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Endothelium-dependent relaxation to acetylcholine in bovine oviductal arteries: mediation by nitric oxide and changes in apamin-sensitive K^+ conductance

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1 Mechamisms underlying the relaxant response to acetylcholine (ACh) were examined in bovine oviductal arteries (o.d. 300-500 μm and i.d. 150-300 μm) *in vitro*. Vascular rings were treated with indomethacin (10 μM) to prevent the effects of prostaglandins.

2 ACh elicited a concentration-related relaxation in ring segments precontracted with noradrenaline (NA), which was abolished by endothelium denudation.

3 The ACh-induced relaxation was attenuated but not abolished by N^G-nitro-L-arginine (L-NOARG, 1 μM-1 mM), an inhibitor of nitric oxide (NO) formation. The inhibition caused by L-NOARG (10 μM) was reversed by addition of excess of L-arginine but not D-arginine (1 mM).

4 In high K^+ (40-60 mM)-contracted rings, ACh was a much less effective vasodilator and its relaxant response was completely abolished by L-NOARG (100 μM).

5 In NA (10 μM)-contracted rings, ACh induced sustained and concentration-dependent increases in cyclic GMP, which were reduced below basal values by L-NOARG (100 μM), while potent relaxation persisted. Similar increases in cyclic GMP were evoked by ACh in high K^+ (50 mM)-treated arteries and under these conditions, both cyclic GMP accumulation and relaxation were L-NOARG-sensitive.

6 S-nitroso-L-cysteine (NC), a proposed endogenous precursor of endothelial NO, also induced cyclic GMP accumulation in NA-contracted oviductal arteries.

7 Methylene blue (MB, 10 μM), a proposed inhibitor of soluble guanylate cyclase, inhibited both endothelium-dependent relaxation to ACh and endothelium-independent response to exogenous NO, whereas relaxation to NC remained unaffected.

8 The L-NOARG-resistant response to ACh was not affected by either ouabain (0.5 mM), glibenclamide (3 μM), tetraethylammonium (TEA, 1 mM) or charybdotoxin (50 nM), but was selectively blocked by apamin (0.1-1 μM). However, apamin did not inhibit either relaxation to ACh in high K^+-contracted rings or endothelium-independent relaxation to either NO or NC.

9 Apamin and MB inhibited ACh-induced relaxation in an additive fashion, suggesting the involvement of two separate modulating mechanisms.

10 These results suggest that ACh relaxes bovine oviductal arteries by the release of two distinct endothelial factors: a NO-like substance derived from L-arginine, which induces cyclic GMP accumulation in smooth muscle, and another non-prostanoid factor acting by hyperpolarization mechanisms through alterations in apamin-sensitive K^+ conductance.

Keywords: Acetylcholine; EDRF; endothelium-derived hyperpolarizing factor (EDHF); nitric oxide; cyclic GMP; apamin; K^+-channels; oviductal arteries

Introduction

Oviductal blood flow may play an important role in fallopian tube fluid formation and hence in fertilization and early embryo development (Leese, 1988). However, little is known about the mechanisms underlying blood flow regulation in the oviduct. The existence of a dense adrenergic innervation (Black, 1974) and the presence of α-adrenoceptors in the vasculature of the oviduct (Costa *et al.*, 1992) provide a contractile mechanism through which reactivity of oviductal vessels can be regulated by the sympathetic nervous system. In the uterine artery, from which the arterial supply of the oviduct is derived, several reports have shown that acetylcholine (ACh) elicits vasodilatation through the activation of muscarinic receptors (Jovanovic *et al.*, 1994a), which in turn, release endothelium relaxing factors, mainly nitric oxide (NO) (Tare *et al.*, 1990; Jovanovic *et al.*, 1994b). However no information is available on the effect of ACh on oviductal arteries. A scanty cholinergic innervation is present in the oviduct (Black, 1974), although ACh and other autacoids can be released by the vascular endothelium in response to various stimuli (Milner *et al.*, 1990). On the other hand, it is known that ACh modulates the vascular tone by releasing endothelial factors (Furchgott & Vanhoutte, 1989). In this way, ACh may contribute to the regulation of oviductal blood flow in an autocrine fashion, through endothelium-dependent mechanisms.

Previous studies have shown that there are more than one endothelium-derived relaxing factor (EDRF) and that ACh may release different EDRFs depending on the vascular bed and animal species (Furchgott & Vanhoutte, 1989; Cowan *et al.*, 1993; Hwa *et al.*, 1994). It has been suggested that one of these EDRFs is NO, formed from L-arginine by the enzyme NO synthase (Palmer *et al.*, 1987), which elicits relaxation by the activation of soluble guanylate cyclase in vascular smooth muscle (Ignarro, 1990). Another unidentified nonprostanoid EDRF, distinct from NO, is a diffusible agent released by ACh and other vasoactive agents, which induces smooth muscle hyperpolarization (Chen *et al.*, 1988). The activity of this factor, referred to as endothelium-derived hyperpolarizing factor (EDHF), is controversial. In some studies, the hyperpolarization induced by ACh is inhibited by ouabain, sug-

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gesting the activation of the Na^+-K^+-ATPase (Brayden & Wellman, 1989; Alonso *et al.*, 1993). On the other hand, a growing body of evidence points to an increase in membrane permeability to K^+ as responsible for the ACh-induced hyperpolarization (Brayden, 1990; Hasunuma *et al.*, 1991; Khan *et al.*, 1993; Cowan *et al.*, 1993; Hwa *et al.*, 1994), although the type of K^+-channel involved is not clear.

The present study was designed to evaluate the relative contribution of EDRF-NO and EDHF in the endothelium-dependent relaxations to ACh of bovine oviductal arteries *in vitro*. Compounds interfering with the synthesis (N^G-nitro-L-arginine) and action (methylene blue) of NO were used together with selective K^+-channel blockers and the Na^+/K^+-ATPase inhibitor, ouabain. The effect of NO and S-nitroso-L-cysteine (NC) were also examined and compared with that of ACh. In addition, guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in response to ACh was also assessed.

Methods

Vascular preparations

Genital tracts from heifers, with macroscopically quiescent ovaries, were collected at the local slaughterhouse and transported to the laboratory in ice-cold Krebs solution (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, ethylenediaminetetraacetic acid disodium salt (EDTA) 0.01 and glucose 11 at pH 7.4). The oviduct and the tip of the uterine horn were separated from the genital tract and placed in a Petri dish containing Krebs solution. Arterial branches (o.d. 300–500 μm and i.d. 150–300 μm) supplying the oviductal isthmus were carefully dissected free from the mesosalpinx and surrounding tissue and cut into rings approximately 1 mm wide.

Recording of mechanical activity

The artery rings were placed in 5 ml organ baths containing Krebs solution warmed at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The segment vessels were gently slipped onto two parallel stainless steel legs (75 μm diameter) of two L-shaped steel hooks, taking care not to damage the endothelium unintentionally. One of the hooks was connected to a Grass FT03C force-displacement transducer, for measurement of isometric tension, and the other was fixed to a moveable unit allowing the adjustment of the vascular diameter. Isometric wall tension was recorded on a Grass model 79D polygraph. Artery rings were stretched in a stepwise fashion to the optimal point of their length-tension relationship (1.75 mN mm^{-1}) as determined in previous studies (Costa *et al.*, 1992). Tissues were allowed to equilibrate for at least 60 min during which time the Krebs solution was replaced every 15 min.

