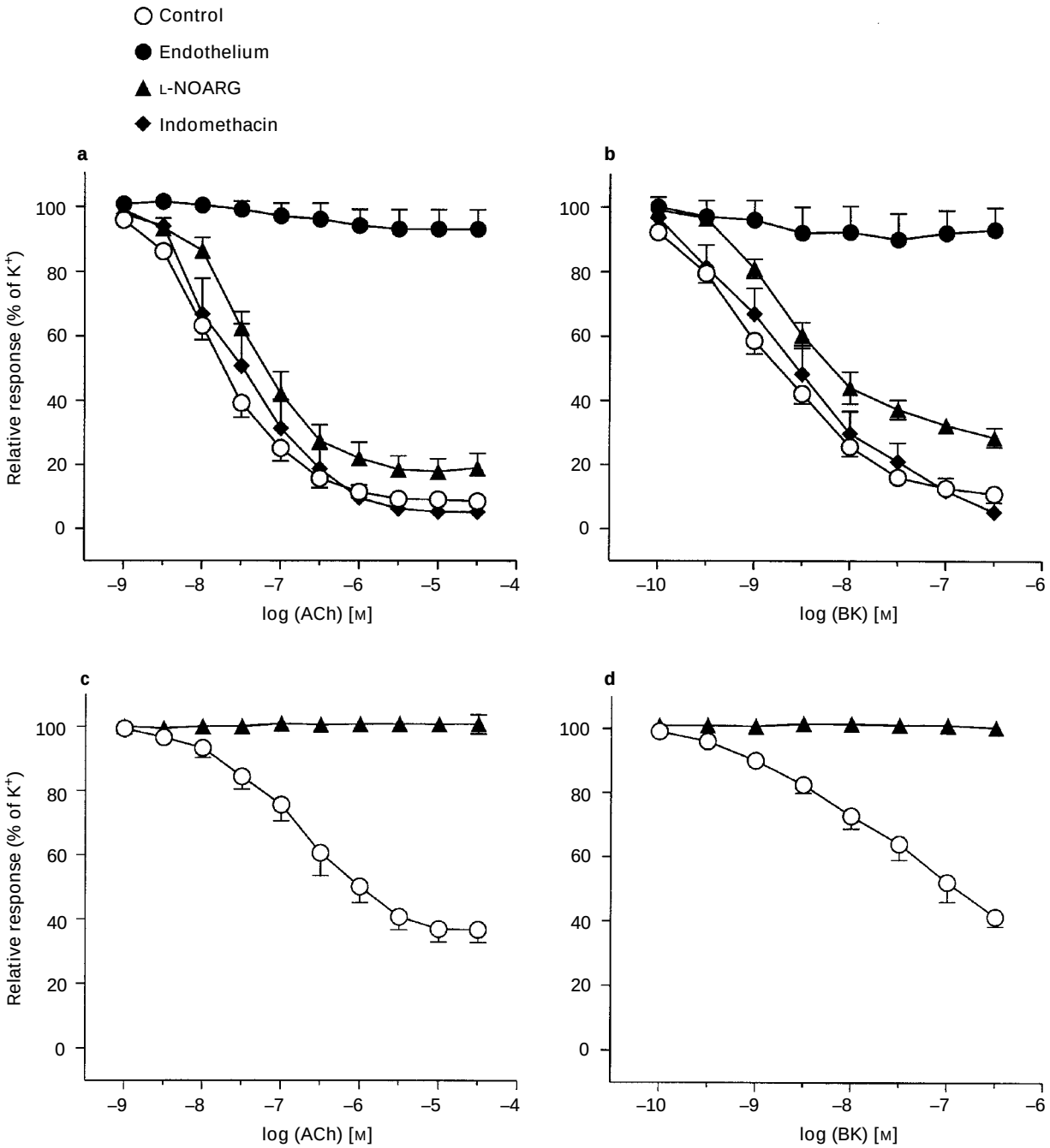

Figure 1 Average concentration-response curves for the relaxant effects of (a,c) acetylcholine (ACh) and (b,d) bradykinin (BK) in horse penile small arteries preconstricted with U46619 (a,b) or K^+ (20–30 mM) (c,d) by about 50% of the K-PSS response. (a,b) Effects of mechanical removal of the endothelium, N^G-nitro-L-arginine (L-NOARG, 100 μM) and indomethacin (3 μM). (c,d) Effect of increasing extracellular K^+ to 20–30 mM in the absence and presence of 100 μM L-NOARG on the relaxations to ACh (c) and BK (d). Responses are expressed as percentage of the contraction elicited by either U46619 or K^+ before addition of either ACh or BK. Points represent mean and vertical lines show s.e.mean of 5–18 experiments.

(U.K.). Stock solutions of indomethacin and glibenclamide were made in 96% ethanol, and that of ODQ in dimethyl sulphoxide (DMSO). These drugs were added in volumes not exceeding 0.3% of the tissue bath volume (10 ml). S-nitroso-cysteine (SNC) was prepared fresh just before use by reacting equimolar concentrations of L-cysteine and sodium nitrite under acidic conditions, as described by Field *et al*. (1978). Preliminary experiments showed that at the final concentration applied, neither ethanol nor DMSO, nor the acid vehicle in which SNC was present, had an effect on penile small arteries.

Calculations and statistics

Mechanical responses of the arteries were measured as force and expressed as active wall tension, ΔT, which is the increase in force, ΔF, divided by twice the segment length. Relaxant responses are given as percentage of the preconstriction level just before the addition of the agonist. By using a computer program (GraphPad, Institute for Scientific Information, San Diego, California, U.S.A.), the concentration-response curves to the different relaxant agents were fitted to the classical Hill-equation, as described earlier (Simonsen *et al*., 1995). Sensitivity to the agonists is expressed in terms of $pD_2 = -\log(EC_{50})$, EC_{50} being the concentration of agonist required to give half-maximal relaxation.

Results are expressed as means ± s.e.mean and *n* represents the number of arteries (1–2 from each animal). Statistical differences between means were determined by Student's *t* test for paired observations. Means of multiple groups were compared by one-way analysis of variance (ANOVA) and Bonferroni method as an *a posterio* test. Probability levels less than 5% were considered significant.

Results

Penile small arteries with a normalized internal lumen diameter of 504 ± 12 μm (*n* = 168) responded to K-PSS with an average contraction of 10.1 ± 0.5 Nm^{-1} (*n* = 168). At concentrations of 3–30 nM, the thromboxane analogue, U46619, elicited contractions of 4.8 ± 0.2 Nm^{-1} (*n* = 168) representing 52 ± 1% of the response to K-PSS.

