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Apamin-sensitive K^+ channels involved in the inhibition of acetylcholine-induced contractions in lamb coronary small arteries

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Abstract

In vitro experiments were designed to investigate the endothelial factors involved in modulation of the contractile response to acetylcholine in lamb coronary small arteries. Endothelial cell removal, and inhibitors of the L-arginine/nitric oxide (NO) pathway increased basal tension and contractions in response to acetylcholine and abolished relaxations in response to the Ca^{2+}-ionophore, 6*S*-[6α(2*S**,3*S**),8β(*R**),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]-*undec*-2-yl]methyl]-4-benzoxazolecarboxylic acid (A23187). N^G-Nitro-L-arginine enhanced acetylcholine-induced contractions in the absence, but not in the presence of the muscarinic M_1 receptor antagonist, telenzepine. In contrast to glibenclamide and charybdotoxin, apamin enhanced the acetylcholine-induced contractions and reduced the relaxations caused by A23187 and exogenously added NO. The combination of 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) and apamin did not further increase the acetylcholine-induced contractions. These results indicate that muscarinic M_1 receptor-released endothelial NO inhibits the contractile responses to acetylcholine in lamb coronary small arteries through activation of guanylate cyclase, followed by an increase in apamin-sensitive K^+ conductance of the smooth muscle. © 1997 Elsevier Science B.V.

Keywords: Endothelium; Nitric oxide (NO); Coronary small artery; Acetylcholine; A23187; Apamin; K^+ channel

1. Introduction

There are great species differences in the vascular reactivity of coronary arteries to cholinergic agonists (Kalsner, 1985). Thus, infused acetylcholine increases coronary blood flow in dogs (Van Winckle and Feigl, 1989), but decreases it in pigs (Cowan and Mckenzie, 1990; Hata et al., 1993). Acetylcholine relaxes rat, rabbit and dog isolated coronary resistance arteries (Nyborg et al., 1991; Angus et al., 1991; Simonsen et al., 1992), while it induces atropine-sensitive contractions of small porcine and lamb coronary arteries (Nakayama et al., 1988; Myers et al., 1991; Tschudi et al., 1991; Simonsen et al., 1993), and of human coronary arteries isolated from the atrial appendage (Angus et al., 1991).

Endothelium-derived nitric oxide (EDNO) is an important factor involved in the acetylcholine-induced relaxations. It is synthesized from L-arginine and oxygen by nitric oxide (NO) synthase, and is released from the endothelium following the binding of acetylcholine to muscarinic receptors (Bassenge and Heusch, 1990). NO diffuses to vascular smooth muscle where it activates soluble guanylate cyclase, resulting in an increase in cyclic GMP levels (Ignarro et al., 1987), although it can also induce relaxation through a cyclic GMP-independent mechanism, which involves opening of K^+ channels and hyperpolarization (Bolotina et al., 1994; Cohen and Vanhoutte, 1995). Thus, in vivo studies showed that inhibition of NO synthase with L-arginine analogues abolishes the vasodilation in response to acetylcholine in the rabbit (Amezcua et al., 1989) and dog (Richard et al., 1991) and increased the contractile response to infused acetylcholine in the pig coronary circulation (Hata et al., 1993). In porcine coronary small arteries, endothelial cells have been suggested either to have no role (Nakayama et al., 1988; Kawamura et al., 1989; Cowan and Mckenzie, 1990) or to inhibit the contractile response to acetylcholine through the release of endothelium-derived relaxing factor (EDRF) (Tschudi et al., 1991; Hata et al., 1993). However, it is not clear whether the latter inhibition is due to basal or agonist-in-

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duced release of endothelial NO, although muscarinic M_1 receptors have been suggested to mediate the vasodilatation in response to acetylcholine in the dog coronary circulation (Pelc et al., 1988) and the inhibition of the contractile responses to acetylcholine in lamb coronary resistance arteries in vitro (Simonsen et al., 1993). In guinea-pig large coronary arteries, acetylcholine-elicited relaxation involves the release of both EDNO, which induces cyclic GMP accumulation (Eckman et al., 1994), and an endothelium-derived hyperpolarizing factor (EDHF) different from NO (Eckman et al., 1994; Parkington et al., 1993, 1995; Wei et al., 1994; Campell et al., 1996). Moreover, NO itself can also induce hyperpolarization of coronary smooth muscle (Parkington et al., 1995). Therefore, since several endothelial factors acting through different mechanisms might be involved in the responses of the coronary circulation to acetylcholine, the purpose of the present study was to determine the specific role of the endothelium, as well as the nature of the factors involved in the modulation of the contractions elicited by acetylcholine in lamb coronary small arteries in vitro. The effects of the inhibitors of the L-arginine/NO pathway and of K^+-channel blockers were compared on the endothelium-dependent relaxant responses to the Ca^{2+} ionophore, A23187.

2. Materials and methods

2.1. Dissection and mounting

Hearts from 3–6-month-old lambs, excised immediately after the animals were killed, were obtained from the local slaughterhouse and placed in ice-cold physiological salt solution (PSS) to reduce cardiac metabolism. Throughout the subsequent dissection, the hearts were bathed in cold PSS (4°C) of the following composition (mmol/l): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.5, ethylenediaminetetraacetic acid (EDTA) 0.026 and glucose 11. The solution was gassed with 5% CO_2 in O_2 to maintain the pH at 7.4.

Third- to fourth-order small subepicardial arteries of the left anterior descending coronary artery, located close to the apex of the left ventricle were dissected as previously described for lamb coronary resistance arteries (Simonsen et al., 1993). Segments (ca. 2 mm long) of the small vessels were subsequently mounted as ring preparations on two 40-μm wires on an isometric double myograph by fixing one of the wires to a force transducer and the second wire to a length displacement device (Mulvany and 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 (Mulvany and Halpern, 1977). The vessels were set to an internal circumference, L_1, given by $L_1 = 0.9 \times L_{100}$. Preliminary experiments has shown that force development is close to maximal at this internal circumference (Simonsen et al., 1993). The effective internal lumen diameter was determined as $l_1 = L_1/\pi$.

