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European Journal of Pharmacology 515 (2005) 150 – 159

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Endothelial mechanisms underlying responses to acetylcholine in the horse deep dorsal penile vein

Ana Cristina Martínez, Dolores Prieto, Medardo Hernández, Luis Rivera, Paz Recio, Albino García-Sacristán, Sara Benedito*

Sección Departamental de Fisiología, Facultad de Farmacia, Universidad Complutense, UCM, 28040 Madrid, Spain

Received 6 January 2005; received in revised form 1 April 2005; accepted 8 April 2005
Available online 13 May 2005

Abstract

This study evaluates the mechanisms underlying endothelium-dependent responses to acetylcholine in horse deep dorsal penile veins. Acetylcholine-induced relaxation was abolished by endothelium removal, the soluble guanylyl cyclase-inhibitor, and the nitric oxide (NO) synthase inhibitors. Acetylcholine-induced relaxation was inhibited by high K^+ concentrations and blockade of large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels, and voltage-dependent potassium (K_v) channels. Relaxations were unaffected by a small-conductance K_{Ca} (SK_{Ca}) channel blocker, or an ATP-sensitive potassium (K_{ATP}) channel blocker. Relaxation in response to a NO donor was unaffected by K_{Ca} channel blockers, but inhibited by high K^+ concentrations and a K_v channel blocker. In the presence of a NO synthase inhibitor, acetylcholine-induced contractions were inhibited by a cyclooxygenase blocker and abolished by endothelial removal. The contractile response was competitively inhibited by muscarinic receptor antagonists, high affinity M_1 and M_3 antagonists, while the M_2 antagonist had no effect. The pharmacological profile suggests that acetylcholine contraction is mediated by muscarinic M_1 receptors. Our findings indicate that acetylcholine-induced relaxation in the horse deep dorsal penile vein is essentially mediated by NO, acting via the cGMP-dependent pathway and opening of K^+ channels. The contraction elicited by acetylcholine is prostanoid-mediated and induced by endothelial muscarinic M_1 receptor activation.

Keywords: Acetylcholine; Nitric oxide; Cyclic GMP; Potassium channel; Prostanoid; Endothelial muscarinic receptor; Horse dorsal penile vein

1. Introduction

It is well known that the presence of an intact endothelium is a determining factor for the vasorelaxation induced by acetylcholine. To date, the dilatory mediators released in response to acetylcholine have been identified as nitric oxide (NO), prostacyclin, and an endothelium-derived hyperpolarizing factor (EDHF). NO and prostacyclin induce vascular smooth muscle relaxation via stimulation of guanylate and adenylate cyclase, respectively (Moncada and Higgs, 1993; Vane et al., 1982). However, the chemical identity of EDHF and the mechanisms through which it produces its biological activity are still controversial issues. It has been reported that the effects of EDHF can be mimicked by increasing the levels of extracellular potassium and cytochrome-P450-derived epoxyeicosatrienoic acids (Edwards et al., 1998; Campbell and Harder, 2001). Further mechanisms proposed to account for EDHF-produced relaxation and hyperpolarization are the activation of Na^+–K^+ ATPase in vascular smooth muscle cells (Edwards et al., 1998; Prieto et al., 1998), the direct transfer of hyperpolarizing current from endothelial cells to smooth muscle cells via myoendothelial gap junctions, or electrical communication in the reverse direction, from vascular smooth muscle cells to the endothelium (Beny and Pacicca, 1994).

Although endothelium-dependent relaxations are largely mediated by NO, the other mechanisms including those regulated by prostacyclin and EDHF, are variable. In fact, in

* Corresponding author. Tel./fax: +34 913941696.
E-mail address: sbenedi@farm.ucm.es (S. Benedito).

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doi:10.1016/j.ejphar.2005.04.012

vessels such as rat hepatic artery (Zygmunt et al., 1998) and rabbit mesenteric artery (Murphy and Brayden, 1995), all three factors contribute to the vasodilatory response evoked by stimulation of the endothelium. However, each endothelial factors may rely on different mechanisms to produce relaxation. NO and prostacyclin are thought to open potassium channels either directly or through their respective second messengers (Félétou and Vanhoutte, 1999) and acetylcholine-induced vasorelaxation has even been associated with NO-independent hyperpolarization (Jiang et al., 2000).

Four muscarinic receptor subtypes (M_1–M_4) have been pharmacologically identified and a fifth subtype (m_5) has been genetically identified (Eglen et al., 1996; Caulfield and Birdsall, 1998). Muscarinic M_1–M_4 receptor subtypes were shown to be expressed in corpus cavernosum tissue and cultured smooth muscle cells; the receptor on smooth muscle was suggested to be of the M_2 subtype, whereas that on the endothelium was subtype M_3 (Traish et al., 1995). Moreover, in a recent report, a muscarinic M_3 receptor was associated with acetylcholine-induced relaxation in horse penile dorsal vein (Martínez et al., 2003). Nevertheless, it has been established that, in some vascular beds, acetylcholine mediates a contractile effect involving a variety of muscarinic receptors whose subtype depends on the vascular setting and the species from which the preparations are derived (Eglen et al., 1996). Further, the paradoxical effects (relaxation/contraction) of acetylcholine in vascular preparations are known to be associated with the activation of receptors present on endothelial cells as well as on smooth muscle cells (Eglen et al., 1996; Norel et al., 1996).