Effects of endothelial cell removal, L-NOARG, indomethacin and raising extracellular K^+ on acetylcholine- and bradykinin-elicited relaxations

In U46619-contracted arteries, both ACh and BK induced concentration-dependent relaxations, BK being one order of magnitude more potent than ACh (Figure 1, Table 1). Mechanical endothelial cell removal increased resting tension by 13 ± 3% (*n* = 12) of the K-PSS response, and abolished the relaxations to ACh and BK (Figure 1a, b; Table 1). Treatment with the NOS blocker, L-NOARG (100 μM) increased basal tone by 6 ± 2% (*n* = 10) and slightly reduced the maximal responses to both vasodilators, and the sensitivity to ACh (Figure 1a, b; Table 1). The cyclo-oxygenase blocker, indomethacin (3 μM) did not have any significant effect on the ACh- and BK-elicited relaxations, either alone or in the presence of L-NOARG (100 μM), but enhanced resting tension by 7 ± 2% (*n* = 8) and 15 ± 4% (*n* = 9), respectively, of the K-PSS contraction (Figure 1a,b; Table 1).

Raising extracellular K^+ to 20–30 mM, induced contractions of penile resistance arteries of 6.4 ± 0.8 Nm^{-1} (*n* = 12), representing 53 ± 5% of the K-PSS-elicited response, and significantly reudced the relaxations to both ACh and BK, compared with those evoked in U46619-precontracted arteries (Figure 1c, d; Table 1). In the presence of 100 μM L-NOARG, in arteries contracted with K^+ by 63 ± 5% (6.0 ± 0.5 Nm^{-1}) of the K-PSS response, both ACh- and BK-elicited relaxations were completely abolished (Figure 1c, d).

In the continuous presence of L-NOARG, ACh dose-response curves constructed at 30 min intervals could be repeated without a significant loss of relaxant response. Thus pD_2 and maximal responses were 7.33 ± 0.09 and 99 ± 1% (*n* = 4), 7.35 ± 0.01 and 98 ± 2% (*n* = 4), and 7.28 ± 0.06 and 97 ± 2% (*n* = 4), in a first, second and third stimulation, respectively.

Table 1 Effect of mechanical endothelial cell removal (-Endo), N^G-nitro-L-arginine (L-NOARG, 100 μM), indomethacin (Indo, 3 μM) and raising extracellular K^+ on the relaxations elicited by acetylcholine and bradykinin in horse penile small arteries

	Acetylcholine				
	pD_2 ($-\log EC_{50}$)	E_{max} (%)	ΔBT (Nm^{-1})	l_1 (μm)	n
Control	7.71 ± 0.09	91.4 ± 1.3	—	553 ± 30	23
-Endo	—	6.9 ± 5.6**	1.6 ± 0.2*	567 ± 65	5
L-NOARG	7.30 ± 0.13*	81.2 ± 4.6	0.6 ± 0.2*	505 ± 35	10
Indo	7.48 ± 0.23	94.7 ± 1.6	0.9 ± 0.3*	561 ± 42	7
L-NOARG + Indo	7.35 ± 0.15	96.7 ± 1.4	1.7 ± 0.4*	436 ± 35	5
K^+ (20–30 mM)	6.76 ± 0.20*	63.4 ± 3.8*	—	556 ± 54	7
	Bradykinin				
	pD_2 ($-\log EC_{50}$)	E_{max} (%)	ΔBT (Nm^{-1})	l_1 (μm)	n
Control	8.80 ± 0.07	89.0 ± 1.6	—	559 ± 35	24
-Endo	—	8.2 ± 7.0**	1.6 ± 0.4*	562 ± 33	5
L-NOARG	8.46 ± 0.14	71.4 ± 2.8**	0.4 ± 0.1*	550 ± 45	6
Indo	8.60 ± 0.20	94.7 ± 2.5	1.3 ± 0.4*	595 ± 50	8
L-NOARG + Indo	8.90 ± 0.10	85.3 ± 3.1	1.0 ± 0.3*	550 ± 41	5
K^+(20–30 mM)	7.53 ± 0.20**	58.7 ± 3.5**	—	570 ± 17	6

Values are mean ± s.e.mean; *n* indicates the number of arteries. pD_2 is $-\log EC_{50}$, EC_{50} being the concentration of agonist required to give half maximal relaxation. E_{max} is the maximal relaxation expressed as percentage of the contractions induced by U46619 or 20–30 mM K^+. ΔBT indicates the increase in resting tension after treatment. l_1 is the normalized diameter at which experiments were performed. Significant differences from control values were analysed by paired or unpaired *t* test, as appropriate. *$P < 0.05$; **$P < 0.01$.