Experimental procedure

After 60 min of equilibration, each ring was initially challenged with 119 mM KCl (by equimolar replacement of NaCl with KCl) as a standard stimulus to check the preparation's viability. Following washout and complete relaxation, the arteries were next constricted to its maximal force with a combination of noradrenaline (NA, 10 μM), 5-hydroxytryptamine (10 μM) and high K^+ (119 mM). The contraction obtained was considered as the reference maximal contraction for each vessel ring. After washout, vascular rings were partially contracted with NA (0.5–3 μM) or with high K^+ (40–60 mM); the contractions obtained were in a range between 45 and 60% of the reference maximal contraction. At the plateau of this partial contraction, tissues were exposed to ACh (10 μM) and only those which responded with 80% or greater relaxations were selected for further study for ACh relaxations. Endothelial cells were removed by gently rolling the artery rings back and forth over the parallel stainless steel legs inside the organ baths. The absence of endothelium was confirmed pharmacologically by loss of relaxant response to ACh (10 μM).

Cumulative concentration-response curves were developed for ACh (0.1 nM to 100 μM), NC (10 nM to 100 μM) and NO (1 nM to 100 μM) in NA precontracted rings. The effects of blocking agents were examined by pretreatment of arterial rings during 30 min. At the end of each experiment, papaverine (100 μM) was added to the organ bath to determine the maximal relaxation. Only one concentration-response curve was performed in each vessel and a control tissue ring from the same animal was run in parallel without antagonist pretreatment. All experiments were done in the presence of indomethacin (10 μM).

Determination of cyclic GMP levels

Cyclic GMP was measured in endothelium-intact arteries incubated in test tubes containing Krebs solution, warmed at 37°C and bubbled with 95%O_2:5%CO_2 (which was replaced every 15 min) as in the organ bath conditions. After 60 min of incubation, vessels were treated with indomethacin (10 μM) for 20 min and subsequently with NA (1 μM) for another 15 min. A group of segments were then frozen to assay for basal levels of cyclic GMP.

Separate groups of vascular segments were subjected to different treatments as follows: NC (1 μM) for 2 min, ACh (1 μM) at different exposure times (from 15 s to 5 min), and ACh at different concentrations (from 0.1 to 10 μM) for 1 min. For inhibition experiments, vascular segments were exposed to L-NOARG (100 μM) for 30 min before incubation with ACh.

In order to compare the ACh-induced relaxations with changes of the cyclic GMP levels, parallel assays were performed in vascular segments from the same animal. In these experiments, vessels were exposed to ACh at 10 μM for 1 min (after indomethacin and NA or indomethacin and high K^+ (50 mM) incubation) with or without L-NOARG (100 μM) pretreatment

The analytical procedure followed is described elsewhere (García-Pascual & Triguero, 1994) with minor modifications. In brief, vessel segments were quickly plunged into liquid nitrogen and stored at −70°C. At assay, frozen segments were homogenized in 1 ml of 6% trichloroacetic acid (TCA) at 4°C with a glass homogeinizer and centrifuged at 2500 *g* for 15 min. Supernatants were collected and TCA extracted four times with 5 ml of water-saturated ether. The final aqueous phase was evaporated to dryness *in vacuo* in a Speed-vac sample concentrator and the residue was reconstituted in 500 μl of 0.05 M sodium acetate buffer, pH 6.2. The concentration of cyclic GMP was assayed in 100 μl aliquots by radioimmunoassay commercial kits following the instructions of the manufacturer (Dupont New England Nuclear) for acetylated samples. The recovery of cyclic GMP was at least 90%. The protein content of the TCA precipitate was quantified (Lowry *et al.*, 1951) with bovine serum albumin used as standard. Cyclic GMP levels were expressed as femtomoles per mg of protein (fmol mg^{-1} prot.).

Drugs and solutions

The following drugs were used: (±)-noradrenaline hydrochloride (Serva, Heidelberg, Germany), acetylcholine chloride, apamin, D-arginine hydrochloride, L-arginine hydrochloride, L-cysteine, charybdotoxin, glibenclamide, indomethacin, methylene blue, N^G-nitro-L-arginine, ouabain octahydrate, papaverine hydrochloride, serotonin creatinine sulphate, sodium nitrite, tetraethylammonium chloride (TEA) (Sigma, St Louis, MO, U.S.A.) and NO (Sociedad Española de Oxígeno, Madrid, Spain). Drugs were dissolved in twice distilled water except indomethacin, which was dissolved in 99% ethanol, and glibenclamide, which was prepared in dimethyl sulphoxide. Stock solutions were dissolved and stored at −20°C and fresh dilutions were prepared daily.

Nitric oxide solutions were prepared by a slight modification of the method described by Garland & McPherson (1992). Sealed vials containing twice distilled water (40 ml) at room temperature (20°C) were bubbled with nitrogen for 60 min. One of the de-aerated vials was then exposed to a stream of NO (for 5 min) enough to produce a saturated solution. Based on the solubility constant for NO in water at 1 atm and 20°C (4.6 ml 100 ml^{-1} H_2O), the resulting NO concentration of the saturated solution was 1.9 mM. Subsequents dilutions of NO were prepared immediately before use in sealed vials by means of a gas-tight syringe which was also used for the NO addition into the organ baths.

S-nitroso-L-cysteine (NC) was prepared by reacting L-cysteine with sodium nitrite under acidic conditions (Field *et al.*, 1978). To a stirred ice-cold reactant solution containing: 1 N HCl: methanol (1:1), equimolar amounts of $NaNO_2$ and L-cysteine were added. Following the reaction, the solution became red and exhibited a visible absorption maximum at 544 nm, characteristic of S-nitrosothiols. The NC concentration was determined spectophotometrically at this wavelength, assuming a molar absorption coefficent of 16.6 (Kowaluk & Fung, 1990). Working dilutions were prepared in twice distilled water immediately before use, kept on ice and added as 10–30 μl aliquots to 5 ml organ baths. A blank reactant solution, containing all the components except L-cysteine was prepared and diluted in an identical manner. This blank reactant solution was without effect up to 100 μM.

Analysis of data

The relaxations induced by each concentration of ACh, NO and NC were expressed as a percentage of the maximum relaxation to papaverine (100 μM) and used in the construction of the concentration-response curves. The IC_{50} (the negative molar concentration required to cause half-maximal relaxation induced by papaverine) was determined by linear interpolation for each concentration-response curve.

The results are expressed as mean ± s.e.mean, where n represents the number of animals studied in each experiment. One-way analysis of variance (ANOVA) followed by Bonferroni's test was used when more than two groups were analysed. Statistical differences between two means were determined by Student's t test (two-tailed) for paired or unpaired observations where appropriate. A value of $P<0.05$ was considered to be statistically significant.

Results

Acetylcholine relaxations in bovine oviductal arteries

ACh evoked concentration-dependent relaxations in bovine oviductal artery rings contracted with NA. NA was applied in various concentrations (between 0.5 and 3 μM), in order to produce a similar-sized contraction in each experiment. ACh was applied against a mean background contraction of 4.5 ± 0.2 mN ($n=70$), corresponding to 45.5 ± 1.1% of the reference maximal response (see Methods).