2.2. Experimental procedure

The following procedure was followed for each small coronary artery to ensure functional integrity of the vessel segments: (1) after normalization, the contractile ability of the vessels was tested by stimulating the arterial rings with KPSS (equivalent to PSS but NaCl replaced by KCl on an equimolar basis, giving a final concentration of 123.7 mM K^+) until reproducible responses were recorded; (2) the presence of spontaneous tonus was measured as the difference between the baseline just after normalization and to the baseline obtained after exchange of PSS for Ca^{2+}-free PSS (similar to PSS except that $CaCl_2$ was omitted and replaced by 10^{-4} M EGTA) or application of 10^{-4} M papaverine as the last step of the protocol; (3) the presence of intact endothelium was evaluated by inducing a stable contraction with either 3×10^{-7}–10^{-6} M acetylcholine or $(1–3) \times 10^{-7}$ M U46619 and then adding either $(3–5) \times 10^{-6}$ M Ca^{2+} ionophore, A23187, or 3×10^{-8} M substance P. A relaxation greater than 50% was taken as evidence of endothelial integrity and segments with a relaxation less than 50% were discarded.

To investigate which factors mediate endothelial modulation of the contractile response to acetylcholine, a first concentration–response curve for this agonist was obtained. The preparations were washed several times, and a stable contraction corresponding to 60% of the maximal response was induced with acetylcholine, and a cumulative concentration–response curve to the Ca^{2+} ionophore, A23187, was obtained. These curves served as control curves. We had observed that concentration–response curves made with this protocol are reproducible (Simonsen et al., 1993). After several washings, the vessels were incubated with inhibitors of either NO synthase, N^G-nitro-L-arginine (L-NOARG, 10^{-5}–10^{-4} M), cyclooxygenase, indomethacin (10^{-5} M), lipoxygenase, nordihydroguaiaretic acid (NDGA, 5×10^{-6} M), guanylate cyclase, methylene blue (10^{-6} M) or 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, 3×10^{-6} M) or with the nitric oxide scavenger, oxyhemoglobin (10^{-5} M). In addition, the effects of several K^+-channel blockers and ouabain (10^{-4} M) were tested on both the contractile response to acetylcholine and the relaxant responses to A23187. If the inhibitors themselves induced an increase in tension or potentiated the contraction evoked by acetylcholine, the added concentration of preconstrictor in a second curve was adjusted to match the level of preconstriction reached in the first concentration–response curve for A23187. In another set of experiments, it was tested whether L-arginine could influence or reverse the effect of L-NOARG. Thus, a

first concentration–response curve for acetylcholine was obtained, a second curve was made in the presence of L-NOARG, and finally the vessel was incubated with L-arginine (10^{-3} M) and L-NOARG and a third concentration–response curve for acetylcholine was obtained. Similarly, three consecutive concentration–response curves for A23187 were obtained, the first curve for A23187 served as control for the second curve made in the presence of L-NOARG and a third curve for A23187 obtained in the presence of both L-arginine and L-NOARG. Finally, the effects of different inhibitors were tested on the responses to exogenous NO, added as acidified sodium nitrite ($NaNO_2$), and NO donor, L-nitrosocysteine.

2.3. Drugs

Acetylcholine chloride, apamin, Ca^{2+} ionophore (6*S*-[6α(2*S**,3*S**),8β(*R**),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]-*undec*-2-yl]methyl]-4-benzoxazolecarboxylic acid or A23187), charybdotoxin, glibenclamide, indomethacin, L-arginine hydrochloride, N^G-nitro-L-arginine (L-NOARG), L-cysteine, methylene blue, nordihydroguaiaretic acid (NDGA), ouabain, papaverine hydrochloride, sodium nitrite ($NaNO_2$) and 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619) were purchased from Sigma (St. Louis, MO, USA). Telenzepine and hexahydro-sila-diphenidol (HHSid) were from Research Biochemicals International, UK. The thromboxane receptor antagonist, SQ30741 ([1*S*-[1α,2α(5*Z*),3α,4α]]-7[[[[[(oxaheptyl)amino]acetyl]amino]methyl]-7oxabicyclo-[2.2.1]hept2-yl]-5-heptenoic acid), a generous gift form Dr. M. Ogletree, Bristol Mayer Squibs, Princeton, NJ, USA, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, Tocris Cookson, MO, USA). Drugs were dissolved in distilled water, except indomethacin, U46619 and SQ30741 which were dissolved in 90% ethanol, and A23187, glibenclamide and ODQ which were dissolved in dimethyl sulphoxide, and further diluted in water. Stock solutions were prepared and stored at −20°C and further fresh dilutions were prepared daily.

Oxyhemoglobin was prepared from a 1 mM solution of commercial hemoglobin (bovine hemoglobin, Sigma) by addition of 10 mM sodium dithionite ($Na_2S_2O_4$, Sigma) (Martin et al., 1985). The reducing agent converting methaemoglobin to oxyhemoglobin was removed by dialysis in 2 l of distilled water and bubbling with N_2 at 4°C. The purity of oxyhemoglobin solutions was determined spectrophotometrically, giving a final concentration of $(5–8) \times 10^{-4}$ M.

$NaNO_2$ was freshly prepared as 1 M stock solutions by adjusting the pH to 2 by adding concentrated HCl (Simonsen et al., 1997). This stock solution was kept cold and protected from air. Further dilutions were made in diluted HCl (pH 2) immediately before use and added in volumes of 5–10 μl. Neither equivalent volumes of the vehicle for $NaNO_2$ nor non-acidified $NaNO_2$ (pH 7.4) up to 10^{-2} M caused relaxation of lamb coronary resistance arteries. L-Nitrosocysteine was made fresh each day according to the method described by Field et al. (1978).

2.4. Analysis of data

The mechanical responses of the vessels were measured as force and expressed as active wall tension, δT, which is the increase in measured force, δF, divided by twice the segment length. Using a computer program (GraphPad, Institute for Scientific Information, San Diego, CA, USA), the concentration–response curves were fitted to the classical Hill equation: $R/R_{max} = A(M)^n/(A(M)^n + EC_{50}(M)^n)$, where R/R_{max} is the relative response to the effective concentration of drug, $A(M)$, and $EC_{50}(M)$ is the concentration of agonist required to give a half-maximal vessel response (R_{max}), where $A(M)$ and $EC_{50}(M)$ are given in molar concentrations. n is a curve-fitting parameter or Hill coefficient. The results are expressed as means ± S.E.M., where n represents the number of animals studied in each set of experiments. The concentration–response curves before and after treatment were compared by analysis of variance (ANOVA) for repeated measures, and by paired test for comparisons of the individual concentrations. Results comparing endothelium-intact and endothelium-denuded preparations were evaluated by analysis of variance (ANOVA) followed by unpaired two-tailed t-test. When multiple comparisons were made with a single control, values were analysed according to a one-way analysis of variance (ANOVA) and Bonferroni method as an a posterio test (Wallenstein et al., 1980). Probability levels under 5% were considered significant.