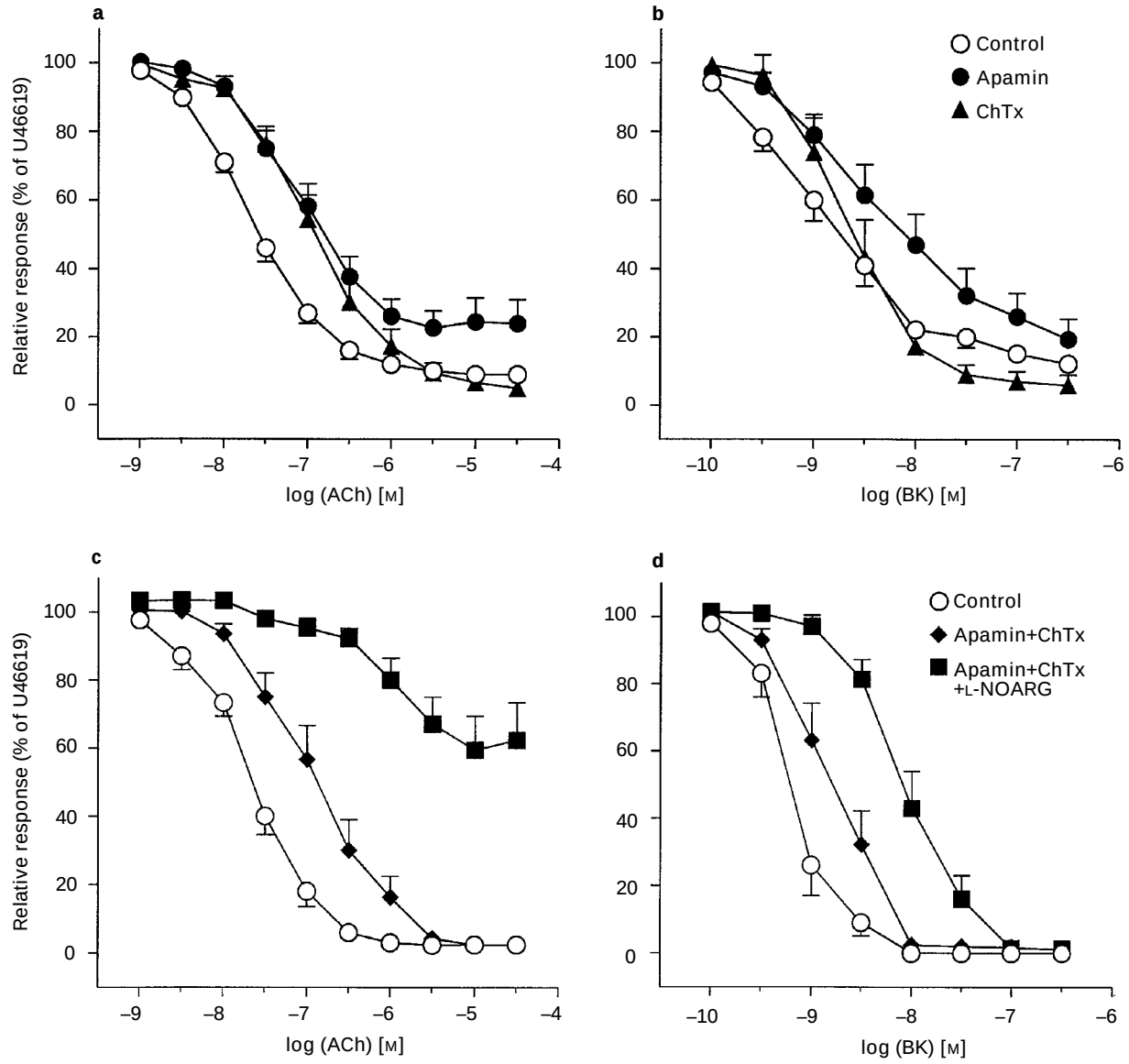

Figure 2 Effect of K_{Ca} channel blockers on the relaxations to (a,c) acetylcholine (ACh) and (b,d) bradykinin (BK) of penile small arteries. Mean concentration-response curves in control conditions and after 20 min treatment with either 0.3 μM apamin (a,b), 30 nM charybdotoxin (ChTx) (a,b) or 0.3 μM apamin plus 30 nM charybdotoxin in the absence and the presence of 100 μM L-NOARG (c,d). Results are expressed as percentage of the contraction to U46619 and points represent mean with vertical lines showing s.e.mean of 5–16 arteries.

Table 2 Effect of blockers of Ca^{2+}-activated K^+ channels in the absence and presence of N^G-nitro-L-arginine (L-NOARG, 0.1 mM) on the relaxations elicited by acetylcholine in horse penile small arteries

	pD_2 ($-\log EC_{50}$)	ΔpD_2	E_{max} (%)	ΔBT (Nm^{-1})	l_l μm	n
Control	7.51±0.16	—	92.6±2.8	—	474±31	6
ChTx 30 nM	7.00±0.17	0.52±0.09	95.2±1.8	2.1±0.9*	—	—
ChTx 30 nM + L-NOARG 0.1 mM	6.14±0.15[a,b]	1.37±0.22	86.6±3.6	3.1±1.2*	—	—
Control	7.80±0.10	—	97.5±0.9	—	411±13	7
Apamin 0.3 μM	7.20±0.18[a]	0.59±0.14	97.1±1.4	0.6±0.2*	—	—
Apamin 0.3 μM + L-NOARG 0.1 mM	6.98±0.05[a]	0.80±0.12	53.0±14.5[a,c]	1.3±0.4*	—	—
Control	7.65±0.08	—	97.6±1.4	—	386±29	6
ChTx 30 nM + apamin 0.3 μM	6.89±0.17[a]	0.77±0.18	97.5±1.4	1.3±0.4*	—	—
ChTx 30 nM + apamin 0.1 μM + L-NOARG 0.1 mM	5.87±0.09[a,d]	1.88±0.11#	37.6±11.1[a,d]	2.7±0.8*	—	—

Values are mean±s.e.mean; n is number of arteries. pD_2 is $-\log EC_{50}$, EC_{50} being the concentration of acetylcholine giving half-maximal relaxation. E_{max} is the maximal relaxation expressed as percentage of the contraction elicited by U46619. ΔBT is the increase in basal tension after treatment. l_l is the normalized diameter at which experiments were performed. ChTx: charybdotoxin. [a] $P<0.05$ versus control; [b] $P<0.05$ versus ChTx alone; [c] $P<0.05$ versus apamin alone; [d] $P<0.05$ versus ChTx plus apamin alone; # $P<0.05$ versus apamin plus L-NOARG; analysed by ANOVA followed by Bonferroni. * $P<0.05$ (t test).

Effects of charybdotoxin, apamin and glibenclamide

In the absence of L-NOARG, charybdotoxin (30 nM) largely increased resting tension, contracting small penile arteries by 16±4% ($n=15$) of the KPSS response. Therefore, contractions to U46619 in the presence of this toxin were matched to those in control conditions, being 3.9±0.4 Nm^{-1} (50±5% of K-PSS, $n=12$) in control and 5.1±0.6 Nm^{-1} (63±4% of K-PSS, $n=12$) in treated arteries. Charybdotoxin did not change the maximal relaxations to either ACh or BK (Figure 2a, b), but significantly reduced the sensitivity to ACh (Table 2). Blockade of small conductance K_{Ca} channels with 0.3 μM apamin increased resting tension by 5±1% ($n=19$) and the contractions to U46619 were 4.7±0.8 Nm^{-1} (45±5% of K-PSS, $n=19$) and 5.6±0.9 Nm^{-1} (51±4% of K-PSS, $n=19$), before and after apamin treatment, respectively. Apamin inhibited significantly the sensitivity to both ACh (Figure 2a, Table 2) and BK (Figure 2b). pD_2 values for BK being 8.78±0.22 ($n=6$) and 8.28±0.21 ($P<0.01$, paired t test, $n=6$), in the absence and the presence of apamin, respectively. Combined treatment with charybdotoxin (30 nM) plus apamin (0.3 μM) increased resting tension by 20±5% ($n=12$) of the K-PSS response and the contractions to U46619 were matched to those in control conditions, being 3.3±0.2 Nm^{-1} (52±5% of K-PSS, $n=12$) and 3.7±0.3 Nm^{-1} (58±6% of K-PSS, $n=12$), before and after incubation with the two toxins, respectively. However, the combination of charybdotoxin and apamin did not cause any further inhibition of the relaxations to ACh (Figure 2c; Table 2), compared with the inhibition by either toxin alone. Combined treatment with the two toxins did not inhibit the relaxations to BK further (Figure 2d; $\delta pD_2=0.39\pm0.07$, $n=6$), compared with the inhibition elicited by apamin alone ($\delta pD_2=0.49\pm0.09$, $n=6$).

In contrast to the effects of K_{Ca} channels blockers, the inhibitor of ATP-sensitive K^+ channels, glibenclamide (3 μM), did not affect the relaxations to either ACh or BK. Thus, pD_2 values and maximal responses for ACh and BK were 7.30±0.25 and 89±2%, and 8.45±0.30 and 75±9%, respectively, in the absence, and 7.43±0.25 and 91±4% ($n=4$), and 8.42±0.36 and 88±6% ($n=5$), respectively, in the presence of glibenclamide.

When penile small arteries were exposed to either apamin or charybdotoxin in the presence of 100 μM L-NOARG, the inhibitory effects of K_{Ca} channel blockers on the relaxations to ACh were significantly enhanced (Table 2). Exposure to both toxins and L-NOARG resulted in the most effective inhibition of the ACh (Figure 2c; Table 2) and BK (Figure 2d) relaxant responses, pD_2 values and maximal relaxations to BK were 9.17±0.09 and 100±0% ($n=6$) and 8.09±0.14 ($P<0.01$, paired t test, $n=6$) and 98±1%, in control conditions and in the presence of all the three blockers, respectively.