The penis can be considered simply as an extension of the vascular system. Vasculogenic erectile dysfunction is the most prevalent condition affecting nearly 80% of patients with organic impotence. Vascular erectile dysfunction comprises penile arterial insufficiency and veno-occlusive dysfunction. Contrary to the accumulated knowledge on corporal smooth muscle, there is only scarce information available on penile large vessels. There is, therefore, a genuine need to extend current understanding in this field. Recently, we evaluated the response of penile dorsal arteries and veins to histamine in view of the possible involvement of this endogenous amine in regulating penile circulation (Martínez et al., 2000a,b,c, 2002). The deep dorsal penile vein is the draining route for the glans, corpus spongiosum, and corpora cavernosa. The main purpose of vasodilation of the deep dorsal vein during penile erection is to allow the vessels to accommodate to the expansion of the corpus cavernosum. This mechanism probably also promotes the recovery of venous flow during detumescence. Hence, a relaxant response to acetylcholine in this vascular preparation would not be unexpected, since large veins would guarantee final draining from the venule plexus by compression during the initial moments of erection (Martínez et al., 2003). Moreover, an increased vascular tone induced by acetylcholine could play a role in aggravating penile dysfunction when endothelial cell damage occurs in disease states such as cardiovascular–vascular disorders.

Acetylcholine causes the endothelium-dependent relaxation of corpus cavernosum tissue, penile arteries, circumflex, and dorsal veins in vitro (Azadzoi et al., 1992; Kirkeby, 1994; Prieto et al., 1998; Simonsen et al., 2001; Martínez et al., 2003), but no specific endothelial mechanisms in large penile veins have yet been described. The aim of the present study was to investigate the mechanisms underlying the endothelium-dependent relaxation and contraction responses to acetylcholine observed in horse deep dorsal penile veins.

2. Methods

2.1. Tissue preparation, dissection, and mounting

Penile tracts of young sexually mature horses were obtained from a local slaughterhouse shortly after the animals were killed. The entire penis was transported to the laboratory in chilled (4 °C) physiological saline solution (PSS). The deep dorsal vein was isolated under a stereomicroscope (Nikon SMZ 2B). Vessel rings of approximately 3 mm in length were transferred to 5 ml organ baths containing PSS at 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2 to maintain the pH at 7.4. The rings were mounted between two parallel L-shaped stainless steel wires. Special care was taken to avoid damage to the endothelium. One wire was fixed to a displacement unit allowing fine adjustment of tension while the other was attached to a force transducer (Grass FT03C). In some experiments, the endothelium of the rings was mechanically removed by gentle rubbing of the intimal surface with a stainless steel wire. The isometric tension of the vessel wall was displayed and recorded using a MacLab data recording system. The preparations were allowed to equilibrate for about 30 min in PSS and washed with fresh (37 °C) PSS at 15 min intervals. After this equilibration period, each ring was stretched in a stepwise fashion to the optimal point of its length-tension ratio (≈ 10 mN) (Martínez et al., 2003).

2.2. Experimental protocol

The contractile capacity of the preparations was tested by exposing the vein rings to a 119 mM potassium-enriched solution (K-PSS, 47.7±4.2 mN; $n=25$). The mechanisms involved in the acetylcholine- and $NaNO_2$-induced relaxations were examined in preparations contracted with phenylephrine (0.5–1 μM) (31.6±2.8 mN; $n=25$), corresponding to 60–70% of the response induced by K-PSS. First, we undertook a relaxation concentration–response curve. The bath solution was changed every 20 min for a period of 80 min and the preparations were then incubated for 30 min with compounds that inhibit NO synthase,

soluble guanylyl cyclase, and cyclooxygenase or block K^+ channels before performing a second relaxation curve. We observed no significant differences in phenylephrine-induced tone in two consecutive relaxation curves.

2.3. Drugs and solutions

The following drugs were used: acetylcholine hydrochloride, 4-aminopyridine, apamin, atropine sulphate, charybdotoxin, glibenclamide, iberiotoxin, indomethacin, methoctramine, N^{ω}-nitro-L-arginine (L-NOARG), N^{ω}-nitro-L-arginine methyl ester (L-NAME), noradrenaline, 1*H*-[1,2,4]oxadiazol [4,3,-α]quinoxalin-1-one (ODQ), phenylephrine, pirenzepine, prostaglandin $F_{2\alpha}$, sodium nitrite ($NaNO_2$), tetraethylammonium and tetrodotoxin (Sigma, U.S.A.), and pFHHSiD (*para*-fluoro-hexahydro-sila-diphenidol) (RBI, U.S.A.). All drugs were dissolved in distilled water except: glibenclamide, indomethacin, and prostaglandin $F_{2\alpha}$, which were prepared in 96% ethanol, and ODQ, which was dissolved in dimethylsulphoxide.

The $NaNO_2$ acidified solution was freshly prepared as a 1 M stock solution by adjusting the pH to 2 adding concentrated HCl. The stock solution was kept cold and protected from air. Further dilutions were made in diluted HCl (pH 2) immediately before use. Preliminary experiments indicated no effect on the preparations of the solvents used.

The composition of PSS was (mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, KH_2PO_4 1.2, and ethylene-diaminetetraacetic acid (EDTA) 0.026. K-PSS was identical to PSS except that NaCl was replaced with KCl on an equimolar basis.

2.4. Calculations and statistics

Responses to acetylcholine and $NaNO_2$ in precontracted veins were expressed as the percentage of phenylephrine-produced contraction. Acetylcholine-induced contractions in vein rings at basal tone were expressed as a percentage of the contractile response produced by 119 mM K-PSS for each vessel. E_{max} refers to the maximum response achieved.

The agonist concentration required to give half-maximal response (EC_{50}) was determined, for each concentration–response curve, by computerized iteration, fitting the responses, and logarithmic concentrations to the Hill equation, using the GraphPad InPlot, non-linear curve fitting programme for personal computers (GraphPad Software, U.S.A.). Sensitivities to the agonists are given in terms of pEC_{50} values, which are defined as the negative logarithm of the EC_{50} for the agonists used. Antagonist potency was assessed by calculating the pA_2 value from the Schild plot analysis. Provided the regression of the Schild plot was linear and the slope was not significantly different from unity, the points were constrained to a slope of unity and a more precise value of the pK_B was calculated.