3. Results

3.1. Effect of muscarinic receptor antagonists, endothelial cell removal and indomethacin

In lamb coronary small arteries with an effective lumen diameter (l_1) of 351 ± 14 μm ($n = 70$), KPSS induced contractions of 1.3 ± 0.1 Nm^{-1} ($n = 70$). Acetylcholine induced relaxation in only 5 out of 25 endothelium-intact lamb coronary resistance arteries precontracted with U46619, whereas it elicited potent concentration-dependent contractions in all lamb coronary arteries examined (Table 1). The concentration–response curves for acetylcholine were shifted to the right in the presence of the muscarinic M_3 receptor antagonist, HHSiD, giving apparent pA_2 values of 8.04 ± 0.20 ($n = 4$), and a slope of 1.27 ± 0.15 (Fig. 1a). In contrast, the muscarinic M_1 receptor antagonist, telenzepine, in small concentrations (10^{-10}–10^{-9} M) induced leftward shifts of the concentration–response curves for acetylcholine in endothelium-intact coronary resistance arteries (Fig. 1b). In endothelium-denuded segments, however, there was no enhancing effect

Table 1
Effects of endothelial cell removal (−E), N^G-nitro-L-arginine (L-NOARG, 10^{-5} M) and its reversal by L-arginine (L-ARG, 10^{-3} M), methylene blue (MB, 10^{-6} M), oxyhemoglobin (Oxyhgb, 10^{-5} M), or 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, 3×10^{-6} M) on basal tension (ΔBT) and contractile concentration–response curves for acetylcholine of lamb coronary small arteries

	n	ΔBT (Nm^{-1})	pD_2 ($-\log(EC_{50})$)	ΔpD_2	E_{max} (Nm^{-1})
Control	45	–	6.07 ± 0.07	–	2.5 ± 0.2
−E	8	0.22 ± 0.07 [a]	6.85 ± 0.14	0.60 ± 0.15 [a]	3.0 ± 0.5
L-NOARG	10	0.32 ± 0.13 [a]	6.55 ± 0.07	0.52 ± 0.07 [a]	2.6 ± 0.3
+L-ARG	6	0.01 ± 0.1	6.34 ± 0.10	0.24 ± 0.10 [b]	2.5 ± 0.5
Oxyhgb	8	0.42 ± 0.10 [a]	6.13 ± 0.14	0.60 ± 0.12 [a]	2.5 ± 0.5
MB	7	0.38 ± 0.11 [a]	6.67 ± 0.15	0.39 ± 0.09 [a]	1.8 ± 0.2
ODQ	6	0.14 ± 0.04 [a]	6.63 ± 0.11	0.46 ± 0.07 [a]	2.9 ± 0.4

Values are means ± S.E.M. *n*, number of vessels. EC_{50} is the concentration of acetylcholine required to produce half-maximal contraction and E_{max} the maximal response to the agonist. $pD_2 = -\log(EC_{50})$ and ΔpD_2 is the difference in pD_2 between a first and a second concentration–response curve obtained in the absence or presence of treatment, respectively.
[a] $P < 0.05$, compared to a first control curve (paired test).
[b] $P < 0.05$, compared to the concentration-response curve for acetylcholine in the presence of L-NOARG.

of telenzepine (10^{-9} M) on the acetylcholine-elicited contractions ($n = 5$).

Mechanical endothelial cell removal increased basal tension, invariably caused leftward shifts in the contractile concentration–response curves for acetylcholine and abolished the relaxations in response to A23187, as previously reported for this preparation (Table 1; Simonsen et al., 1993).

The inhibitor of cyclooxygenase, indomethacin (10^{-5} M), did not affect either basal tension or the responses to acetylcholine or to A23187. Thus, acetylcholine induced contractions with pD_2 values and maximal responses of 6.30 ± 0.13 and 2.9 ± 0.4 Nm^{-1}, and 6.39 ± 0.06 and 3.1 ± 0.5 Nm^{-1} ($n = 8$) in the absence and the presence of 10^{-5} M indomethacin, respectively, while A23187 induced relaxations with a $pD_2 = 6.06 \pm 0.11$ and maximal relaxations of 81.5 ± 10.4% ($n = 9$) and 5.88 ± 0.13 and 69.3 ± 11.7% ($n = 9$), in the absence and the presence of indomethacin, respectively. The thromboxane receptor antagonist, SQ30741 (10^{-6} M), also did not affect the contractions of lamb coronary small arteries in response to acetylcholine ($n = 4$). The inhibitor of lipoxygenase, NDGA (5×10^{-6} M), increased basal tension, while the maximum response to acetylcholine was reduced in 4 segments, although this suppression did not reach significance. Thus, acetylcholine induced contractions with pD_2 values and maximal responses of 5.78 ± 0.16 and 2.7 ± 0.4 Nm^{-1} ($n = 6$), and 5.86 ± 0.16 and 2.0 ± 0.3 Nm^{-1} ($n = 6$) in the absence and the presence of NDGA, respectively. This inhibitor did not affect the relaxations in response to A23187 ($n = 4$) at the concentration applied.

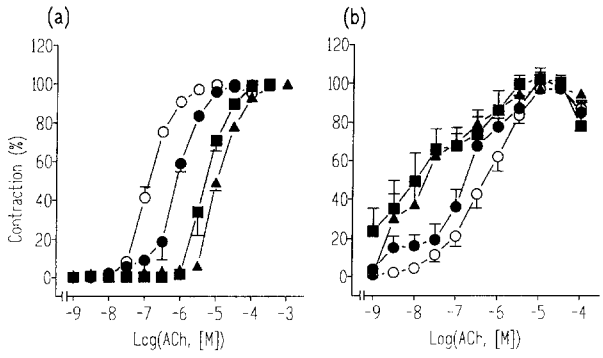

Fig. 1. Cumulative concentration–response curves for acetylcholine (ACh) in endothelium-intact lamb coronary arteries: in the absence (open circles) and (a) the presence of increasing concentrations of the muscarinic M_3 receptor antagonist, hexahydrosiladiphenidol (HHSiD, 3×10^{-8} M, closed circles; 10^{-7} M, closed squares; 3×10^{-7} M, closed triangles), and (b) the presence of the muscarinic M_1 receptor antagonist, telenzepine (10^{-10} M, closed circles; 3×10^{-10} M, closed squares; 10^{-9} M, closed triangles). Each point is the mean ± S.E.M. for 4–6 vessels.