In the presence of L-NOARG (100 μM), glibenclamide did not have any inhibitory effect on the relaxations to either ACh or BK, neither did it produce any additional blockade when it was combined with charybdotoxin plus apamin ($n=4$, not shown), compared to the inhibitory effect induced by the two toxins alone.

Effect of inhibition of guanylate cyclase

The specific inhibitor of guanylate cyclase, ODQ (5 μM), contracted penile resistance arteries by 7±3% ($n=16$) of the K-PSS response and enhanced the preconstriction induced by U46619, that was matched to that in controls: 4.8±0.4 Nm^{-1} and 5.3±0.4 Nm^{-1} ($n=12$), representing 43±4% and 47±4% of K-PSS, in the absence and presence of 5 μM ODQ,

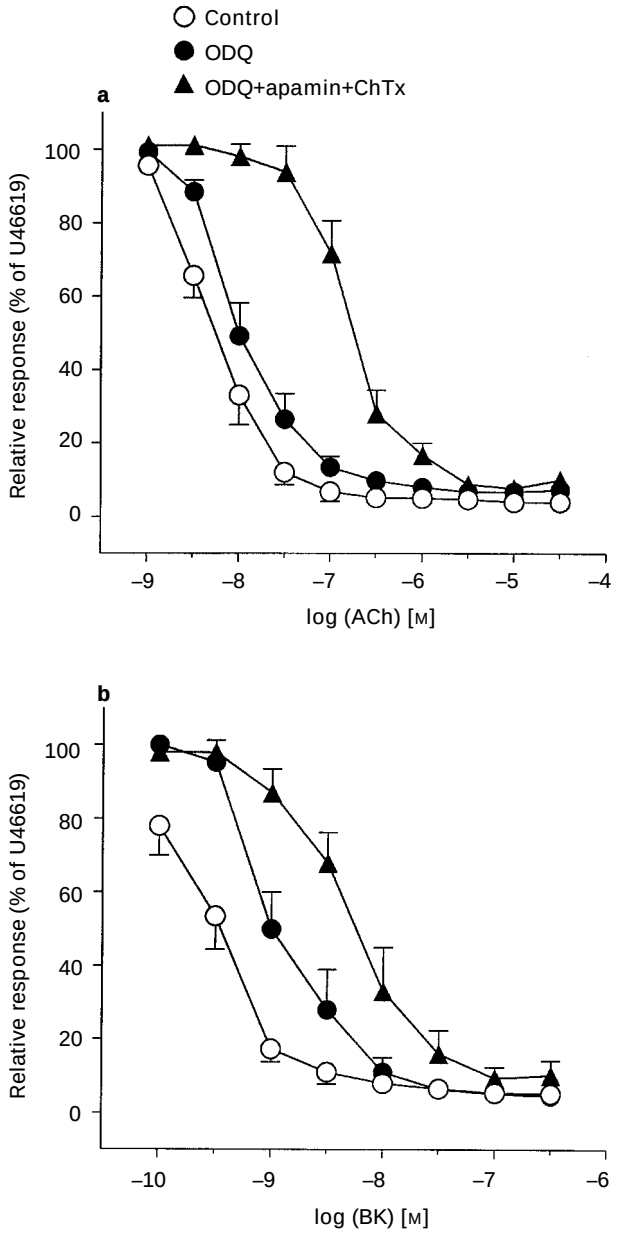

Figure 3 Effect of the selective guanylate cyclase blocker, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM) alone or combined with 0.3 μM apamin plus 30 nM charybdotoxin (ChTx), on the average concentration-relaxation response curves to (a) acetylcholine (ACh) and (b) bradykinin (BK) of penile small arteries. Results (mean with vertical lines showing s.e.mean of 6–8 experiments) are expressed as percentage of the contraction elicited by U46619.

respectively. ODQ did not change the maximal responses to either ACh or BK (Figure 3), but it significantly inhibited the sensitivity to both agonists, this inhibitory effect being more pronounced for BK (Figure 3b). Thus, pD_2 values for the relaxations to ACh and BK were 8.23±0.09 ($n=6$) and 9.54±0.12 ($n=6$), respectively in control, and 7.98±0.09 ($P<0.05$, paired t test, $n=6$) and 8.90±0.14 ($P<0.01$, paired t test, $n=6$), respectively, in ODQ-treated arteries.

Combined treatment with ODQ (5 M) and charybdotoxin (30 nM) plus apamin (0.3 μM) produced an additional inhibiton of the relaxations to both ACh and BK (Figure 3), compared to that in the presence of either ODQ or charybdotoxin plus apamin alone: δpD_2 values for the

relaxations to ACh and BK were 0.77 ± 0.18 ($n=6$) and 0.39 ± 0.07 ($n=6$), respectively, in the presence of charybdotoxin plus apamin, and 1.39 ± 0.09 ($n=6$, $P<0.05$, unpaired t test) and 1.29 ± 0.11 ($n=6$, $P<0.01$, unpaired t test), respectively, in the presence of ODQ, charybdotoxin and apamin.

Effect of K^+ channel blockers and ODQ on the relaxations to nitric oxide and S-nitroso-cysteine

Exogenous NO added as acidified sodium nitrite (1–300 μM) or SNC (10 nM–30 μM) produced nearly full relaxation of penile small arteries, SNC being about two orders of magnitude more potent than NO (Figure 4). Charybdotoxin displaced to the right the relaxation curves for both NO and SNC, and also slightly but significantly reduced their maximal responses (Figure 4a,b). In the absence of this blocker, pD_2 and maximal relaxations for NO and SNC were 4.88 ± 0.09 and $76 \pm 4\%$ ($n=5$), and 6.88 ± 0.12 and $85 \pm 10\%$ ($n=5$), respectively, whereas in the presence of 30 nM charybdotoxin they were 4.50 ± 0.06 ($P<0.01$, $n=5$) and $66 \pm 7\%$ ($P<0.05$, paired t test, $n=5$) (Figure 4a), and 6.38 ± 0.09 ($P<0.01$, $n=5$) and 64 ± 11 ($P<0.05$, paired t test, $n=5$) (Figure 4b), respectively. Treatment with apamin (0.3 μM) reduced sensitivity (pD_2 6.90 ± 0.15 in control vs 6.53 ± 0.11, $P<0.05$, paired t test, $n=5$, in apamin-treated arteries) and maximum relaxation ($88 \pm 8\%$ in control vs $65 \pm 9\%$ in treated arteries, $P<0.05$, paired t test, $n=5$) to SNC (Figure 4b), without changing the relaxant responses to NO (Figure 4a). Combined treatment with charybdotoxin (30 nM) plus apamin (0.3 μM) did not further inhibit the NO-elicited relaxations compared with the inhibition elicited by charybdotoxin alone (Figure 4a). Combination of charybdotoxin plus apamin did not cause an additional inhibition of the relaxations to SNC, compared to that produced by either toxin alone (Figure 4b).