Results are given as the mean±standard error of the mean (S.E.M.) or 95% confidence limits. Statistical determinations were performed using the Student's *t*-test for paired and unpaired observations where appropriate. Differences were considered significant with a probability level of $P<0.05$.

3. Results

3.1. Acetylcholine-induced relaxation in the horse deep dorsal penile vein

Acetylcholine (1 nM–1 μM) caused the concentration-dependent relaxation of phenylephrine-contracted vein rings ($pEC_{50}=7.39\pm0.05$; $E_{max}=76.5\pm2.5\%$; $n=20$) (Fig. 1). This relaxant effect was reproducible in two consecutive concentration–response curves. Acetylcholine had no effect on the basal tone of the preparations.

3.2. Effects on acetylcholine-induced relaxation of endothelial removal, nitric oxide (NO) synthase inhibition, soluble guanylyl cyclase (sGC), and prostanoids synthesis blockade

Acetylcholine-induced relaxation was abolished by mechanical removal of the endothelium and incubation with the soluble guanylyl cyclase inhibitor ODQ (3 μM) (Fig. 1). In endothelium-intact preparations, the NO synthase inhibitors, L-NAME (100 μM) and L-NOARG (100 μM), also abolished relaxations induced by acetylcholine and unmasked an acetylcholine-mediated vasoconstriction at higher concentrations in phenylephrine-contracted veins ($pEC_{50}=5.91\pm0.14$ and $E_{max}=71.5\pm16.3\%$ for L-NAME; $pEC_{50}=6.05\pm0.12$; $E_{max}=78.3\pm13.9\%$ for L-NOARG; $n=8$) (Fig. 1). The cyclooxygenase inhibitor, indomethacin (3 μM), failed to modify the acetylcholine concentration–relaxation curve ($pEC_{50}=7.22\pm0.05$ and

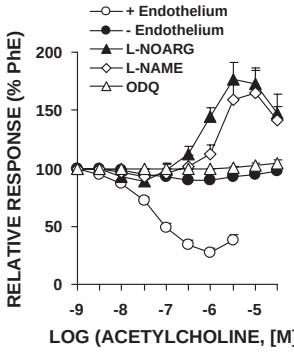

Fig. 1. Concentration–response curves for the effects of acetylcholine on endothelium-intact veins contracted with phenylephrine (0.5–1 μM PhE) in: endothelium-denuded preparations, the presence of L-NOARG (100 μM), L-NAME (100 μM), and ODQ (3 μM). Each point represents the mean±S.E.M. of $n=8$–10 experimental determinations.

E_{max} = 66.5 ± 2.4% for controls; pEC_{50} = 7.23 ± 0.10 and E_{max} = 72.5 ± 3.4% for indomethacin; n = 7).

3.3. Effect of raising the extracellular K^+ concentration on acetylcholine-induced relaxation

Contractions induced by 80 mM KCl were 31.4 ± 2.8 mN (n = 12), representing 64.1 ± 2.7% of the 119 mM K-PSS-elicited contraction. These were comparable to the contraction induced by 1 μM phenylephrine (35.5 ± 3.1 mN representing 65.1 ± 8.7% of the 119 mM K-PSS-elicited response). Raising extracellular K^+ (80 mM) led to a significant decrease in the sensitivity of the acetylcholine-induced relaxation and in the maximum response achieved compared to the response recorded for veins precontracted with phenylephrine (Fig. 2A, Table 1). When concentration–response curves of the effects of acetylcholine on the contraction induced by potassium were repeated in the presence of L-NOARG (100 μM), relaxation was abolished in these preparations and further contraction was also observed.

3.4. Effects on acetylcholine-induced relaxation of blockers of Ca^{2+}-activated K^+ (K_{Ca}) channels, voltage-dependent K^+ (K_V) channels, and ATP-sensitive K^+ (K_{ATP}) channels

Pretreatment with the non-selective K_{Ca} channel blocker, tetraethylammonium (1 mM), produced a rightward shift in the concentration–response curve for acetylcholine, without affecting the maximal response (Fig. 2B, Table 1). The