3.2. L-Arginine / nitric oxide pathway

In quiescent endothelium-intact coronary resistance arteries, the inhibitor of NO synthase, L-NOARG (10^{-7}–10^{-5} M), evoked slow contractions which reached a maximum of 0.28 ± 0.11 Nm^{-1} ($n = 7$). This effect was not observed in endothelium-denuded vessels ($n = 5$) (Fig. 2a). L-NOARG (10^{-5} M) caused leftward shifts of the concentration–response curves for acetylcholine in the lamb coronary small arteries, and the shifts were partially reversed in the presence of L-arginine (Table 1). There was no additional effect of 10^{-4} M L-NOARG on the response to acetylcholine ($n = 4$). In the presence of telenzepine (10^{-9} M), L-NOARG (10^{-5} M) did not produce additional enhancement of the acetylcholine-induced contractions compared to the effect of L-NOARG alone (Fig. 2b). In addition, L-NOARG concentration dependently inhibited the relaxations in response to A23187 (Fig. 2c, Table 2). Incubation with L-arginine (10^{-3} M) partially reversed the L-NOARG inhibition of the relaxations caused by A23187. Thus, A23187 caused relaxations with pD_2 values and maximal responses of 5.26 ± 0.05 and 41.9 ± 8.2%, and 5.59 ± 0.07 ($P < 0.05$, $n = 6$) and 57.3 ± 3.9% ($P < 0.01$, $n = 6$, paired *t*-test) in the presence of L-NOARG (10^{-4} M) or L-NOARG and L-arginine (10^{-3} M), respectively.

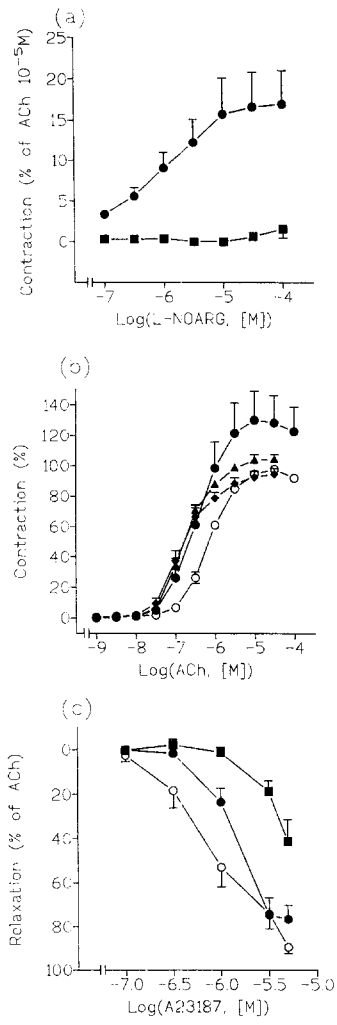

Fig. 2. The effect of N^G-nitro-L-arginine (L-NOARG) in isolated lamb coronary small arteries. (a) Effect of increasing concentrations of L-NOARG on the resting tension of endothelium-intact (closed circles) and -denuded coronary segments (closed squares). (b) Contractions in response to acetylcholine (ACh) in the absence (open circles) and presence of L-NOARG (10^{-5} M, closed circles), the muscarinic M_1 receptor antagonist, telenzepine (10^{-9} M, closed diamonds) or the presence of both L-NOARG (3×10^{-5} M) and telenzepine (closed triangles). (c) Relaxations in response to A23187 in the absence (open circles) and the presence of 10^{-5} M L-NOARG (closed circles) or 10^{-4} M L-NOARG (closed squares). The relaxations in response to A23187 are expressed as percentages of the precontractions with acetylcholine ($(1-3)\times10^{-6}$ M), which were 1.8 ± 0.2 Nm^{-1} in the absence, and 2.0 ± 0.3 Nm^{-1} and 1.5 ± 0.3 Nm^{-1} in the presence of 10^{-5} M and 10^{-4} M L-NOARG, respectively. Each point represents the mean±S.E.M. for 5–10 vessel segments.

The nitric oxide scavenger, oxyhemoglobin (10^{-5} M), produced contractions of endothelium-intact lamb coronary small arteries and caused leftward shifts in the concentration–response curves for acetylcholine (Fig. 3a, Table 1). The inhibitors of NO-sensitive guanylate cyclase, methylene blue and ODQ increased basal tension in endothelium-intact, but not in endothelium-denuded preparations. However, when the increase in basal tension was subtracted, the effect on the concentration–response curve for

Table 2
Effects of N^G-nitro-L-arginine (L-NOARG, 10^{-5} M and 10^{-4} M), methylene blue (MB, 10^{-6} M), oxyhemoglobin (Oxyhgb, 10^{-5} M), or 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, 3×10^{-6} M) on the relaxations induced by the Ca^{2+} ionophore, A23187, in lamb coronary small arteries

	n	pD_2 ($-\log(EC_{50})$)	ΔpD_2	Maximum relaxation (%)
Control	37	5.90±0.08	–	82.5±3.2
L-NOARG 10^{-5} M	8	5.80±0.10	0.30±0.20	80.5±5.8
L-NOARG 10^{-4} M	10	5.24±0.06	0.81±0.19 [a]	40.9±8.2 [a]
OxyHgb	6	5.24±0.12	0.84±0.10 [a]	32.0±4.6 [a]
MB	7	5.34±0.09	0.37±0.10 [a]	55.9±9.6 [a]
ODQ	6	5.31±0.08	0.67±0.12 [a]	55.5±11.5 [a]

Values are means±S.E.M. *n*, number of vessels. EC_{50} is the concentration of A23187 required to produce half-maximal relaxation and the maximum relaxation is the response obtained at the highest concentration (5×10^{-6} M) of A23187 applied. $pD_2 = -\log(EC_{50})$ and ΔpD_2 is the difference between a first and a second concentration–response curve obtained in the absence and presence of treatment, respectively.
[a] $P < 0.05$, compared to a first control curve (paired test).