In the absence of indomethacin, the action of ACh was variable: stable vasodilatations in some experiments and vasodilatation followed by transient contraction at higher concentrations in others. However, in the presence of indomethacin (10 μM), ACh induced more sustained and significantly higher relaxations (the average relaxation induced by ACh (1 μM) was 75 ± 4.5% in the absence and 86.5 ± 3.2% in the presence of indomethacin, $P<0.05$, $n=6$). To minimize the influence of endogenously formed prostaglandins, indomethacin (10 μM) was routinely included in all experiments.

Repeated ACh concentration-response curves produced desensitization. IC_{50} values for ACh were significantly decreased from 7.4 ± 0.2 in the first curve to 6.8 ± 0.1 in the second application ($P<0.05$, $n=10$), although the maximal relaxation was not significantly different in the first (91.4 ± 1.6%) and second (87.1 ± 2.7%) concentration-response curve. For this reason, a single concentration-response curve was performed in each vascular preparation.

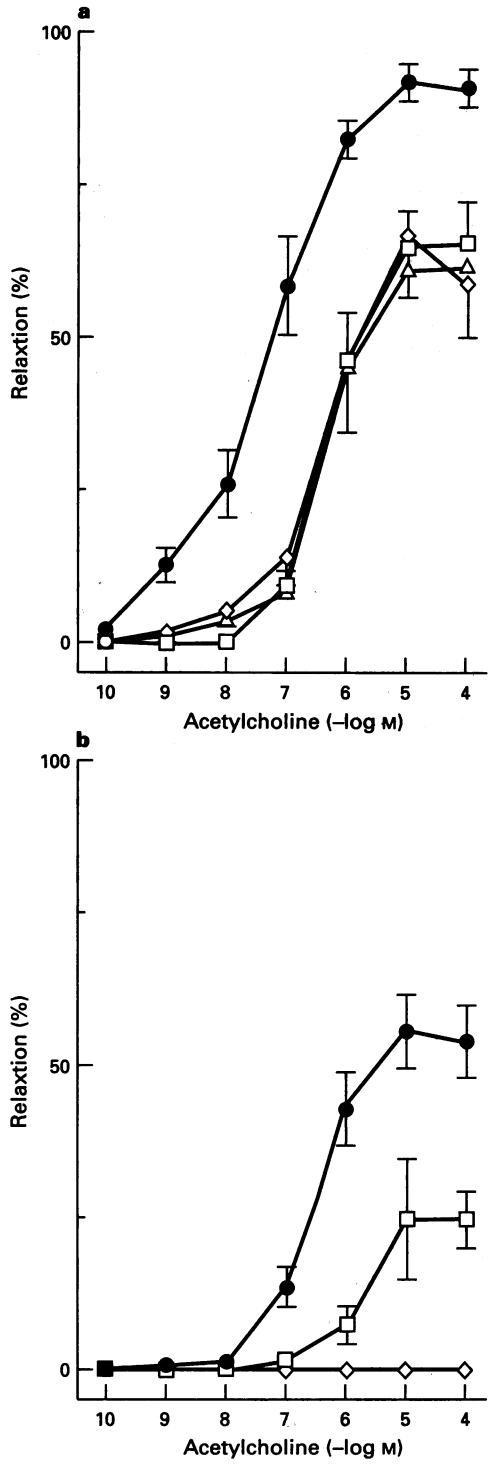

Figure 1 Effect of N^G-nitro-L-arginine (L-NOARG) on relaxations to acetylcholine in bovine oviductal arteries with intact endothelium, contracted with noradrenaline (a) or high K^+ (b). The experiments were performed in the presence of indomethacin (10 μM). The preparations were pretreated with L-NOARG for 30 min. Data are expressed as percentage of the maximal relaxation to papaverine (100 μM) and given as means ± s.e.mean. (a) Mean absolute values in control and L-NOARG (10 μM, 100 μM and 1 mM)-treated rings were 5.0 ± 1.1 mN ($n=10$), 4.4 ± 0.8 mN ($n=8$), 4.08 ± 0.9 mN ($n=5$) and 4.3 ± 1.1 mN ($n=6$), respectively. (b) Those in control and L-NOARG (10 and 100 μM)-treated rings were 4.6 ± 0.7 mN ($n=10$), 4.3 ± 1.4 mN ($n=5$) and 5.1 ± 1 mN ($n=6$), respectively. (●) Control; (□) L-NOARG (10 μM); (◇) L-NOARG (100 μM); (△) L-NOARG (1 mM).

In rings without endothelium ACh did not induce relaxation.

Effect of L-arginine analogues on acetylcholine-dependent relaxation

Figure 1 shows the comparison of ACh concentration-response curves and the effect of L-NOARG under the conditions of NA vs. K^+ depolarization. The contractions caused by NA and K^+ (40–60 mM) were not significantly different (47±5% vs. 51±2% of the reference maximal contraction, respectively). ACh was clearly a much less effective vasodilator with a condition of high K^+ induced contraction as compared to a NA-induced contraction. The IC_{50} and maximal relaxation were significantly lower in high K^+-contracted rings (5.7±0.2 and 55.6±6%) compared with NA-contracted preparations (7.4±0.2 and 91.4±1.6%) ($P<0.001$, $n=10$). Relaxation to ACh was inhibited to a greater degree by L-NOARG with K^+-induced depolarization than with NA. In high K^+- contracted rings; maximal relaxation to ACh was reduced to 28±11% by L-NOARG (10 μM) and blocked completely by L-NOARG (100 μM) (Figure 1b). Although L-NOARG (10 μM) significantly inhibited relaxations to ACh in NA-contracted preparations, potent relaxation persisted. Furthermore, 10 and 100 fold higher concentrations of L-NOARG (100 μM and 1 mM) did not significantly inhibit the ACh concentration-response further (Figure 1a; Table 1). The inhibitory effect of L-NOARG (10 μM) was reversed by the addition of L-arginine (1 mM) but not by D-arginine (1 mM) (Table 1).

In the continous presence of L-NOARG (100 μM), repeated ACh concentration-response curves showed a more pronounced tachyphylactic effect than in the absence of the drug. IC_{50} values and maximal relaxation were reduced from 6.4±0.1 and 78±7.1% in the first concentration-response curve to 4.6±0.4 and 50.5±8.9% in the second curve ($P<0.01$, $n=9$).

Treatment with L-NOARG (100 μM) evoked a small increase in vessel tone in only 11 out of 40 preparations averaging 10.5±2.7% of the reference maximal contraction, and augmented the contractile response to NA and high K^+, so that lower doses were needed to achieve the same level of constriction.

Effects of ouabain and K^+-channel blockers on acetylcholine relaxations resistant to L-NOARG

In order to ascertain the contribution of membrane K^+ conductance and Na^+/K^+-ATPase activity to ACh-induced vasodilatation, we tested the effect of ouabain and several K^+-channel blockers (TEA, glibenclamide, charybdotoxin and apamin) on the residual relaxation to ACh under treatment with L-NOARG (100 μM) in NA-contracted vessels. Ouabain (0.5 mM), TEA (1 mM), glibenclamide (3 μM) or charybdotoxine (50 nM) did not affect the response to ACh in the presence of L-NOARG (Table 1). However, apamin produced significant and concentration-dependent inhibition of the L-NOARG-resistant response (Figures 2a and 3; Table 1).