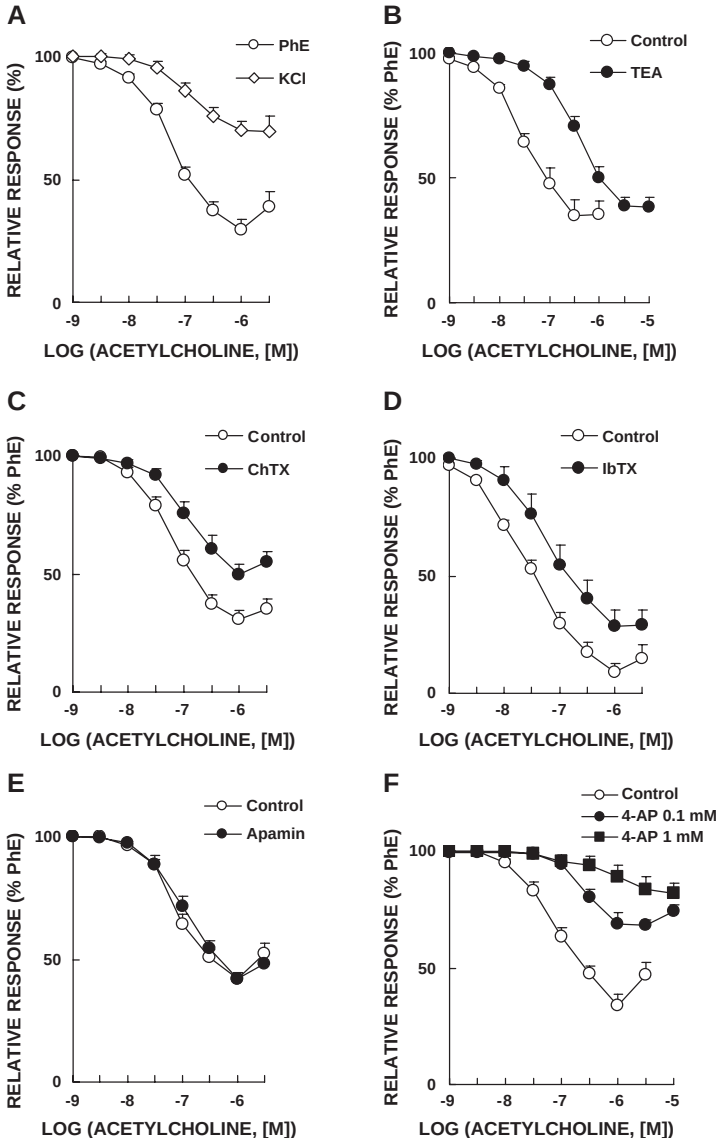

Fig. 2. Concentration–response curves for the effects of acetylcholine on (A) phenylephrine (PhE)- and 80 mM KCl-contracted vessels; concentration–response curves for the effects of acetylcholine in the presence of (B) tetraethylammonium (TEA, 1 mM), (C) charybdotoxin (ChTX, 0.1 μM), (D) iberiotoxin (IbTX, 0.1 μM), (E) apamin (0.5 μM), and (F) 4-aminopyridine (4-AP, 0.1 and 1 mM) on dorsal penile veins. Each point represents the mean ± S.E.M. of n = 6–14 experimental determinations.

Table 1
Effects of high extracellular K^+ concentrations and K^+ channel blockers on relaxation responses to acetylcholine

	n	pEC_{50}	E_{max} (%)
Phenyleprine	8	7.30±0.08	72.8±3.4
KCl (80 mM)	8	6.42±0.19[a]	44.2±5.9[a]
Control	10	7.38±0.10	69.9±3.3
Tetraethylammonium (1 M)	10	6.26±0.11[a]	66.3±3.2
Control	12	7.22±0.06	70.9±3.1
Charybdotoxin (0.1 μM)	12	6.88±0.14[b]	51.2±4.5[b]
Control	8	7.57±0.17	90.8±4.4
Iberiotoxin (0.1 μM)	8	7.02±0.19[b]	76.9±5.7[b]
Control	8	7.07±0.08	58.4±3.1
Apamin (0.5 μM)	8	7.21±0.17	59.9±2.9
Control	9	7.23±0.09	75.7±5.1
Charybdotoxin+Apamin (0.5 μM)	9	6.74±0.17[b]	49.0±4.5[b]
Control	14	7.02±0.07	66.6±4.4
4-Aminopyridine (0.1 mM)	6	6.50±0.05[b]	36.5±2.8[b]
4-Aminopyridine (1 mM)	8	5.86±0.15[a]	19.4±4.9[a]
Control	7	7.13±0.11	65.3±3.6
Glibenclamide (1 μM)	7	6.99±0.20	69.5±7.5

Results are expressed as the mean±S.E.M. of n experiments.
[a] $P<0.01$ vs. before drug treatment according to the Student's paired t-test.
[b] $P<0.05$ vs. before drug treatment according to the Student's paired t-test.

large-conductance K_{Ca} (BK_{Ca}) channel blockers, charybdotoxin (0.1 μM) and iberiotoxin (0.1 μM), also inhibited vasodilatory responses to acetylcholine (Fig. 2C,D, Table 1). Conversely, the acetylcholine response was not significantly affected by 0.5 μM apamin, a blocker of small-conductance K_{Ca} (SK_{Ca}) channels (Fig. 2E, Table 1). Treatment with charybdotoxin plus apamin caused no further enhancement of the inhibition of the acetylcholine relaxant response beyond that observed when charybdotoxin was added separately (Table 1). Blockade of K_v channels with 0.1 and 1 mM 4-aminopyridine increased basal tension (29.5±2.6%, $n=6$ and 43.9±2.9%, $n=8$, of the 119 mM K-PSS contraction, respectively) and inhibited relaxations produced in response to acetylcholine (Fig. 2F, Table 1). Blockade of K_{ATP} channels with glibenclamide (1 μM) did not alter the relaxations elicited by acetylcholine (Table 1).

3.5. Relaxation induced by an NO donor ($NaNO_2$)

To test whether NO released from the vein endothelium is able to modulate K^+ channel activity, we assessed the effects of exogenous NO added as acidified $NaNO_2$. This $NaNO_2$ acidified solution (1–300 μM) induced a dose-dependent relaxation (Fig. 3, Table 2). Relaxations were similar in preparations in which the endothelial cells had been mechanically removed (Fig. 3, Table 2). The relaxation provoked by $NaNO_2$ was almost abolished by ODQ, and unmodified by L-NOARG (Fig. 3, Table 2).

Concentration–relaxation curves for $NaNO_2$ in vessels contracted with 80 mM KCl were inhibited with respect to the contraction caused by phenylephrine (Fig. 4A, Table 2). These relaxations were unaffected by the addition of tetraethylammonium (1 mM), charybdotoxin (0.1 μM), iberiotoxin (0.1 μM), or apamin (0.5 μM) (Fig. 4B–E, Table 2). However, the relaxation induced by $NaNO_2$ was reduced by 0.1 and 1 mM 4-aminopyridine (Fig. 4F, Table 2).

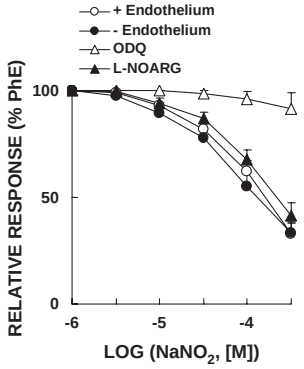

Fig. 3. Concentration–response curves for the effects of $NaNO_2$-acidified solution on endothelium-intact veins contracted with phenylephrine (0.5–1 μM PhE), and on endothelium-denuded preparations in the presence of ODQ (3 μM) or L-NOARG (100 μM). Each point represents the mean±S.E.M. of $n=6–8$ experimental determinations.