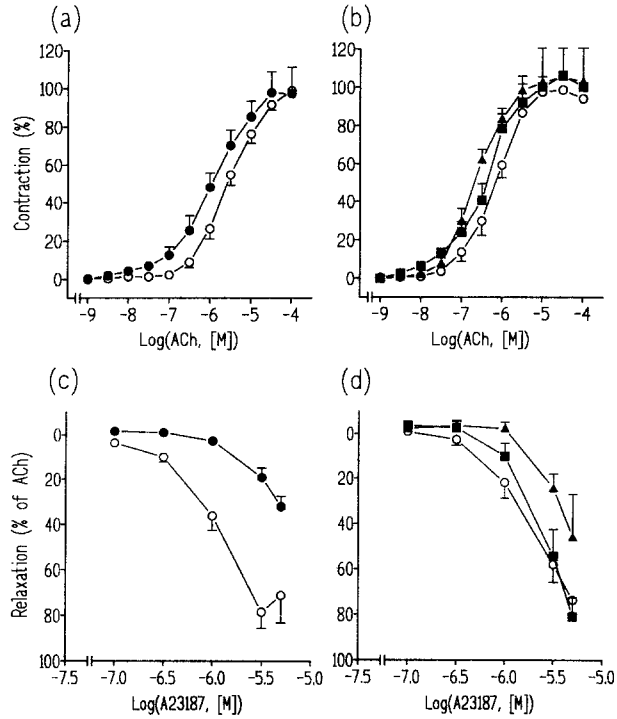

Fig. 3. (a, b) Cumulative concentration–contraction curves for acetylcholine (ACh) and (c, d) concentration-relaxation curves for the Ca^{2+} ionophore, A23187, in endothelium-intact lamb coronary resistance arteries. Control responses (open circles) and responses in the presence of either 10^{-5} M oxyhemoglobin (closed circles), 10^{-6} M methylene blue (closed squares) or 3×10^{-6} M 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, closed triangles). The relaxations induced by A23187 are expressed as percentages of the contractions with acetylcholine ($(0.5-3)\times10^{-6}$ M), which were 1.7 ± 0.2 Nm^{-1} under control conditions, and 1.9 ± 0.2 Nm^{-1}, 1.7 ± 0.3 Nm^{-1}, and 1.4 ± 0.4 Nm^{-1} in the presence of oxyhemoglobin, methylene blue or ODQ, respectively. Each point represents the mean±S.E.M. for 6–8 vessel segments.

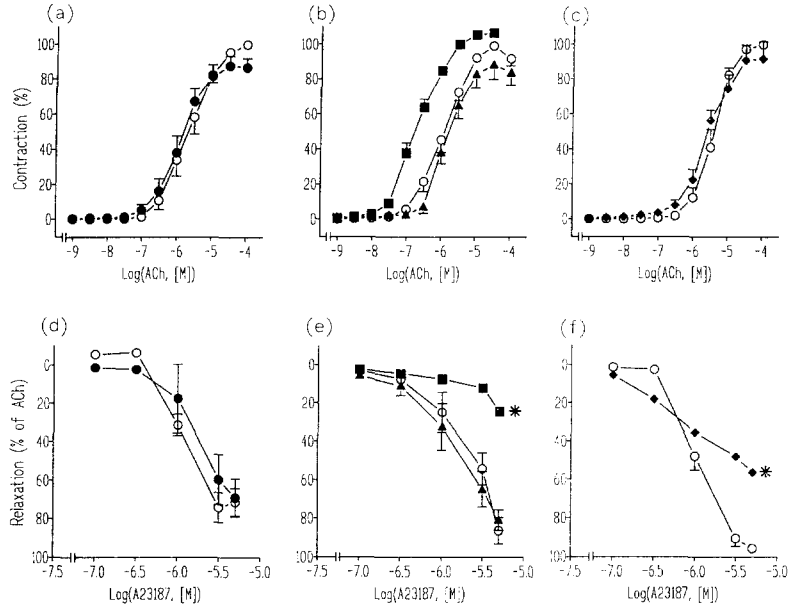

Fig. 4. Effect of K^+-channel blockers and ouabain on (a, b, c) contractions in response to acetylcholine (ACh), and (d, e, f) relaxations in response to the Ca^{2+} ionophore, A23187, in endothelium-intact lamb coronary resistance arteries. Control responses (open circles), in the presence of the ATP-sensitive K^+-channel blocker, 3×10^{-6} M glibenclamide (closed circles), the small Ca^{2+}-activated K^+-channel blocker, 5×10^{-7} M apamin (closed squares), the large Ca^{2+}-activated K^+-channel blocker, 3×10^{-8} M charybdotoxin (closed triangles), or the Na^+/K^+-ATPase inhibitor, ouabain (10^{-4} M, closed diamonds). The differences in concentrations of acetylcholine needed to produce half-maximal contractions in the absence and the presence of treatment were: for glibenclamide ($\Delta pD_2 = 0.10 \pm 0.12$), apamin ($\Delta pD_2 = 0.40 \pm 0.06$, $P < 0.05$, paired t-test), charybdotoxin ($\Delta pD_2 = 0.03 \pm 0.16$), and for ouabain ($\Delta pD_2 = 0.21 \pm 0.12$). The relaxations with A23187 are expressed as percentages of the precontractions caused by acetylcholine, which were 1.4 ± 0.3 Nm^{-1} and 1.3 ± 0.3 Nm^{-1} in the absence and presence of glibenclamide, 1.3 ± 0.2 Nm^{-1}, 1.3 ± 0.1 Nm^{-1} and 0.7 ± 0.3 Nm^{-1} in the absence and the presence of apamin or charybdotoxin, and 2.0 ± 0.2 Nm^{-1} and 1.0 ± 0.1 Nm^{-1} ($P < 0.05$, $n = 5$) in the absence and presence of ouabain, respectively. Significantly different responses compared to the maximum of the control curve, analysis of variance for repeated measures: $^*P < 0.05$. Each point represents the mean ± S.E.M. for 5–6 vessel segments.

acetylcholine was less pronounced (Table 1, Fig. 3b). The relaxations caused by A23817 were significantly reduced in the presence of oxyhemoglobin, methylene blue and ODQ (Fig. 3c,d, Table 2).