Although ouabain *per se* evoked a slight increase in basal tone (3.2±0.6% of the reference maximal contraction) and augmented the response to NA, the precontraction level was matched to that in the absence of the inhibitor. On the other hand, TEA, glibenclamide, charybdotoxin or apamin were all without effect on the basal tone and the contractile response to NA.

In contrast to NA contraction, relaxant responses to ACh were not affected by apamin with high K^+-induced contraction (Figure 2b). The IC_{50} and maximal relaxation were 4.4±0.2 and 46.5±5.3% in the presence and 4.1±0.5 and 41±5.1% in the absence of apamin (0.5 μM). Precontraction levels in high K^+-contracted rings were analogous to those in NA-contracted preparations (45±3% vs. 49±3% of the reference maximal contraction, respectively).

Effect of methylene blue and apamin on nitric oxide-, S-nitroso-L-cysteine- and acetylcholine-induced relaxations

In control experiments, endothelium-dependent relaxation to ACh reached 92.1±2.1% ($IC_{50}=6.9\pm0.1$, $n=6$). Both, MB (10 μM) and apamin (0.5 μM) caused inhibition of the ACh-

Table 1 Effects of L-arginine analogues, ouabain and K^+-channel blockers on relaxation to acetylcholine in bovine oviductal arteries with intact endothelium

Treatment	Contraction (mN)	IC_{50} –log M	E_{max} (% max to Pap)	n
A *With indomethacin pretreatment*				
Control	5.0±1.1	7.32±0.18	91.7±2.9	10
L-NOARG (10 μM)	4.0±0.8	5.49±0.28**	65.6±6.5**	8
L-NOARG (100 μM)	4.0±0.9	5.90±0.21**	66.8±10.3*	10
L-NOARG (1 mM)	4.3±1.1	5.60±0.55*	61.6±9.6**	6
L-Arg (1 mM) +L-NOARG (10 μM)	4.3±0.7	6.97±0.21	96.3±3.6	6
D-Arg (1 mM) +L-NOARG (10 μM)	5.0±0.7	5.56±0.29**	67.3±6.0**	9
B *With indomethacin plus L-NOARG pretreatment*				
Control	4.8±0.4	5.99±0.27	68.2±8.2	9
Ouabain (0.5 mM)	4.9±0.8	6.31±0.16	82.1±5.5	7
Glibenclamide (3 μM)	4.2±0.7	5.83±0.38	68.7±7.8	7
TEA (1 mM)	4.4±0.8	5.79±0.15	82.8±2.8	7
Charybdotoxin (50 nM)	4.6±0.8	5.97±0.23	70.3±7.8	6
Apamin (0.1 μM)	5.1±0.9	5.23±0.41	48.8±0.5	7
Apamin (0.5 μM)	4.6±1.0	c	34.8±8.4*	7
Apamin (1 μM)	4.8±0.6	c	20.8±6.7**	6

Data are means±s.e.mean. IC_{50} values are negative log molar concentration of ACh, which caused 50% of the maximal relaxation to paraverine (Pap, 100 μM). E_{max}, the maximal relaxation to ACh is expressed as a percentage of maximal relaxation to papaverine. Arteries were precontracted with noradrenaline. *n* denotes number of animal tested. Indomethacin (10 μM) (**A**) or indomethacin plus N^G-nitro-L-arginine (L-NOARG, 100 μM) (**B**) were present throughout the experiments. Agents tested were added 30 min before the preparations were exposed to ACh. c: ACh failed to produce 50% relaxation. *$P<0.05$, **$P<0.01$, significant differences from control values by one way analysis of variance followed by Bonferroni's method.

induced relaxation (Figure 4a). In the presence of apamin, maximal relaxation was reduced to 47.4±14% ($P<0.05$, $n=5$). Treatment with MB significantly decreased the IC_{50} value of ACh to 5.7±0.3 ($P<0.05$, $n=5$), although the maximal relaxation (58.6±9.2%) was not significantly different from controls. The combination of MB and apamin was more effective than either agent alone and almost completely abolished ACh-induced relaxation (maximum response 22±11%, $P<0.01$, $n=5$) (Figure 4a).

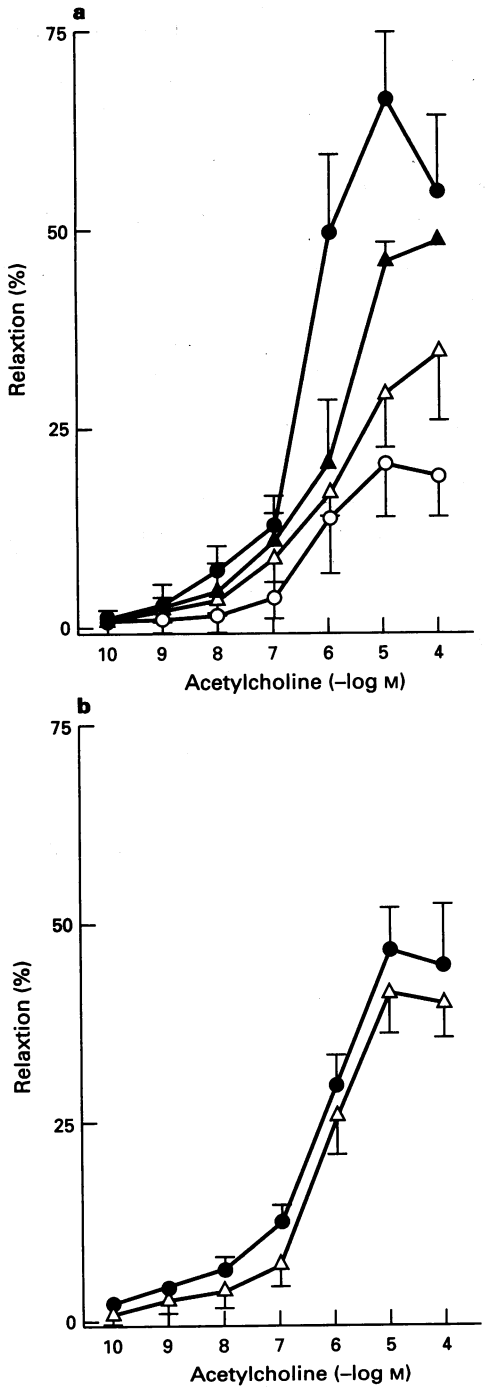

Figure 2 Effect of apamin on relaxations to acetylcholine in bovine oviductal arteries with intact endothelium, contracted with noradrenaline (a) and high K^+ (b). The experiments were performed in the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (100 μM) in (a) or indomethacin alone in (b). Apamin was added 30 min before acetylcholine and present throughout the experiment. Data are expressed as percentage of the maximal relaxation to papaverine (100 μM) and given as mean±s.e.mean. (a) Mean absolute values in control and apamin (0.1, 0.5 and 1 μM)-treated rings were 4.7±0.5 mN ($n=10$), 5.1±0.9 mN ($n=7$), 4.7±0.3 mN ($n=7$) and 4.8±0.6 mN ($n=6$), respectively. (b) Those in control and apamin (0.5 μM)-treated rings were 5.0±0.7 mN ($n=6$) and 4.8±0.7 mN ($n=6$), respectively. (●) Control; (▲) apamin (0.1 μM); (△) apamin (0.5 μM) and (○) apamin (1 μM).