3.6. Contractile effect of acetylcholine

In endothelium-intact preparations, pretreatment with L-NOARG (100 μM) unmasked an acetylcholine-mediated vasoconstrictor effect on the basal tone of vein rings ($pEC_{50}=5.71±0.09$; $E_{max}=59.9±4.4\%$; $n=23$). The isometric force recording showing the magnitude of these L-NOARG-unveiled contractions in response to acetylcholine is provided in Fig. 5A. In vein specimens with an intact endothelium, the maximal relaxation and maximal contrac-

Table 2
Effects of endothelium removal, L-NOARG, high extracellular K^+ concentrations, and K^+ channel blockers on $NaNO_2$-induced relaxations

	n	pEC_{50}	E_{max} (%)
+ Endothelium	8	3.77±0.13	66.9±4.8
− Endothelium	8	3.97±0.09	66.8±5.0
L-NOARG (100 μM)	8	3.84±0.11	65.7±3.9
Phenyleprine	8	4.80±0.15	84.5±5.6
KCl (80 mM)	8	4.05±0.08[a]	64.4±4.2[a]
Control	8	4.45±0.10	87.3±4.8
Tetraethylammonium (1 M)	8	4.37±0.11	77.8±3.7
Control	6	4.59±0.20	88.6±4.0
Charybdotoxin (0.1 μM)	6	4.80±0.10	77.8±6.0
Control	7	4.18±0.07	73.3±1.8
Iberiotoxin (0.1 μM)	7	4.20±0.07	68.3±4.7
Control	6	4.61±0.12	93.8±2.5
Apamin (0.5 μM)	6	4.60±0.14	86.3±4.9
Control	12	4.38±0.03	86.9±3.3
4-Aminopyridine (0.1 mM)	6	4.11±0.16	62.7±6.1[b]
4-Aminopyridine (1 mM)	6	3.57±0.13[b]	51.4±7.2[a]

Results are expressed as the mean±S.E.M. of n experiments.
[a] $P<0.01$ vs. before drug treatments according to the Student's paired t-test.
[b] $P<0.05$ vs. before drug treatments according to the Student's paired t-test.

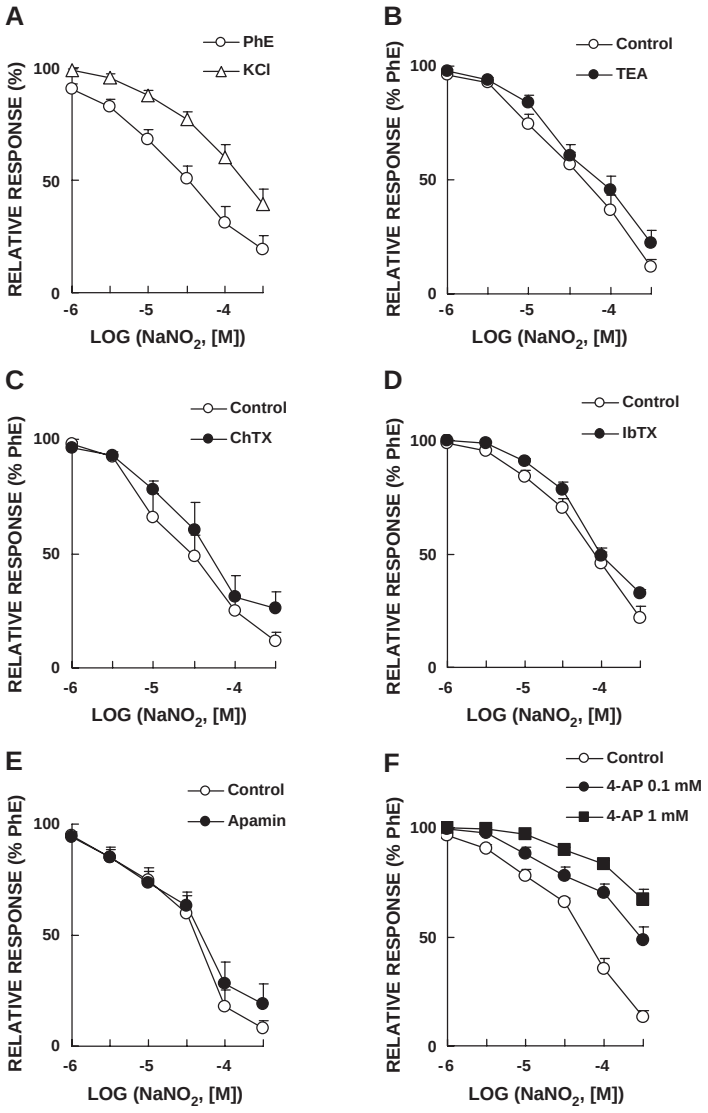

Fig. 4. Concentration–response curves for the effects of $NaNO_2$-acidified solution on (A) phenylephrine (PhE)- and 80 mM KCl-contracted vessels; concentration–response curves for the effects of $NaNO_2$ in the presence of (B) tetraethylammonium (TEA, 1 mM), (C) charybdotoxin (ChTX, 0.1 μM), (D) iberiotoxin (IbTX, 0.1 μM), (E) apamin (0.5 μM), and (F) 4-aminopyridine (4-AP, 0.1 and 1 mM) on dorsal penile veins. Each point represents the mean±S.E.M. of $n=6–12$ experimental determinations.

tion induced by acetylcholine were linearly correlated (slope=0.95±0.11; $r=0.96$; $n=11$). Moreover, endothelium removal did not induce the acetycholine contraction produced in the presence of L-NOARG ($n=8$).