3.3. Effect of glibenclamide, charybdotoxin, apamin and ouabain

The blocker of ATP-sensitive K^+ channels, glibenclamide, had no effect on either basal tension, contractions induced by acetylcholine, or relaxations in response to A23187 (Fig. 4a,d). Inhibition of large conductance Ca^{2+}-activated K^+ channels with 3×10^{-8} M charybdotoxin neither induced an increase in basal tension, nor influenced the acetylcholine-induced contractions or the relaxations in response to A23187 (Fig. 4b,e). However, the blocker of small conductance Ca^{2+}-activated K^+ channels, apamin, did not affect basal tension, but induced significant leftward shifts in the concentration–response curves for acetylcholine and significantly reduced the relaxations with A23187 (Fig. 4b,e).

The inhibitor of Na^+/K^+-ATPase, ouabain (10^{-4} M), did not change either basal tension or the acetylcholine-induced contractions, but it did significantly reduce the relaxations in response to 5×10^{-6} M A23187 (Fig. 4c,f).

In the presence of the selective inhibitor of NO-sensitive guanylate cyclase, ODQ (3×10^{-6} M), apamin (5×10^{-7} M) did not produce additional increases of the acetylcholine-induced contractions or inhibition of the relaxations with A23187 (Fig. 5).

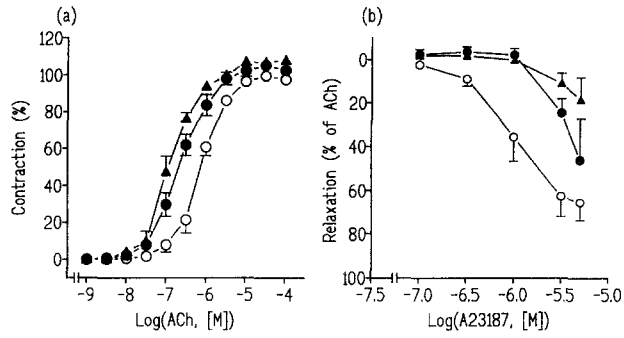

Fig. 5. Combined effect of the guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ), and the blocker of small Ca^{2+}-activated K^+ channels, apamin, on the contractions in response to (a) acetylcholine (ACh) and (b) the relaxations in response to the Ca^{2+} ionophore (A23187) in endothelium-intact lamb coronary small arteries. Control responses (open circles), in the presence of 3×10^{-6} M ODQ (closed circles), and in the presence of both ODQ and 5×10^{-7} M apamin (closed triangles). The relaxations induced by A23187 are expressed as percentages of the precontractions with acetylcholine ($(1–3) \times 10^{-6}$ M), which were 1.4 ± 0.4 Nm^{-1}, 1.2 ± 0.1 Nm^{-1} and 1.5 ± 0.2 Nm^{-1} in the absence and the presence of apamin and apamin plus ODQ, respectively. Each point represents the mean ± S.E.M. for 6 vessel segments.

3.4. Effect of ODQ and apamin on exogenously added NO

Exogenous NO, added as acidified sodium nitrite ($NaNO_2$) and the NO donor, L-nitrosocysteine, induced concentration-dependent relaxations of lamb coronary small arteries. Relaxations caused by NO were abolished in the presence of 10^{-5} M oxyhemoglobin ($n = 8$), and significantly reduced by ODQ, the maximum response to NO being 81 ± 5% ($n = 6$) in the absence, and 30 ± 7% ($P < 0.05$, $n = 6$, paired t-test) in the presence of 3×10^{-6} M ODQ (Fig. 6a and Fig. 7a). NO- and L-nitrosocysteine-induced maximal relaxations were significantly reduced in the presence of apamin (5×10^{-7} M). Thus, in acetylcholine-contracted coronary small arteries, NO added as acidified $NaNO_2$ caused relaxations with pD_2 values and maximal responses of 4.61 ± 0.17 and 88 ± 3%, and 4.24 ± 0.14 ($P < 0.05$, $n = 4$, paired t-test) and 61 ± 6% ($P < 0.05$, $n = 4$, paired t-test) in the absence and the presence of 5×10^{-7} M apamin (Fig. 6b and Fig. 7a), while L-nitrosocysteine induced relaxations with pD_2 values and maximal responses of 6.48 ± 0.14 and 87.6 ± 4.0%, and 6.21 ± 0.07 ($P < 0.05$, $n = 6$, paired t-test) and 73.8 ± 4.0% ($P < 0.05$, $n = 6$, paired t-test) in the absence and presence of apamin, respectively. The relaxations caused by NO were abolished by combined treatment with ODQ plus apamin (Fig. 7a).

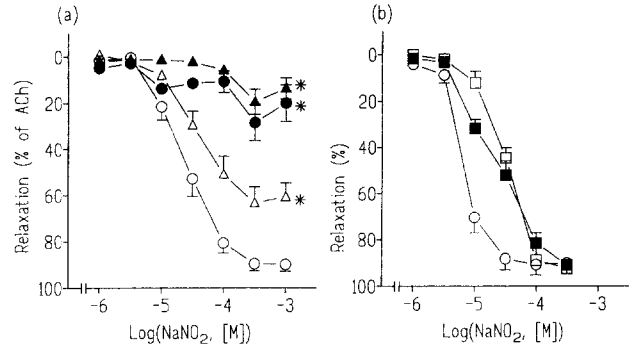

Fig. 7. Average concentration–relaxation curves for exogenous NO (in acidified sodium nitrite, $NaNO_2$) in endothelium-intact lamb coronary small arteries contracted with acetylcholine (ACh). (a) Control responses in segments contracted with acetylcholine (open circles), and in the presence of 5×10^{-7} M apamin (open triangles), 3×10^{-6} M 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, closed circles) or both ODQ and 5×10^{-7} M apamin (closed triangles). The relaxations with NO are expressed as percentages of the precontractions with acetylcholine, which were 1.8 ± 0.4 Nm^{-1} (control), 2.1 ± 0.7 Nm^{-1} (apamin), 2.2 ± 0.2 Nm^{-1} (ODQ) and 2.8 ± 0.3 Nm^{-1} (apamin plus ODQ). (b) Relaxations induced by $NaNO_2$ in acetylcholine-contracted (open circles), 80 mmol K^+-contracted (closed squares), and in the presence of 5×10^{-7} M apamin in 80 mmol/l K^+-contracted (open squares) vessels. The precontractions were 1.6 ± 0.4 Nm^{-1} (acetylcholine), 1.5 ± 0.5 Nm^{-1} (80 mmol/l K^+) and 1.9 ± 0.7 Nm^{-1} (apamin in 80 mmol/l K^+). Each point represents the mean ± S.E.M. for 5–6 vessel segments. Significantly different responses compared to the maximum of the control curve, analysis of variance for repeated measures: $^*P < 0.05$.