NO and NC, two putative endothelium-derived relaxing factors from L-arginine, induced concentration-dependent relaxations in rings without endothelium, contracted with NA (Figure 4b and c). At the highest concentration tested (100 μM), the maximum effect was 97.6±0.8% and 95.2±1.4% for NO and NC, respectively. The IC_{50} values for NO and NC were also similar (6.6±0.1 vs. 6.9±0.2). MB (10 μM) significantly inhibited dose-dependent relaxations induced by exogenous NO, reducing its maximum effect to 45.6±6.1% ($P<0.001$, $n=6$) (Figure 4b). In contrast, at the same concentration, MB failed to affect dose-dependent relaxant responses to NC (Figure 4c). Apamin (0.5 μM), under these conditions, had no effect on the relaxations to NO and NC, as can be seen from Figure 4b and c. Combined treatment with MB and apamin had no additive inhibitory effect on relaxations to either NO or NC (Figure 4b and c).

Like L-NOARG, treatment with MB (10 μM) induced a slight increase in resting tension in 13 out of 23 preparations amounting to 10.8±3% and potentiated contractile responses to NA. However, the effect of MB was endothelium-independent.

Cyclic GMP levels

The control value for cyclic GMP content of the endothelium-intact oviductal arteries contracted with NA (1 μM) was 34±6.4 fmol mg^{-1} prot ($n=5$). The exposure for 1 min to increasing concentrations of ACh induced dose-dependent increases in cyclic GMP values (Figure 5). At the highest concentration examined (10 μM), cyclic GMP levels were about 10.8 fold larger than those in unstimulated preparations

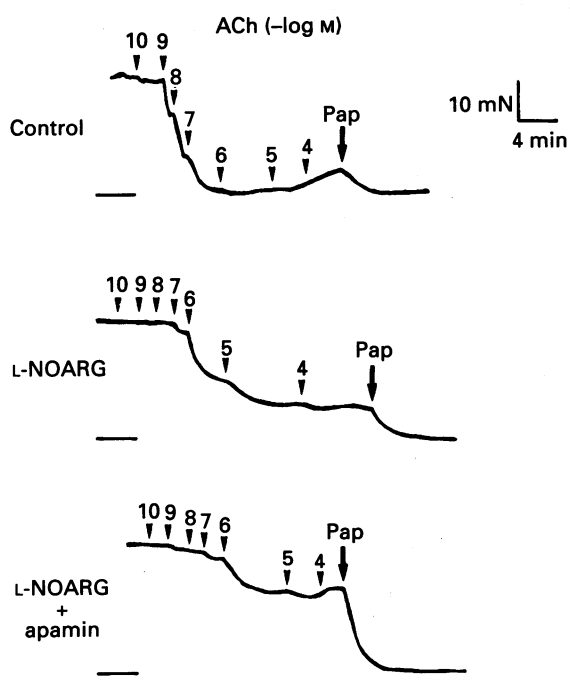

Figure 3 Typical recordings of the response to acetylcholine (ACh, −log M) of bovine oviductal artery rings with intact endothelium, obtained from the same animal. Responses were obtained in control conditions (Control) or after treatment with N^G-nitro-L-arginine (L-NOARG, 100 μM) or L-NOARG plus apamin (0.5 μM). The experiment was performed in the presence of indomethacin (10 μM). The rings were precontracted with noradrenaline (NA). Horizontal lines represent the level before addition of NA. Pap represents 100 μM papaverine which produced maximal relaxation.

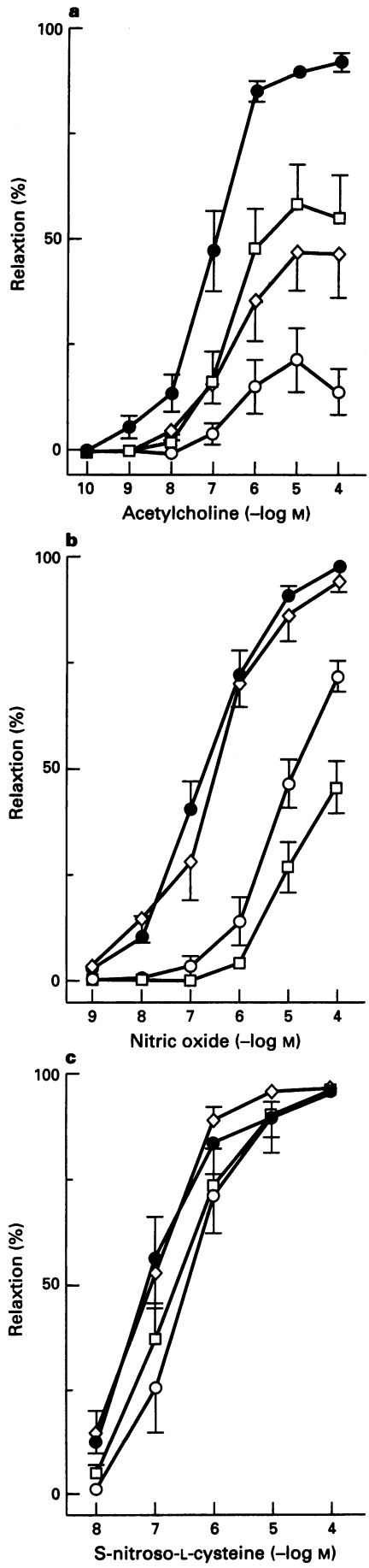

Figure 4 The effect of methylene blue (MB) alone, apamin alone and their combination on relaxations to acetylcholine (a), nitric oxide (b) and S-nitroso-L-cysteine (c) in bovine oviductal arteries with intact endothelium (a) or without endothelium (b and c), contracted with noradrenaline. The experiments were performed in the presence of indomethacin (10 μM). The preparations were pretreated with MB, apamin or both for 30 min. Data are expressed as percentage of the maximal relaxation to papaverine (100 μM) and given as means ± s.e.mean. (a) Mean absolute values in control and MB-, apamin- and MB + apamin-treated rings were 4.6 ± 0.8 mN (n = 7), 5.0 ± 1.2 mN (n = 5), 4.9 ± 0.9 mN (n = 5) and 4.8 ± 1.2 mN (n = 5), respectively. (b) Those in control and MB-, apamin- and MB + apamin-treated rings were 4.4 ± 0.6 mN (n = 10), 4.5 ± 0.9 mN (n = 6), 4.4 ± 0.7 mN (n = 8) and 4.2 ± 1.0 mN (n = 6), respectively. (c) Those in control, MB-, apamin- and MB + apamin-treated rings were 5.2 ± 1.0 mN (n = 5), 4.1 ± 0.4 mN (n = 5), 4.8 ± 1.0 mN (n = 5) and 4.5 ± 1.1 mN (n = 5), respectively. (●) Control; (□) MB (10 μM); (◇) apamin (0.5 μM) and (○) MB + apamin.

(367 ± 72 fmol mg^{-1} prot, n = 6). On exposure to NC (1 μM) for 2 min, a higher increase in cyclic GMP was measured (18 fold from control values) reaching 626 ± 121 fmol mg^{-1} prot (n = 7) (Figure 5).