ODQ (5 μM) shifted to the right the relaxations curves to both NO (Figure 4c) and SNC (Figure 4d). Thus, pD_2 values for NO and SNC were 4.95 ± 0.11 and 6.79 ± 0.10, respectively, in the absence, and 4.21 ± 0.07 ($P<0.05$, ANOVA followed by

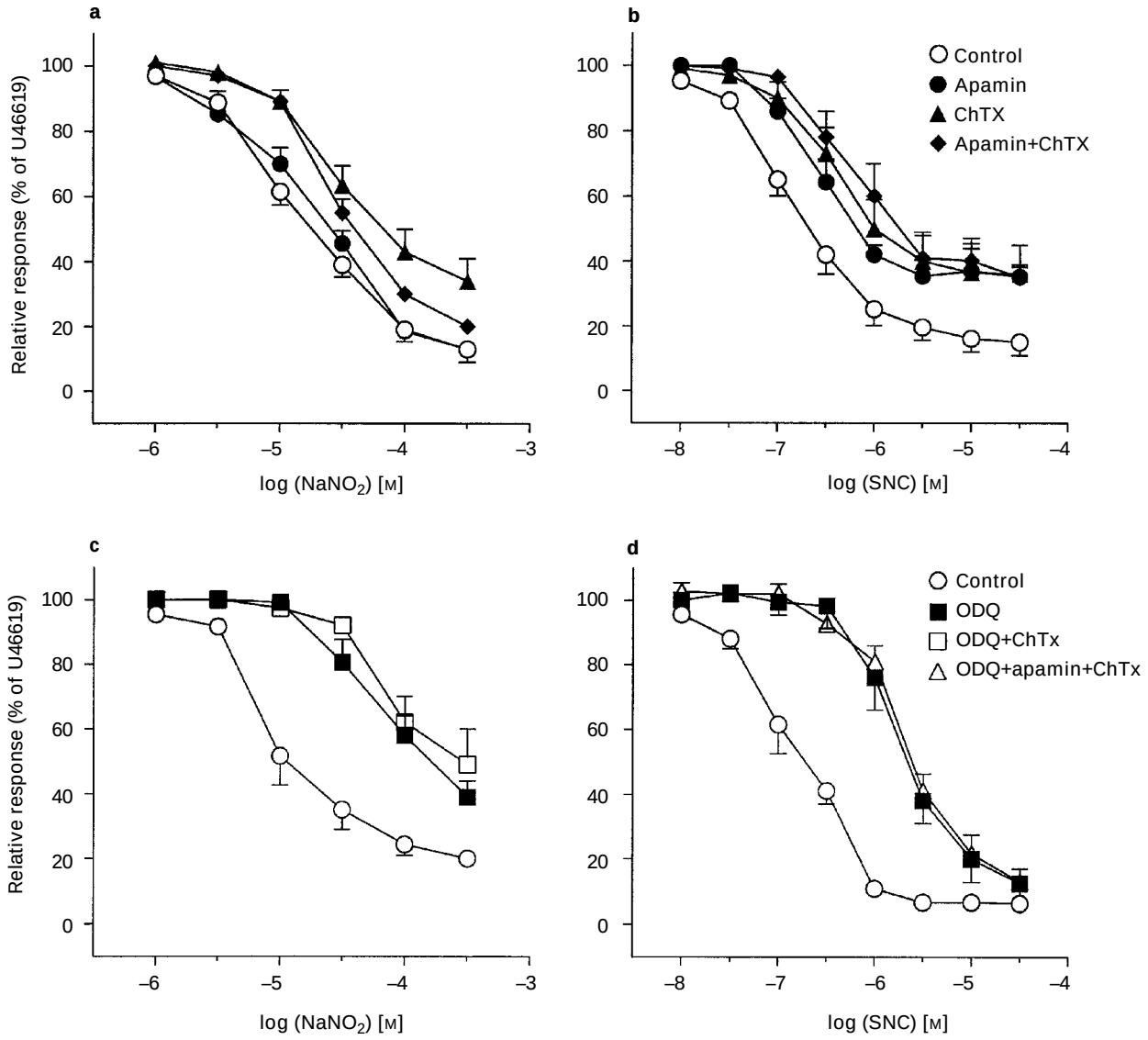

Figure 4 Mean concentration-relaxation response curves for (a,c) acidified sodium nitrite ($NaNo_2$) and (b,d) S-nitroso-cysteine (SNC) in horse penile small arteries. Effects of 0.3 μM apamin (a,b), 30 nM charybdotoxin (ChTx) (a,b), 0.3 μM apamin plus 30 nM charybdotoxin (a,b) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM) alone (c,d) or combined with either 30 nM ChTx (c) or 0.3 μM apamin plus 30 nM ChTx (d). Results are mean and vertical lines show s.e.mean of 5–6 experiments and are expressed as percentage of the U46619 response.

Bonferroni, $n=5$) and 5.76 ± 0.09 ($P<0.05$, ANOVA followed by Bonferroni, $n=5$), respectively, in the presence of ODQ. Combination of ODQ (5 μM) with either charybdotoxin (Figure 4c) or charybdotoxin plus apamin (Figure 4d), did not cause further inhibition of the responses to either NO or SNC, respectively. Thus, pD_2 values and maximum response for NO in the presence of ODQ plus charybdotoxin were 4.04 ± 0.18 ($P<0.05$ vs controls, $n=5$) and $50\pm11\%$ ($P<0.05$ vs controls, ANOVA followed by Bonferroni, $n=5$), respectively (Figure 4c). pD_2 and maximum response for SNC in the presence of ODQ and charybdotoxin plus apamin were 5.70 ± 0.04 ($P<0.05$ vs controls, ANOVA followed by Bonferroni, $n=5$) and $87\pm4\%$ ($n=5$), respectively (Figure 4d).