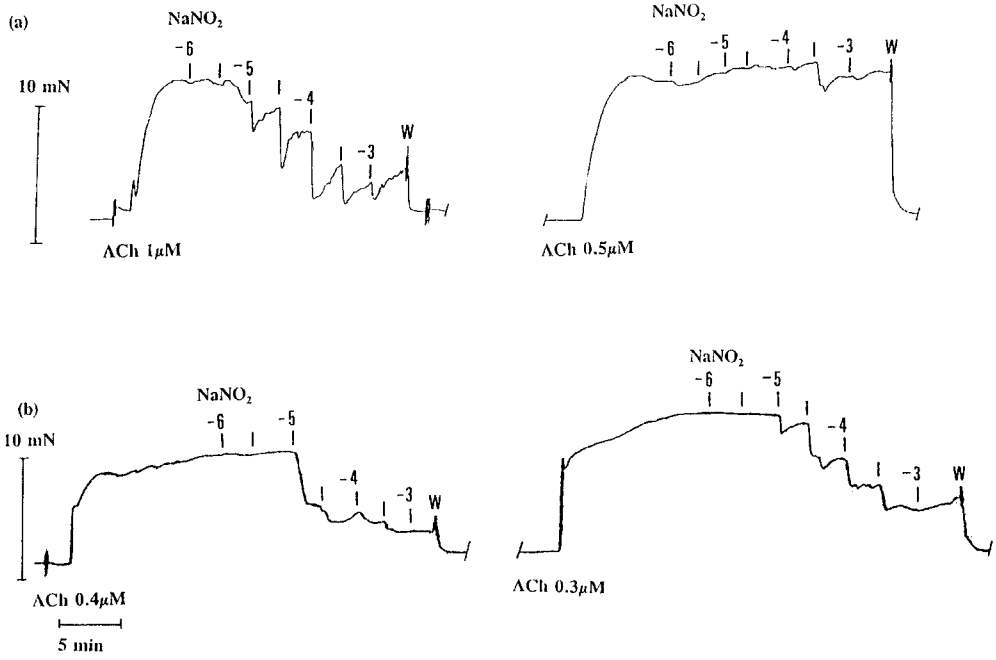

Fig. 6. Isometric force recordings showing the effect of an inhibitor of guanylate cyclase, [1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) and a blocker of small conductance Ca^{2+}-activated K^+ channels, apamin, on the relaxations induced by exogenous nitric oxide (NO) added as acidified sodium nitrite ($NaNO_2$) in coronary small arteries precontracted with acetylcholine (ACh). (a) Relaxations by acidified $NaNO_2$ of coronary segment contracted with acetylcholine in the absence (left) and the presence of 3×10^{-6} M ODQ (right). (b) Relaxations by acidified $NaNO_2$ of coronary segment contracted with acetylcholine in the absence (left) and the presence of 5×10^{-7} M apamin (right). Note the contractions were matched by applying a lower concentration of acetylcholine in the presence of ODQ. W: washout. Horizontal bar shows force and vertical bar, time.

NO added as acidified sodium nitrite caused a significantly smaller relaxation of preparations contracted by 80 mmol/l K^+ than of acetylcholine-contracted preparations (Fig. 7b). Moreover, apamin did not have any additional inhibitory effect on the relaxations caused by NO in coronary segments contracted by 80 mmol/l K^+ (Fig. 7b). L-Nitrosocysteine also caused significantly smaller relaxation in arteries contracted with 80 mmol/l K^+ than in preparations contracted by acetylcholine. Apamin also had no additional effect on the relaxations in response to L-nitrosocysteine in 80 mmol/l K^+-contracted preparations. Thus, L-nitrosocysteine relaxed with pD_2 and maximal values of 6.84 ± 0.20 and $85 \pm 3\%$ the acetylcholine-contracted, 5.91 ± 0.15 ($P < 0.05$, versus control, $n = 6$, Bonferroni t-test) and $78 \pm 2\%$ the 80 mmol/l K^+-contracted, and 6.01 ± 0.13 ($P < 0.05$, versus control, $n = 6$, Bonferroni t-test) and $84 \pm 2\%$ in the presence of 5×10^{-6} M apamin the 80 mmol/l K^+-contracted coronary small arteries.

4. Discussion

The findings of the present study suggest that muscarinic M_1 receptor-released NO can suppress the contractions induced by acetylcholine in lamb coronary small arteries through a mechanism which involves guanylate cyclase activation and opening of small conductance Ca^{2+}-activated K^+ channels.

Acetylcholine only relaxed 5 out of 25 lamb small coronary arteries contracted with U46619, in contrast to what has been reported for the same type of arteries in rat, rabbit and dog (Angus et al., 1991; Myers et al., 1989; Nyborg et al., 1991; Simonsen et al., 1992), where the muscarinic agonist consistently induced dose-dependent relaxation. This discrepancy may be ascribed to a concomitant activation by acetylcholine of contractile muscarinic receptors on the smooth muscle of lamb coronary small arteries, a notion which is supported by the results we now obtained with the muscarinic M_3 receptor antagonist. Thus, HHSiD competitively antagonized the responses to acetylcholine confirming the presence of contractile muscarinic M_3 receptors in lamb coronary small arteries (Simonsen et al., 1993). Endothelial cell removal increased basal tension and induced leftward shifts in the concentration–response curves to acetylcholine, suggesting the release of an endothelium-derived relaxing factor which inhibits the muscarinic M_3 receptor-mediated contractions to acetylcholine. This inhibition could be due to a basal release of NO (Bassenge and Heusch, 1990). However, in endothelium-intact coronary small arteries, incubation with telenzepine in concentrations selective for muscarinic M_1 receptors induced leftward shifts of the concentration–response curves for acetylcholine, indicating the presence of endothelial inhibitory muscarinic M_1 receptors. The density of muscarinic M_1 receptors leading to endothelium-dependent relaxations is low compared to the density of contractile muscarinic M_3 receptors, as indicated by the predominance of contractions in response to acetylcholine. Therefore, the role of the endothelium-derived relaxing factors released either basally or after activation of muscarinic M_1 receptors is probably modulation of the contractile responses to acetylcholine.