Only one acetylcholine contractile concentration–response curve per vein segment at resting tone could be obtained using consecutive segments from the same animal as controls. Incubation with the cyclooxygenase inhibitor indomethacin (3 μM) significantly inhibited the acetylcholine-induced contractions ($pEC_{50}=5.43\pm0.13$ and $E_{max}=63.7\pm7.5\%$ for controls; $pEC_{50}=4.81\pm0.18$, $P<0.05$ and $E_{max}=40.7\pm4.5\%$, $P<0.01$ for indomethacin; $n=9$) (Fig. 5B). At the same concentration, 3 μM, indomethacin did not alter the contraction induced by either 10 μM prostaglandin $F_{2\alpha}$ ($E_{max}=44.66\pm5.12\%$ before treatment with indomethacin and $E_{max}=48.53\pm6.56\%$ after treatment, $n=8$) or 1 μM noradrenaline ($E_{max}=89.71\pm9.29\%$ before treatment with indomethacin and $E_{max}=84.30\pm11.38\%$ after treatment). This rules out the possibility that indomethacin (3 μM) could show Ca^{++}-antagonistic properties.

The neural voltage-gated sodium channel blocker tetrodotoxin (1 μM) showed no effect on the acetylcholine-elicited contractions ($pEC_{50}=5.63\pm0.20$ and $E_{max}=78.9\pm12.2\%$ for controls; $pEC_{50}=5.40\pm0.23$ and $E_{max}=79.1\pm15.4\%$ for tetrodotoxin; $n=8$).

Contraction in response to acetylcholine was competitively inhibited by atropine (1 nM–0.1 μM), a muscarinic receptor antagonist, pirenzepine (0.01–0.1 μM) a high, intermediate and low affinity antagonist for muscarinic M_1, M_3, and M_2 receptors, respectively, and pFHHSiD (0.1 μM–1 μM), a high affinity muscarinic M_3 receptor anta-

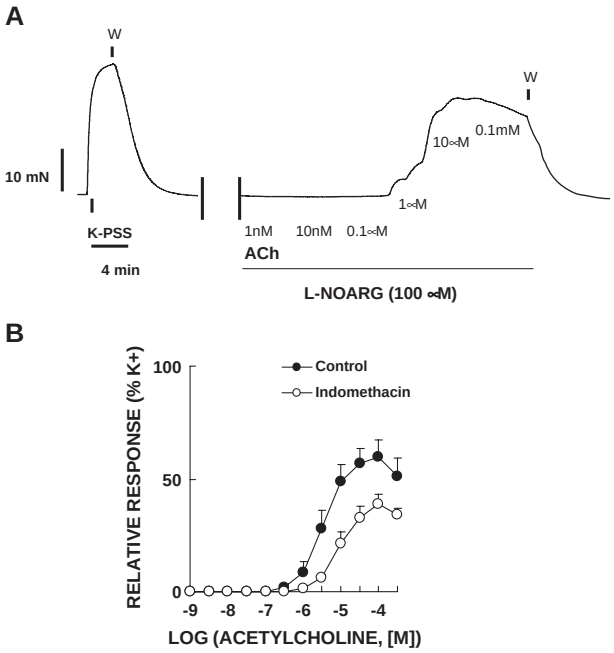

Fig. 5. (A) Isometric force recordings showing the contraction response to acetylcholine (ACh) on the basal tone of penile veins. Vertical bar shows tension in mM and horizontal bar time in min. Numbers indicate nano- or micromolar concentrations in the organ bath. W: wash out. (B) Concentration–response curves for the effects of acetylcholine on the basal tone of penile veins in the presence of L-NOARG (100 μM) or L-NOARG (100 μM) plus indomethacin (3 μM). Each point represents the mean±S.E.M. of $n=9$ experimental determinations.

gonist (Table 3). However, methoctramine (0.1 μM), a high affinity muscarinic M_2 receptor antagonist, showed no significant effect on the response to acetylcholine ($pEC_{50}=5.67\pm0.2$ and $E_{max}=42.9\pm5.4\%$ for controls; $pEC_{50}=5.68\pm0.09$ and $E_{max}=46.2\pm6.7\%$, for methoctramine; $n=6$).

4. Discussion

This study was designed to evaluate the mechanisms underlying the endothelium-dependent relaxation and contraction produced in response to acetylcholine in the horse deep dorsal penile vein. In this study, we demonstrated that: (1) the relaxation induced by acetylcholine was mainly mediated by activation of the NO-cGMP pathway; (2) this relaxation was inhibited by high extracellular K^+ concentrations and by BK_{Ca} and K_v channel blockers, suggesting the involvement of both types of channel in regulating the endothelium-dependent relaxation induced by acetylcholine; (3) the lack of effect of K_{Ca} channel blockers on exogenous NO-induced vasodilation precludes a role for these channels in the NO-induced relaxation; (4) the inhibitory effect of the K_v channel blocker, on the contrary, suggests the direct stimulatory action of the NO donor on K_v channel activity; and (5) in the presence of NO synthase blockers, a prostanoid-mediated contraction response to acetylcholine – mediated by activation of an endothelial muscarinic M_1 receptor – was unmasked.

It is widely accepted that acetylcholine interacts with endothelial receptors to induce relaxation through the release of factors such as NO, and with muscle receptors to induce contraction. We recently reported that the vasodilation response to acetylcholine observed in horse penile veins was mediated by muscarinic M_3 receptors and was fully dependent on an intact endothelium (Martínez et al., 2003). To our knowledge, this is the first attempt at elucidating the mechanisms involved in the endothelium-dependent responses to acetylcholine shown by the deep dorsal penile vein.

The endogenous production of prostanoids regulates penile smooth muscle tone, since arachidonic acid analogues induce relaxation in human corpus cavernosum strips and in penile resistance arteries, and this relaxation is blocked by the cyclooxygenase inhibitor indomethacin (Angulo et al., 2002). This finding is consistent with recent in vitro studies performed on bovine penile small arteries suggesting that acetylcholine-induced relaxation is not only mediated by NO but also by prostanoids (Simonsen et al., 2001). In contrast, we found no evidence of the involvement of endothelial prostanoids since indomethacin did not alter the relaxation provoked by acetylcholine.