To compare relaxation and cyclic GMP accumulation stimulated by ACh, relaxation and cyclic GMP were measured in parallel preparations from the same animals, as described under Methods (Figure 6). Control rings contracted with NA relaxed 83.3 ± 4.3% during exposure to ACh (10 μM) (Figure 6a). In these arteries, stimulated cyclic GMP levels during 1 min exposure to ACh were 387 ± 76 fmol mg^{-1} prot. The increase in cyclic GMP evoked by ACh was completely inhibited by treatment with L-NOARG (100 μM), falling to 23 ± 5 fmol mg^{-1} prot, while rings treated with L-NOARG still relaxed 65.1 ± 5.9% (Figure 6a). In arterial preparations contracted with high K^+ (50 mM), relaxation to ACh (10 μM) was reduced to 37.8 ± 8.6%, while cyclic GMP levels (284 ± 66 fmol mg^{-1} prot) were not significantly different from those evoked in NA-contracted arteries (Figure 6b). However, in these high K^+-contracted preparations, both cyclic GMP accumulation and relaxation induced by ACh were completely abolished by L-NOARG (100 μM) treatment (Figure 6b).

To determine if an early rise in cyclic GMP, which might have returned to baseline by 60 s, could account for the relaxation that persisted in the presence of L-NOARG, cyclic GMP was determined over the time course of 0, 15, 30, 60 and 300 s exposure to ACh (1 μM) (Table 2). As can be seen, ACh stimulated a rapid rise in cyclic GMP to near maximal levels at

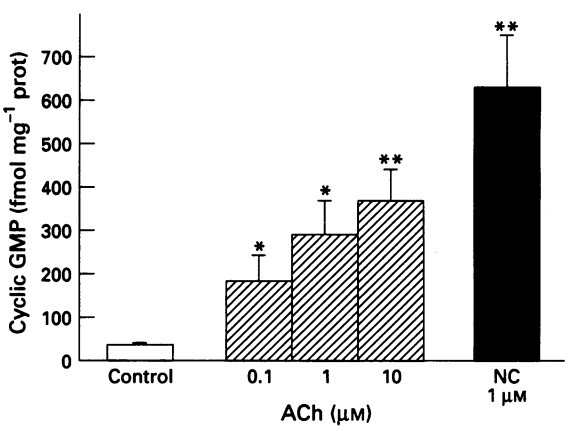

Figure 5 Effect of acetylcholine (ACh) and S-nitroso-L-cysteine (NC) on the cyclic GMP formation in bovine oviductal arteries with intact endothelium. Preparations were contracted with noradrenaline (1 μM, for 15 min) and frozen immediately or after 1 min exposure to ACh (0.1, 1 or 10 μM) or 2 min exposure to NC (1 μM). Indomethacin (10 μM) was present throughout the experiment. Each column is the mean ± s.e.mean of 4–7 experiments. $^*P<0.05$, $^{**}P<0.01$ significantly different from control values.

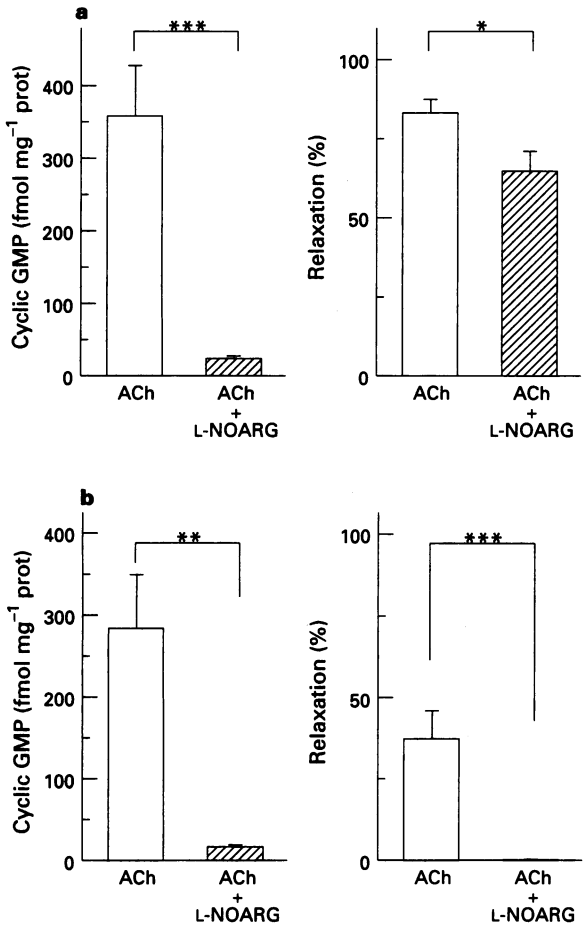

Figure 6 Effect of N^G-nitro-L-arginine (L-NOARG, 100 μM) on cyclic GMP accumulation and relaxation induced by acetylcholine (ACh, 10 μM) in oviductal arteries with intact endothelium, contracted with noradrenaline (1 μM) (a) or high K^+ (50 mM) (b). For cyclic GMP determinations, tissues were quick frozen after 1 min addition of ACh. Preparations were incubated with L-NOARG for 30 min. Indomethacin (10 μM) was present throughout the experiment. Cyclic GMP is expressed as fmol mg^{-1} of protein and relaxation is expressed as percentage of the maximal relaxation to papaverine (100 μM). Each column is the mean ± s.e.mean of 6–8 experiments. $^*P<0.05$, $^{***}P<0.001$ significantly different from ACh in the absence of L-NOARG.

Table 2 Time course of acetylcholine stimulation of cyclic GMP accumulation and effect of N^G-nitro-L-arginine (L-NOARG)

	Time				
Treatment	*0 s*	*15 s*	*30 s*	*60 s*	*300 s*
ACh	33 ± 6 (5)	120 ± 3* (3)	171 ± 25* (5)	209 ± 75* (6)	182 ± 56* (4)
L-NOARG		19 ± 3•* (6)		15 ± 3•* (6)	

Values are means ± s.e.mean of cyclic GMP (fmol mg^{-1} protein) after indicated time of exposure to acetylcholine (ACh, 1 μM) in bovine oviductal arteries with intact endothelium, contracted with noradrenaline. L-NOARG (100 μM) was added 30 min before ACh. Indomethacin (10 μM) was present throughout the experiment. The number of experiments is given in parentheses. $^*P<0.05$ significant differences following ACh compared with basal. $^•P<0.05$ significant differences between control and L-NOARG-treated preparations (unpaired t test).

15 s, that was maintained through 300 s. Treatment with L-NOARG (100 μM) prevented the increase in cyclic GMP at 60 s as well as the early rise observed at 15 s. The ACh-induced cyclic GMP levels in the presence of L-NOARG were significantly less than cyclic GMP levels under basal conditions (Table 2).