Effect of ouabain on the relaxations to acetylcholine, bradykinin and nitric oxide

In the presence of 3 μM phentolamine, ouabain (1–100 μM) produced concentration-dependent contractions of penile small arteries. We chose a concentration of ouabain, 5 μM, causing a contraction of $50\pm6\%$ (3.7 ± 0.4 Nm^{-1}, $n=17$) of the K-PSS response, to evaluate the effects of inhibition of the Na^+-K^+ATPase on the relaxations to ACh, BK and exogenous NO. Ouabain (5 μM) enhanced the contractions to U46619 that were matched to those in the absence of the drug, being 3.8 ± 0.3 Nm^- ($52\pm4\%$ of K-PSS, $n=18$) and 4.4 ± 0.3 Nm^{-1} ($63\pm5\%$ of K-PSS, $n=12$) before and after 8–10 min incubation with 5 μM ouabain, respectively. Ouabain did not significantly affect the relaxations to either ACh (pD_2 and maximum response 8.05 ± 0.07 and $94\pm3\%$ in controls vs 7.93 ± 0.08 and $87\pm7\%$, $n=6$, after ouabain treatment) (Figures 5a,b and 6a), BK (9.09 ± 0.12 and $98\pm2\%$ in control vs 9.14 ± 0.17 and $82\pm7\%$, $n=5$, in treated-arteries) (Figure 6b) or NO (4.99 ± 0.07 and $92\pm2\%$ in control vs 5.10 ± 0.07 and $84\pm3\%$, $n=6$, after ouabain treatment) (Figures 5d, e and 6c). However, when combined with the NOS inhibitor L-NOARG (100 μM), ouabain (5 μM) reduced the ACh- and BK-elicited relaxations to $16\pm5\%$ ($P<0.01$, ANOVA followed by Bonferroni, $n=6$) (Figures 5a, c and 6a) and $13\pm3\%$ ($P<0.01$, ANOVA followed by Bonferroni, $n=5$) (Figure 6b), respectively, in arteries which were relaxed by papaverine (50 μM) by $91\pm5\%$ ($n=6$) and $83\pm6\%$ ($n=5$), respectively. Moreover, combined treatment with ODQ (5 μM) and ouabain (5 μM) produced a further inhibition of the relaxations to NO compared to that produced by either blocker alone (Figures 5d, f and 6c). The maximum relaxation elicited by NO in the presence of ouabain plus ODQ was reduced to $26\pm3\%$ ($P<0.01$, ANOVA followed by Bonferroni, $n=6$) in arteries which relaxed to papaverine by $91\pm2\%$ ($n=6$).

Discussion

The present study demonstrates that the receptor-mediated endothelium-dependent vasodilatations of penile small arteries involve the release of EDRF-NO and a non-prostanoid non-

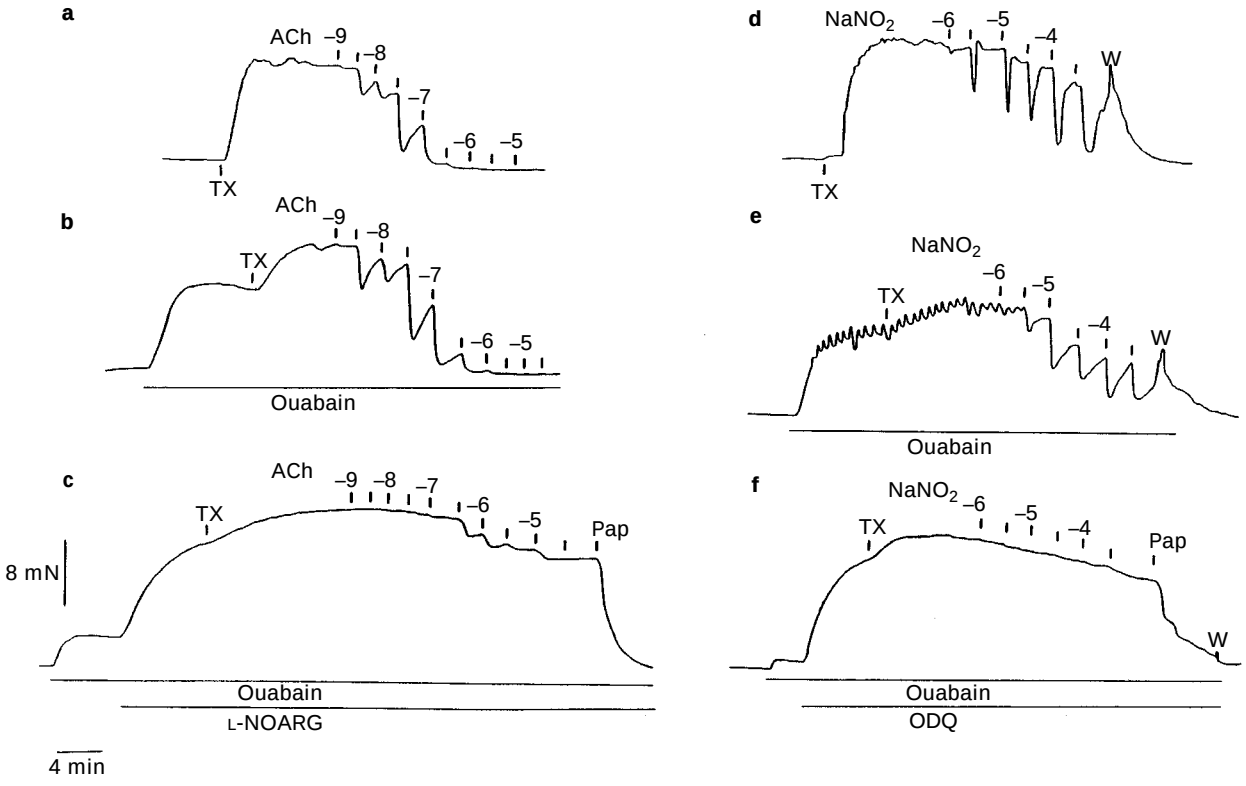

Figure 5 Isometric force recordings showing the effects of ouabain alone or ouabain combined with either L-NOARG or ODQ on the relaxations to (a,b,c) acetylcholine (ACh) or (d,e,f) exogenous nitric oxide (NO) added as acidified $NaNO_2$, respectively, of 2 penile small arteries. (a,b,c) Relaxation to ACh in an artery preconstricted with the thromboxane analogue, U46619 (TX), in control conditions (a), after incubation with 5 μM ouabain (b) or after combined application of ouabain and 100 μM L-NOARG (c). (d,e,f) Relaxations to acidified $NaNO_2$ in the absence (d) and presence of 5 μM ouabain (e) or ouabain plus ODQ (5 μM) (f). Contractions to U46619 in the presence of ouabain or ouabain plus L-NOARG or ODQ were matched to those in controls by applying a lower concentration of the drug. The concentrations of U46619 were 15 nM (a,d), 0.5 nM (b), 0.2 nM (c), 1.5 nM (e) and 0.8 nM (f). Vertical bar shows force and horizontal bar shows time.