Evidence of an essential role for NO in erection is very strong. NOS inhibitors substantially reduce erection induced either by acetylcholine or electrical stimulation (Ignarro et al., 1990). Acetylcholine causes endothelium-dependent relaxation in penile arteries (Liu et al., 1991; Prieto et al., 1998; Simonsen et al., 2001), penile veins (Kirkeby, 1994; Martínez et al., 2003), and the cavernous trabeculae (Azadzoi et al., 1992), while blockade of NO synthesis almost completely abolishes acetylcholine relaxation in large penile arteries and cavernous trabeculae (Liu et al., 1991; Azadzoi et al., 1992) and NO synthase inhibitors hardly affect this response in penile small arteries (Prieto et al., 1998) or completely fail to affect the same responses in the circumflex veins (Kirkeby, 1994). Our study demonstrates that the vasodilatory effect of acetylcholine was abolished by L-NAME and L-NOARG, indicating that NO is involved in the endothelium-dependent relaxation response to acetylcholine shown by large penile veins. NO produced by endothelial cells is known to promote vascular relaxation by activating soluble guanylate cyclase and increasing the production of cGMP in smooth muscle (Ignarro, 1990). In our experiments, activation of this

Table 3
Potency of muscarinic antagonists in horse penile dorsal veins established according to their ability to inhibit acetylcholine (ACh)-evoked contractions

	n	pA_2	Slope	r	pK_B
Atropine/ACh	8	9.87±0.26	1.02±0.12	0.88	10.07±0.19
Pirenzepine/ACh	6	8.56±0.05	1.13±0.16	0.89	8.66±0.13
p-FHHSiD/ACh	6	7.20±0.11	0.92±0.19	0.77	6.68±0.17

Values are the mean±S.E.M. of n experiments.

enzyme and subsequent cGMP accumulation is the principal mechanism ultimately leading to acetylcholine- and NO-induced relaxations. Acetylcholine-elicited relaxations are mediated by cGMP mechanisms in varying measure, including those produced in the horse penile small artery (Prieto et al., 1998) and piglet femoral artery (Støen et al., 2003).

Here, we initially assessed the possibility that acetylcholine induces hyperpolarization, by comparing relaxant responses in preparations contracted with phenylephrine to those contracted with a high K^+ solution. The latter was used to abolish the driving force for K^+ efflux and subsequent membrane hyperpolarization. High extracellular K^+ significantly inhibited the acetylcholine-induced dilatation, this being completely prevented by L-NOARG, confirming previous observations in the carotid artery of eNOS-deficient mice that the relaxation response to acetylcholine is fully mediated by NO (Chataigneau et al., 1999). Since K^+-evoked contractions matched those induced by phenylephrine, it is unlikely that the inhibition of relaxation is due to functional antagonism. These results initially would rule out an endothelium-derived hyperpolarizing factor (EDHF) different to NO. However, the inhibitory effect of high K^+ concentrations probably points either to the involvement of hyperpolarizing mechanisms in the release and/or action of NO in response to acetylcholine or to an additional EDHF. In the latter case, the large contraction caused by acetylcholine in the presence of the NOS inhibitors may overcome any response to EDHF.

The hyperpolarizing component of the relaxation response to acetylcholine can normally be attributed to activation of K^+ channels (Zygmunt et al., 1998; Prieto et al., 1998; Quignard et al., 2000), although the types of K^+ channels identified are heterogeneous. The BK_{Ca} channel is among the most prominent types in cultured corporal smooth muscle (Fan et al., 1995). Furthermore, in penile arteries, BK_{Ca} channels are involved in NO-mediated neurogenic (Simonsen et al., 1995) and endothelium-dependent relaxations (Prieto et al., 1998; Kun et al., 2003). In the present study, the non-selective K_{Ca} channel blocker, tetraethylammonium, and the BK_{Ca} channel blocker, charybdotoxin, reduced acetylcholine-induced relaxation. Since tetraethylammonium and charybdotoxin have also been described to inhibit certain K_V channels (Shieh et al., 2000), we tested the selective BK_{Ca} channel blocker, iberiotoxin, which does not block K_V channels. Iberiotoxin reduced acetylcholine-evoked relaxations, indicating that activation of BK_{Ca} channels is partly involved in the acetylcholine response.

In rabbit mesenteric arteries, EDHF-mediated responses are virtually abolished by apamin (Murphy and Brayden, 1995), regarded as a classic type of SK_{Ca} channel blocker (Nelson and Quayle, 1995), suggesting the involvement of this K^+ channel. In our study, apamin did not modify the acetylcholine-elicited relaxation, ruling out the participation of SK_{Ca} channels in this response. In rat hepatic and mesenteric arteries, apamin plus charybdotoxin completely inhibited endothelial cell hyperpolarization preventing the vasodilatory action exerted by acetylcholine (Edwards et al., 1998). In the present investigation, the mixture of both toxins caused no further inhibition of the acetylcholine-induced relaxation compared to the effect of charybdotoxin alone.

To clarify the precise mechanism by which NO causes the endothelium-independent relaxation of penile veins, we used a NO donor. Although recent evidence suggests that both NO and nitrovasodilators can activate BK_{Ca} channels and may contribute to vessel relaxation (Khan et al., 1998), such a mechanism appears not to be significant in the penile vein. We found that exogenous NO-induced relaxation was not inhibited by tetraethylammonium, charybdotoxin, iberiotoxin, or apamin. Our data are in agreement with the findings observed in the smooth muscle cells of the airways where no direct action of NO on K_{Ca} channels could be observed (Yamakage et al., 1996). Thus, in the absence of an effect on responses to NO, the inhibition of BK_{Ca} channels in acetylcholine-induced relaxation again suggests an action on endothelial K^+ channels inhibiting the synthesis/release of NO or to an additional EDHF.