Discussion

EDRF-NO is formed by oxidation of one guanidino-nitrogen atom (N^G) of L-arginine (Palmer *et al.*, 1987) and L-arginine analogues with a chemically altered quanidino moiety inhibit the oxidative L-arginine pathway in intact cells (Rees *et al.*, 1989). Of these L-arginine analogues, N^G-nitro-L-arginine (L-NOARG) has been proved to be the most potent and specific inhibitor of the cytosolic NO formation, showing a 300 fold higher inhibitory potency against constitutively expressed NO synthase isozymes, compared to inducible NO synthase from macrophages (Furfine *et al.*, 1993). In the present study, the incubation of oviductal arteries with L-NOARG inhibited the endothelium-dependent ACh-induced relaxation and this inhibitory effect was reversed by the addition of excess L-arginine but not D-arginine. Furthermore, ACh produced, in endothelium-intact oviductal arteries, a dose-dependent and sustained increase in cyclic GMP levels, which was abolished in the presence of L-NOARG. In addition, MB, considered to be an inhibitor of guanylate cyclase (Martin *et al.*, 1985), induced a quantitatively similar inhibition to L-NOARG of the relaxant response to ACh. Taken together, these findings provide evidence that the L-arginine-NO pathway is constitutively expressed in this tissue and seems to contribute to endothelium-dependent ACh relaxation through a mechanism involving guanylate cyclase activation and accumulation of cyclic GMP in smooth muscle. NO-dependent mechanisms mediating, in variable degree, ACh responses have been widely reported in several vascular preparations, including human uterine artery (Jovanovic *et al.*, 1994b), rat mesenteric arteries (Hwa *et al.*, 1994), guinea-pig carotid artery (Suzuki *et al.*, 1992), rabbit (Müggе *et al.*, 1991) and cat (Alonso *et al.*, 1993) femoral arteries, human pulmonary arteries (Crawley *et al.*, 1990) and gastric submucosal arterioles (Chen *et al.*, 1993).

We have shown that treatment of endothelium-intact vascular rings with L-NOARG enhanced the vasoconstrictor effect of NA, although it did not modify the resting vascular tone in most preparations. L-NOARG also significantly reduced cyclic GMP content below basal levels in NA-contracted vessels. Since stretch-activated ion channels exist in endothelial cells as mechanotransducers (Lansman *et al.*, 1987), EDRF-NO might be released in higher amounts with increases in tension, while when the tissue is at baseline tension no, or very little, EDRF-NO is released. Thus, release of EDRF-NO by physical stimuli, such as stretching of the vessel wall, may represent a physiological mechanism for the regulation of vascular tone in these vessels. MB induced a similar effect to L-NOARG on NA-vasoconstriction and basal tone of oviductal arteries, although its action was endothelium-independent. Similarly, in the human uterine artery, MB caused an endothelium-independent contraction and shifted the concentration-response curve to phenylephrine, even in rings with endothelium (Jovanovic *et al.*, 1994a). Non specific effects of MB, such as depolarization of smooth muscle or release of NA from intramural nerves (Martin *et al.*, 1985), could be involved in this action.

Vascular EDRF-NO may not be identical to NO. Several NO-containing species have been suggested as the native EDRF (Busse *et al.*, 1993). Among these, NC is a very attractive candidate for a tissue-derived vasoactive mediator (Myers *et al.*, 1990). Ample evidence exists in support of the view that low molecular weight thiols react rapidly with oxides of nitrogen to form S-nitrosothiols, which are significantly more stable than NO itself (Kowaluk & Fung, 1990). In the present study, both exogenous NO and NC induced potent

dose-dependent relaxations of endothelium-denuded oviductal arteries. However, MB showed dissimilar effects on relaxations induced by NO and NC. At concentrations which induced a parallel rightward shift in the concentration-response curve to NO, NC-dependent relaxation was not affected by MB. The fact that NC effectively induced cyclic GMP accumulation in oviductal vascular preparations argues against MB being a specific inhibitor of soluble guanylate cyclase as is generally assumed. Generation of O_2^-, which induces direct chemical inactivation of extracellular NO has been shown to mediate the inhibition of NO-dependent relaxations by MB in some tissues (Wolin *et al.*, 1990; García-Pascual & Triguero, 1994). Nitrosothiols, such as NC can yield NO directly to the cytoplasm of smooth muscle cells following denitrosation at the membrane (Kowaluk & Fung, 1990), being less sensitive to extracellular inactivation by O_2^-. The fact that ACh- and NO-induced relaxations were similarly inhibited by MB suggest that EDRF-NO and NO might be identical. Recently, it has been shown that MB is a potent inhibitor of NO-synthase and other iron containing enzymes (Mayer *et al.*, 1993). Alternatively, MB-induced inhibition of endothelium-dependent relaxation to ACh might be related to NO synthesis blockade rather than to guanylate cyclase inhibition.

Our study also demonstrated the contribution of other EDRF(s), different from EDRF-NO in the ACh-induced endothelium-dependent relaxation of oviductal arteries. This is supported by the following observations: first, endothelium-dependent relaxations to ACh were only partially affected by L-NOARG and MB. An incomplete inhibition of the L-arginine pathway is unlikely, as even 10 and 100 fold higher concentrations of L-NOARG caused no further inhibition of the response. Second, L-NOARG blocked ACh-stimulated accumulation of cyclic GMP while potent relaxation persisted, indicating that relaxation in the presence of L-NOARG is independent of EDRF-NO and cyclic GMP. The contribution to the relaxation of rapid rises in cyclic GMP levels, which might have returned to baseline by 1 min, is also rejected because of the complete blockade by L-NOARG treatment of both the early (15 s) and sustained (60 s) increases in cyclic GMP-induced by ACh.

It is noteworthy that in the human uterine artery, endothelium-dependent responses to ACh are mainly due to EDRF-NO release from endothelial cells (Jovanovic *et al.*, 1994a). Several studies have shown that the relative importance of EDRF-NO in ACh-dependent relaxations is dependent on vessel size, being less important in resistance than in conduit arteries (Hwa *et al.*, 1994). Since the arterial blood supply to the oviduct derives from uterine and ovarian arteries, it could be suggested that, in the female genital tract also, the smaller the artery, the greater the tendency to L-NOARG-resistant responses to occur. However, species differences between human and bovine arteries in the endothelium-dependent responses to ACh cannot be excluded. Relaxation responses resistant to L-NOARG could be due to the release of a preformed pool of EDRF-NO or an L-arginine-derived intermediate (Busse *et al.*, 1993). Our observation that repeated concentration-response curves to ACh cause diminishing vascular relaxations and that this tachyphylactic effect increases in the presence of L-NOARG favours the view that ACh could cause the release of EDRF-NO from a depletable store. However, the fact that L-NOARG-resistant relaxations occur with cyclic GMP levels below basal ones argue against this hypothesis and suggest that the involvement of a factor other than EDRF-NO is a more likely explanation. A contribution of prostacyclin or any other cyclo-oxygenase products can be ruled out since indomethacin was present at all times. Moreover, in preliminary experiments indomethacin was able to increase rather than to decrease endothelium-dependent relaxation to ACh.