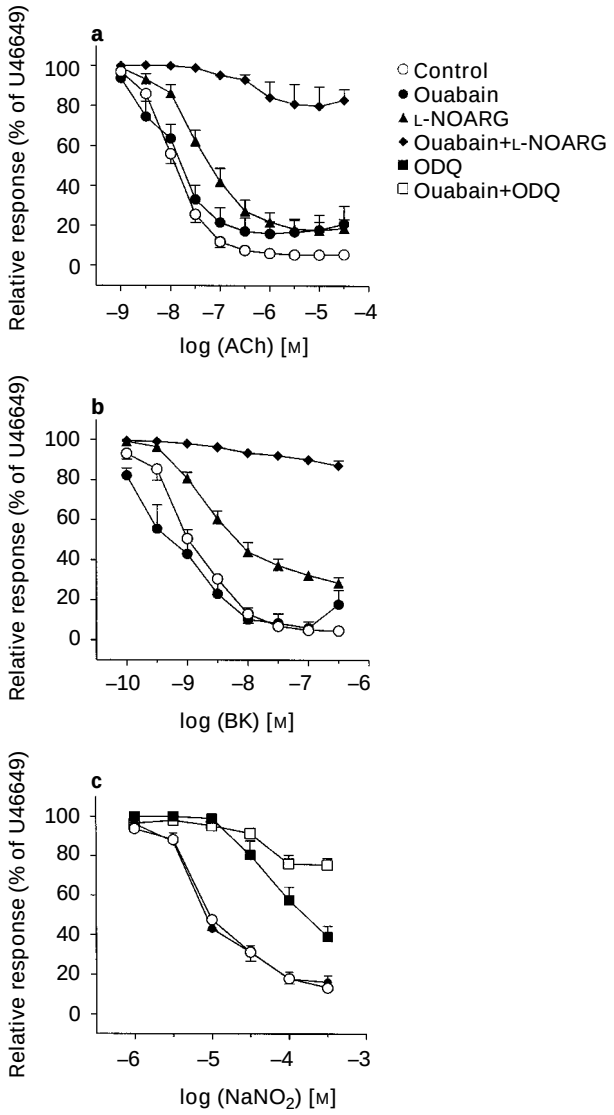

Figure 6 Effect of ouabain on the relaxations to (a) acetylcholine (ACh), (b) bradykinin (BK) and (c) exogenous nitric oxide (NO), added as acidified $NaNO_2$, of penile small arteries. Average concentration-response curves in control conditions (a,b,c), and after incubation with 5 μM ouabain alone (a,b,c), N^G-nitro-L-arginine (L-NOARG, 100 μM) alone (a,b). ODQ (5 μM) alone (c), ouabain plus L-NOARG (a,b) and ouabain plus ODQ (c). Results are mean and vertical lines show s.e.mean of 6–16 arteries and are expressed relative to the U46619-elicited contractions.

NO endothelial factor(s) which relax(es) the underlying smooth muscle by activating apamin- and charybdotoxin-sensitive K_{Ca} channels and the Na^+-K^+ ATPase. Moreover, NO itself appears to activate also charybdotoxin-sensitive K_{Ca} channels and the electrogenic pump by cyclic GMP-dependent and independent mechanisms, respectively.

The ACh- and BK-elicited endothelium-dependent relaxations were only attenuated by L-NOARG. This is in contrast to that found for other erectile tissues of the penis, such as the dorsal penile artery (Liu *et al*., 1991) and the cavernous trabeculae (Azadzoi *et al*., 1992), where blockade of NOS inhibited most of the relaxations to ACh. The present findings thus support the view that in the periphery, the contribution of a L-NOARG-resistant factor to the endothelium-dependent vasodilatations is more relevant in small resistance than in large conductance arteries, this factor playing a significant role in the determination of peripheral vascular resistance (Nagao *et al*., 1992; Hwa *et al*., 1994; Garland *et al*., 1995; Shimokawa *et al*., 1996). Moreover, indomethacin did not change the relaxant responses to either ACh or BK and combined inhibition of prostanoids and NO did not have any additional inhibitory effect compared to that of L-NOARG alone, thus excluding a participation of one factor in the absence of the other. The possibility that ACh and BK exert their vasorelaxant effects by mobilization of preformed pools of NO-containing compounds (Davisson *et al*., 1996) seems also unlikely, since consecutive dose-response curves for ACh in the presence of L-NOARG remained unaltered.

In the present study both ACh- and BK-elicited relaxations were largely reduced when extracellular K^+ was increased, and completely prevented by L-NOARG in the presence of high extracellular K^+. Since K^+-evoked contractions were matched to those induced by U46619, it is unlikely that inhibition of the relaxations is due to functional antagonism. Therefore, these results indicate that the endothelium-dependent responses of penile small arteries are mediated by at least two different factors, namely, NO and a non-prostanoid non-NO factor which might relax smooth muscle by increasing K^+ conductance. Unlike experiments with rat small mesenteric arteries (Plane & Garland, 1996), we did not find a marked inhibition of the relaxations to ACh in penile arteries precontracted with U46619 after NOS blockade. This discrepancy may be ascribed to the different vascular bed and animal species examined and perhaps also to the concentration of U46619 used, which was about 100 fold lower in the present study.

Multiple endothelial hyperpolarizing factors may exist or, alternatively, several types of K^+ channels are involved in the hyperpolarization to EDHF, this heterogeneity depending on the vascular bed and animal species (Brayden, 1990; Van Voorde *et al*., 1992; Eckman *et al*., 1994; Hecker *et al*., 1994; Murphy & Brayden, 1995a). The fact that both charybdotoxin and apamin, but not glibenclamide, partially inhibited the ACh- and BK-elicited relaxations of penile small arteries indicates that K_{Ca} channels may play a role in these responses. Since the inhibitory effect of either charybdotoxin or apamin and that of L-NOARG were additive, it can be suggested the involvement of an endothelial factor different from NO, the release and/or action of which involves an increased apamin- and charybdotoxin-sensitive K^+ conductance. It has been recently shown that whereas neither charybdotoxin nor apamin alone had an effect on the L-NOARG/indomethacin-resistant responses induced by ACh, the combination of the two toxins abolished both the relaxation (Zygmunt & Högestätt, 1996) and the hyperpolarization (Corriu *et al*., 1996) elicited by the cholinoceptor agonist in rat hepatic and guinea-pig carotid artery, respectively. However, in penile small arteries, the combination of the two K_{Ca} channel blockers did not further inhibit the relaxations to either ACh, BK, NO or SNC, compared to either toxin alone, which indicates that charybdotoxin and apamin may be interacting with a single K^+ channel type. In fact, besides blocking large-conductance K_{Ca} channels, charybdotoxin is known to inhibit other types of K^+ channels, such as intermediate- conductance K_{Ca} voltage-dependent K^+ channels (Nelson & Quayle, 1995) and a small-conductance K_{Ca} channel type, also selectively blocked by apamin in rat glomerular arterioles (Gebremedhim *et al*., 1996).

The possibility that charybdotoxin and apamin may affect K^+ channels at the endothelial cells, thus interfering with the synthesis/release of EDHF (Groschner *et al*., 1992), cannot be ruled out from the present experiments. However, this seems unlikely since both toxins also inhibited the relaxations to

exogenous NO and SNC. Therefore, the present results suggest an important role of K_{Ca} channels in the increased K^+ conductance underlying the non-NO non-prostanoid-mediated endothelium-dependent relaxations of penile small arteries.