Our results indicate that 4-aminopyridine-sensitive K_V channels play a role in preserving basal tone in penile veins, since this blocker significantly increased resting tension. This is consistent with recent findings suggesting that K_V channels are involved in the modulation of myogenic tone in rat penile arteries (Kun et al., 2003). Interestingly, K_V channels seem to control the membrane potential level of cavernosal smooth muscle cells (Malysz et al., 2001), resembling their role in other vascular smooth muscle cells (Nelson and Quayle, 1995). The K_V channel blocker, 4-aminopyridine, substantially inhibits both acetylcholine- and exogenous NO-induced relaxation The present data show that modulation of K_V channels significantly contributes to the mechanisms whereby NO relaxes the penile vascular bed, as a previous study reported in isolated pulmonary artery (Zhao et al., 1997).

K_{ATP} channels are present in erectile smooth muscle and these channels have been suggested as a potential therapeutic target for erectile dysfunction (Rubio et al., 2004). In mesenteric arteries, glibenclamide-sensitive hyperpolarizations in response to acetylcholine have been described, implying the participation of these K_{ATP} channels (Murphy and Brayden, 1995). The lack of effect of glibenclamide on the vasodilation response to acetylcholine in penile veins demonstrates that the effect is not mediated by the opening of K_{ATP} channels, which is in agreement with that reported by Prieto et al. (1998) for horse penile small arteries.

Vascular endothelial cells also play an important role in modifying vasoconstrictor responses to various endogenous substances. At basal tone, human isolated pulmonary arteries contract in the presence of acetylcholine and this response is amplified when the endothelium is removed

(Norel et al., 1996). In the present study, acetylcholine induced no contractile effect in endothelium-free preparations, suggesting that all muscarinic receptors are located in the endothelium, including contractile receptors. Contraction due to muscarinic receptor activation only occurred in the presence of NO synthase inhibitors such as L-NAME and L-NOARG, in agreement with results reported by Librizzi et al. (2000) for guinea-pig cerebral vessels and by Minhas et al. (2001) for rabbit corpus cavernosum.

Under several pathological conditions including hypertension, the impairment of endothelium-dependent vasodilation is the result of the generation of endothelium-derived contracting factor or a reduced bioavailability of NO (Vanhoutte and Boulanger, 1995). Our results seem to indicate that the abolition of the relaxant effect of acetylcholine mediated by NO pathway-dependent muscarinic M_3 receptors unmasks the contractile response produced when ACh binds to endothelial muscarinic M_1 receptors. A likely candidate for mediating endothelium-dependent contractions is a cyclooxygenase product (Vanhoutte and Boulanger, 1995; Yang et al., 2003). Herein, we also reveal the existence of an arachidonic acid derivative with contractile activity, which could oppose the relaxing action of NO. This endothelium-derived contracting factor appears to be masked by NO under normal conditions and to be released by acetylcholine, because contractions were only observed when the NO synthase inhibitor was added to the incubation media, and the presence of indomethacin reduced the acetylcholine-induced contraction. Our experimental findings are in agreement with observations in rabbit corpus cavernosum (Minhas et al., 2001) and suggest that endothelium-dependent contraction plays an important role in endothelial NO pathway dysfunction.

Previously, we were able to identify muscarinic M_3 receptor that mediates the relaxant response to acetylcholine of dorsal penile veins (Martínez et al., 2003). Here, the contractile muscarinic receptor subtype was also characterized by examining the effect to acetylcholine at basal tone. This response was antagonized by atropine, pirenzepine, and pFHHSiD. Pirenzepine showed high affinity for the muscarinic M_1-subtype receptor mediating acetylcholine contraction, as indicated by a calculated pA_2 value of 8.56. This order of potency is consistent with the high affinity of pirenzepine described in previous reports (7.8–8.5) (Caulfield and Birdsall, 1998). In tissue or cloned cells, where only the muscarinic M_3 receptor is expressed, the range of affinity values for pFHHSiD is 7.5–7.9 (Eglen et al., 1996). In the present study, pFHHSiD showed a low affinity for the muscarinic M_3 receptor subtype, with a calculated pA_2 value of 7.20, which argues against the involvement of this receptor subtype and is consistent with the presence of the muscarinic M_1 receptor. A general concept is that smooth muscle contraction generally reflects muscarinic M_3 receptor pharmacology (Eglen et al., 1996; Norel et al., 1996) but many vascular preparations have also been reported to show acetylcholine-induced contraction involving endothelial muscarinic M_1 subtype receptors (Simonsen et al., 1993; Watson et al., 1995).

In summary, we suggest that the main mechanism of acetylcholine-mediated relaxation in the penile vein endothelium is activation of a NO/cGMP-dependent pathway. This mechanism could be modulated by the opening of K_V channels and – to a lesser extent – by NO-independent activation of BK_{Ca}. The contraction induced by acetylcholine, only unmasked in the presence of NO synthase inhibitors, is due to muscarinic receptor activation involving an endothelial muscarinic M_1 subtype receptor. Cyclooxygenase activation plays a key role in the endothelium-dependent contractions evoked by acetylcholine in the dorsal penile vein. Since vascular diseases such as hypertension and diabetes may be associated with a decreased availability of NO, presumably because of diminished NO synthesis and increased NO catabolism, our findings may explain the dramatic reduction in the vasorelaxant capacity of these vessels and/or the upregulation of vasoconstrictor effects.

Acknowledgements

This study was supported by a grant from the Spanish Ministry of Education (PM 98-0088). The authors thank the local slaughterhouse (Guadalajara and Segovia) for kindly supplying fresh tissue and Mr. Francisco Puente and Mr. Manuel Perales for their technical assistance.

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