Our results show that depolarization of oviductal arteries with 40–60 mM K^+ markedly reduces ACh-induced relaxation, which is further abolished by L-NOARG pretreatment. In addition, ACh treatment in high K^+-depolarized oviductal arteries increases cyclic GMP up to the levels observed in NA-treated preparations, which in turn are completely abolished by L-NOARG. Thus under these conditions only EDRF-NO is released. These data suggest that L-NOARG-resistant ACh-induced relaxation in NA-contracted rings is mediated by membrane hyperpolarization. Several studies in cerebral (Standen *et al.*, 1989; Hasunuma *et al.*, 1991); femoral (Alonso *et al.*, 1993); carotid (Suzuki *et al.*, 1992); mesenteric (Khan *et al.*, 1993; Hwa *et al.*, 1994) and pulmonary (Brayden, 1990) arteries have suggested that endothelium-dependent relaxation in response to ACh is mediated in part by an endothelium-dependent hyperpolarizing factor (EDHF). An alternative possibility is that hyperpolarization of endothelial cells induced by ACh may be propagated, through gap junctions, to the arterial media (Brunet & Beny, 1989). However, recent studies (Beny & Pacicca, 1994) have shown that electrical signals can be transmitted from the smooth muscle to the endothelium by pure electrotonic spreading, while electrotonic propagation in the reverse direction is negligible. Thus, transmission of information from the endothelium to the underlying smooth muscle must be by diffusible factors, such as the putative EDHF.

The mechanism of action of the EDHF is considered to be a result of the opening of K^+ channels (Brayden, 1990; Hasunuma *et al.*, 1991; Khan *et al.*, 1993; Cowan *et al.*, 1993; Hwa *et al.*, 1994) or the activation of the electrogenic Na^+-K^+ pump (Brayden & Wellman, 1989; Alonso *et al.*, 1993). However, the latter does not seem to be the case in the oviductal arteries because ouabain did not alter L-NOARG-resistant ACh-induced vasodilatation, indicating that ACh might produce increases in membrane K^+ conductance in a manner independent of Na^+-K^+-ATPase activity. K^+ channel blockers were used to determine whether K^+ channels played a functional role in the relaxation to ACh. Glibenclamide was used as a specific blocker of ATP-sensitive K^+ channels (K_{ATP} channels) (Standen *et al.*, 1989), TEA and charybdotoxin as nonselective and selective blockers, respectively, of large-conductance Ca^{2+}-activated K^+ channels (large-conductance K_{Ca} channels), while apamin was chosen for its high selectivity towards small-conductance Ca^{2+}-activated K^+ channels (small-conductance K_{Ca} channels) (Kolb, 1990).

Some studies of cerebral arteries suggested that K_{ATP} channels could provide an important mechanism for producing hyperpolarization and subsequent relaxation in response to ACh (Standen *et al.*, 1989; Hasunuma *et al.*, 1991). However, studies in other vascular preparations failed to support this hypothesis (Chen *et al.*, 1991; McPherson & Angus, 1991). In the present study, glibenclamide had no effect on relaxation induced by ACh in the presence of L-NOARG, suggesting that activation of K_{ATP} channels does not contribute to the ACh-induced hyperpolarization.

Perhaps the most striking observation in the present study was the profound inhibitory effect of apamin on the vasodilator action of ACh, while TEA and charybdotoxin were without effect. To date, three types of K_{Ca} channels have been described: large conductance K_{Ca} channels (100–250 pS), blocked by low concentrations of TEA (0.1–1 mM), barium and nanomolar concentrations of charybdotoxin; intermediate-conductance K_{Ca} channels (18–60 pS), also sensitive to charybdotoxin; and small-conductance K_{Ca} channels (6–14 pS), selectively blocked by apamin but insensitive to TEA and charybdotoxin (Kolb, 1990). ACh-induced relaxations sensitive to charybdotoxin have recently been described in the abdominal aorta and carotid artery (Cowan *et al.*, 1993) and in rat (Hwa *et al.*, 1994) and rabbit (Khan *et al.*, 1993) mesenteric arteries, leading to the suggestion that large conductance K_{Ca} channels mediate the action of EDHF. On the other hand, it has been shown that charybdotoxin also inhibits relaxation responses to exogenous NO and nitroglycerin (Khan *et al.*, 1993). In fact, exogenous NO and native EDRF have been demonstrated to induce hyperpolarization of smooth muscle (Tare *et al.*, 1990; McPherson & Angus, 1991) by a direct interaction with K_{Ca} channels, without requiring cyclic GMP

(Bolotina *et al.*, 1994). Alternatively, K_{Ca} channels can also be opened after activation of cyclic GMP-dependent protein kinase (Robertson *et al.*, 1993). Thus, direct hyperpolarizations induced by EDRF-NO should be rejected before assessing a role for any type of K^+ channels in the EDHF-mediated hyperpolarization. In the present study, both MB and apamin inhibited endothelium-dependent relaxation to ACh and its combination had an additive effect, suggesting the inhibition of two separate modulating mechanisms. In addition, the lack of effect of apamin either on relaxation to ACh in high K^+-contracted rings or on exogenous NO- and NC-induced relaxations suggest that EDRF-NO is not an activator of apamin-sensitive K^+ channels.

Although electrophysiological experiments are needed to clarify the role of K^+ channels in the relaxant response to ACh of oviductal arteries, taken together, the high selectivity of apamin and the lack of effect of both TEA and charybdotoxin support the concept that the L-NOARG-resistant relaxation is mediated by membrane hyperpolarization via activation of the low-conductance type of K_{Ca} channels. Apamin-sensitive relaxations in response to ACh have also been shown in rat perfused mesenteric arteries (Adeagbo & Malik, 1990). However, in this study, ACh-induced relaxation was also inhibited by other distinct K^+ channel blockers: crude scorpion venom, procaine, quinidine and TEA. In the same vascular bed, apamin was also able to inhibit endothelium-dependent vasodilatation to arachidonic acid (Adeagbo & Malik, 1991). Thus, heterogeneity exists in the K^+ channels mediating the effect of hyperpolarizing vasodilators between different vascular beds. Low-conductance K_{Ca} channels have been implicated in the regulation of endothelial membrane potential and its responses to vasodilator agonists (Groschner *et al.*, 1992). Thus alternatively, K^+ channel blockade can cause endothelial depolarization and reduction of the electrochemical gradient for Ca^{2+} entry, leading to reduced formation of both EDRF-NO and EDHF. The present results do not support the view that apamin acts by inhibiting EDRF-NO release or action, although reduction in EDHF release by inhibition of ACh-induced endothelial hyperpolarization cannot be ruled out. Further studies are needed to determine whether the effect of K^+ channel blockers is on the endothelium, the smooth muscle or both. The lack of effect of the K^+ channel blockers used in the present study on either resting tension or contractile responses to NA does not favour a role for K^+ channel regulation of intrinsic tone in oviductal arteries.

Oviductal arteries from immature heifers, in which an influence of the sex hormones can be ruled out, were used in the present study. Recently it has been demonstrated that NO-synthase is subject to regulation by oestrogen (Weiner *et al.*, 1994). Furthermore, the L-arginine-NO system present in the human myometrium has been shown to be upregulated during pregnancy and inhibited at term (Yallampalli *et al.*, 1994). Thus, the role of steroid hormones in regulating endothelium-dependent vasodilatation and changes in oviductal blood flow needs to be elucidated to understand further the pathophysiology of the oviduct fluid formation.

In conclusion, the results of the present study have demonstrated for the first time that ACh relaxes oviductal arteries through the release of two distinct endothelial factors: NO or a related substance, namely EDRF-NO, and EDHF. Our data also suggest that EDRF-NO causes relaxation by a mechanism involving guanylate cyclase activation and cyclic GMP accumulation in smooth muscle cells, while EDHF acts by hyperpolarization mechanisms through alterations in apamin-sensitive K^+ conductance.

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