In addition to EDHF, in some vascular beds prostacyclin and NO may also contribute to the endothelium-dependent hyperpolarization (Komori *et al*., 1988; Tare *et al*., 1990; Bolotina *et al*., 1994; Murphy & Brayden, 1995b). Accordingly, the present results show that the relaxations induced by exogenous NO, released from either acidified $NaNO_2$ or SNC, are reduced by charybdotoxin, which confirms our previous observations that neurally-released endogenous NO relaxes penile small arteries through charybdotoxin-sensitive channels (Simonsen *et al*., 1995). In contrast to the relaxations elicited by ACh and SNC, which were inhibited by both charybdotoxin and apamin, those evoked by NO were not changed by apamin, which probably rules out a role for small-conductance K_{Ca} channels in these responses, unlike that recently found for the NO relaxations in coronary resistance arteries (Simonsen *et al*., 1997a). The differential inhibitory action of apamin on the relaxant responses of SNC, but not those of NO, would be consistent with the ability of this S-nitrosothiol to activate apamin-sensitive channels, as demonstrated in the gastric fundus by means of electrophysiological techniques (Kitamura *et al*., 1993).

NO is generally believed to stimulate soluble guanylate cyclase with a subsequent accumulation of intracellular cyclic GMP levels at the smooth muscle (Ignarro, 1990). The finding that the combination of ODQ and the K_{Ca} channel blockers did not cause an additive inhibition of the relaxations to either NO or SNC suggests that activation of K_{Ca} channels by NO in penile small arteries is accounted for by a cyclic GMP-dependent mechanism, as shown for coronary arteries (George & Shibata, 1995; Simonsen *et al*., 1997a), and not by two different pathways leading to stimulation of soluble guanylate cyclase and activation of charybdotoxin-sensitive K^+ channels, respectively (Bolotina *et al*., 1994; Plane *et al*., 1996). However, the fact that residual relaxations to NO still persisted after blockade of guanylate cyclase with ODQ indicates that NO might also relax penile small arteries through a cyclic GMP-independent mechanism.

Endothelium-dependent relaxations resistant to L-NOARG and indomethacin have been shown to be mediated by cyclic GMP-independent mechanisms (Eckman *et al*., 1994; Cohen & Vanhoutte, 1995). Accordingly, the selective inhibitor of guanylate cyclase, ODQ (Garthwaite *et al*., 1995), only attenuated the relaxations to ACh and BK in penile small arteries, which suggests that these relaxant responses can be almost fully accounted in the absence of increased cyclic GMP levels. Combined application of ODQ and apamin plus charybdotoxin had an additive inhibitory effect on the ACh and BK relaxant responses, compared to that of either ODQ or the two toxins alone. These data indicate that at least two different signal transduction pathways underlie the endothelium-dependent vasodilatations of penile arteries: one probably leads to intracellular cyclic GMP accumulation at smooth muscle and is sensitive to ODQ, and the other induces an increased membrane K^+ conductance and is sensitive to apamin and charybdotoxin. The first pathway probably corresponds to the action of EDRF-NO, since the relaxant responses to exogenous NO were largely inhibited by ODQ, whereas the second pathway may correspond to the action of an unidentified EDHF which probably hyperpolarizes smooth muscle by opening K_{Ca} channels in a cyclic GMP-independent fashion. In contrast to that found for larger arteries (Zygmunt & Högestätt, 1995; Corriu *et al*., 1996) and the rabbit mesenteric small artery (Murphy & Brayden, 1995a), where the L-NOARG/indomethacin-resistant hyperpolarization and relaxation evoked by ACh were completely abolished by K_{Ca} channel blockers, in penile small arteries significant relaxant responses still persisted after blockade of either NOS or guanylate cyclase and K_{Ca} channels. This finding suggests that a third signal transduction mechanism, also independent of cyclic GMP accumulation, may be involved in the endothelium-dependent relaxations of penile small arteries. Moreover, the finding that the relaxant responses to either ACh or BK were totally abolished when extracellular K^+ was increased in the presence of L-NOARG, indicates that this signalling pathway includes a hyperpolarization of vascular smooth muscle.

In the presence of phentolamine to block a possible effect of neurally-released noradrenaline (Simonsen *et al*., 1997c), ouabain elicited pronounced concentration-dependent contractions of penile small arteries. This suggests that a basal activity of the Na^+-K^+ ATPase is probably involved in the maintenance of penile arterial tone, as shown previously for vascular (DeMey & Vanhoutte, 1980; Blaustein, 1993) and corpus cavernosum (Gupta *et al*., 1995) smooth muscle. Blockade of NOS with L-NOARG unmasked a powerful inhibitory effect of ouabain on the relaxations to both ACh and BK. The possibility of this being unspecific, due to a depolarizing effect of ouabain on smooth muscle that could in turn inhibit the effects of EDHF, seems unlikely. Firstly, contractions elicited by U46619 in the presence of ouabain were matched to those in controls, the concentration of ouabain used was one eliciting submaximal contractions and the time of incubation (10–15 min) would not be expected to produce an excessive accumulation of Na^+ within smooth muscle cells. Finally, in similar conditions of preconstriction, concentration and incubation time, but in the absence of NOS blockade, ouabain did not affect the relaxations to either ACh or BK. Therefore, the present results indicate the release of an L-NOARG/indomethacin resistant endothelial factor upon stimulation with ACh or BK which appears to hyperpolarize penile arterial smooth muscle by stimulating the Na^+-K^+ ATPase. Although we cannot rule out an effect of ouabain on the synthesis/release of EDHF, this seems unlikely, since ouabain also inhibited the relaxations to exogenous NO. The present findings are consistent with earlier data showing that ouabain blocks the ACh-elicited endothelium-dependent relaxation (DeMey & Vanhoutte, 1980) and hyperpolarization (Feletou & Vanhoutte, 1988) of canine femoral and coronary arteries, respectively. Whether the endothelial factor activating K_{Ca} channels is the same as that stimulating the Na^+-K^+ ATPase in penile small arteries, as well as its chemical identity(s), remains to be determined.

Whereas ouabain alone did not significantly affect the relaxations to NO, blockade of the Na^+-K^+ ATPase in the presence of ODQ unmasked an inhibitory effect of ouabain, which suggests guanylate cyclase-independent activation of the Na^+-K^+ ATPase as an alternative pathway underlying the NO-elicited relaxations in penile arterial smooth muscle. These data are consistent with earlier findings in vascular smooth muscle (Gupta *et al*., 1994) and with a recent study showing that NO stimulates Na^+-K^+ ATPase activity without increasing intracellular cyclic GMP levels in human corpus cavernosum smooth muscle (Gupta *et al*., 1995).

In conclusion, the present results suggest that the endothelium-dependent relaxations of penile small arteries are mediated by both EDRF-NO and L-NOARG/indomethacin

resistant factor(s) which probably hyperpolarize smooth muscle by increasing membrane K^+ conductance and stimulating the Na^+-K^+ ATPase. Whereas the actions of EDHF(s) appear to be independent of cyclic GMP accumulation, NO can activate charybdotoxin-sensitive K^+ channels and the electrogenic pump by guanylate cyclase-dependent and -independent mechanisms, respectively. Therefore, as depicted from the present data, EDRF-NO and EDHF share common mechanisms of vasodilatation in the same vascular bed, i.e. activation of K^+ channels and stimulation of the Na^+-K^+ ATPase, that might reinforce or modulate each other in order to control penile vascular resistance.

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