4.1. Influence of basally released endothelial NO on the contractions to acetylcholine

The increase in basal tension in lamb coronary resistance arteries after endothelial cell removal suggests basal release of a relaxant factor, as was demonstrated for coronary resistance arteries from rat, rabbit and pig (Nyborg et al., 1991; Tschudi et al., 1991; Simonsen et al., 1992). Inhibition of cyclooxygenase with indomethacin, however, did not alter resting tension, in contrast to the indomethacin-induced contractions in large porcine (Myers et al., 1991) and lamb (Simonsen, unpublished observations) coronary arteries, which indicates the release of a vasodilating prostanoid. Lipoxygenase metabolites are synthesized in the coronary circulation (Piomelli et al., 1987; Rosolowsky et al., 1990) and both the leukotrienes and monohydroxyeicosatetraenoic acids have contractile effects (Piomelli et al., 1987), but a lipoxygenase derivate which causes relaxation has recently been isolated (Pfister et al., 1996). NDGA, considered as a potent inhibitor of the lipoxygenase enzyme at the concentration applied (Förstermann et al., 1988), increased resting tension of lamb coronary resistance arteries, but neither changed the relaxations in response to A23187 nor enhanced the contractions in response to acetylcholine. These results do not exclude the possibility of spontaneous release of a lipoxygenase derivate reducing resting tension of lamb coronary small arteries.

Results of earlier studies have suggested that basally released NO might modulate agonist-induced contractions in the coronary circulation. Thus, L-arginine analogues inhibiting NO synthase and the NO scavenger, oxyhemoglobin, increased the resting tension of isolated porcine coronary small arteries (Tschudi et al., 1991) and coronary resistance after intracoronary infusion (Amezcua et al., 1989; Richard et al., 1991; Hata et al., 1993; Motterlini and McDonald, 1993). In the presence of endothelium, L-NOARG, oxyhemoglobin, methylene blue and ODQ induced contractions, but had no significant contractile effect in endothelium-denuded lamb coronary segments. Interestingly, the potent and specific inhibitor of soluble guanylate cyclase, ODQ, was less effective to increase resting tension than was L-NOARG or methylene blue. L-NOARG is a selective inhibitor of NO synthase, while methylene blue inhibits both guanylate cyclase and NO synthase (Mayer et al., 1993). Inhibition of NO synthase can unmask the production by the endothelium of contractile prostanoids which are involved in the maintenance of vascular resting

tension (Nakaike et al., 1995; Prieto et al., 1995). This might explain the greater effect of L-NOARG and methylene blue than of ODQ on the resting tension of lamb coronary small arteries. Thus, the present results suggest an endothelium-dependent release of NO, increasing the cyclic GMP levels in the underlying smooth muscle and leading to inhibition of basal tension of lamb coronary resistance arteries. On the other hand, neither K^+-channel blockers nor ouabain had an effect on the resting tone, which indicates that hyperpolarization does not appear to play a role in the production of relaxation by basally released NO.

4.2. Muscarinic receptor-mediated inhibition of the contractions to acetylcholine

The increase in acetylcholine-induced contractions of endothelium-intact lamb coronary resistance arteries in the presence of low concentrations of telenzepine indicates that endothelial muscarinic M_1 receptors are activated by acetylcholine and counteract the contractile response elicited by activation of muscarinic M_3 receptors in the smooth muscle. In the presence of telenzepine, addition of L-NOARG did not further increase the contractions with acetylcholine, suggesting that a NO-containing compound formed by NO synthase is released on activation of muscarinic M_1 receptors and mediates the endothelium-dependent inhibitory effect on the contractile response to acetylcholine. In addition, these results indicate that basally released NO is not important for the modulation of acetylcholine-induced contractions, since the muscarinic M_1 receptor antagonist does not influence the basal release of NO. The NO scavenger, oxyhemoglobin, and the inhibitors of NO-sensitive guanylate cyclase, methylene blue (Gruetter et al., 1981) and ODQ (Garthwaite et al., 1995), caused significant leftward shifts in the concentration–response curves for acetylcholine, inhibited the major part of the relaxations induced by A23187 and abolished the relaxations induced by exogenous NO in the same arteries. These results suggest that EDNO mediates the relaxations with A23187 and is released by activation of the endothelial muscarinic M_1 receptors, thus inhibiting the acetylcholine-induced contractions through activation of guanylate cyclase in lamb coronary small arteries.

Several studies have demonstrated a hyperpolarization followed by relaxation in response to endothelium-dependent agonists, including acetylcholine, in large coronary arteries of the guinea-pig (Eckman et al., 1994), pig (Nagao and Vanhoutte, 1992; Hecker et al., 1994) and human (Stork and Cocks, 1994). The endothelium-dependent factor causing hyperpolarization in response to acetylcholine in these studies is not sensitive to the inhibition of NO synthase (Eckman et al., 1994; Hecker et al., 1994; Stork and Cocks, 1994). Several candidates have been suggested, such as nitrosothiols (Myers et al., 1990), arachidonic acid converted by the cytochrome *P*-450 pathway to epoxyeicosatrienoic acids (Hecker et al., 1994; Campell et al., 1996), or C-type natriuretic peptide (Wei et al., 1994). Moreover, recent studies have indicated that EDNO is also capable of inducing hyperpolarization of vascular smooth muscle through activation of K^+ channels through both a cyclic GMP-dependent protein kinase (Khan et al., 1993; Robertson et al., 1993) and a cyclic GMP-independent pathway (Bolotina et al., 1994; Vanheel et al., 1994). Thus, in lamb coronary resistance arteries, the endothelium-derived NO-containing factor released by A23187 or muscarinic M_1 receptor activation might induce hyperpolarization, causing relaxation and inhibition of the acetylcholine-induced contractions, respectively.

Nicorandil, pinacidil and cromakalim, which are believed to relax smooth muscle through activation of ATP-sensitive K^+ channels, relaxed canine and porcine coronary arteries, and these relaxations were abolished in the presence of glibenclamide (Yanagisawa et al., 1990; Satoh et al., 1991). Moreover, NO has been demonstrated to induce hyperpolarization of large guinea-pig coronary arteries through glibenclamide-sensitive channels, although the relaxations caused by NO were unaltered in the presence of this blocker (Parkington et al., 1995). In the lamb coronary resistance arteries, glibenclamide did not affect either the relaxations with A23187 or the contractions with acetylcholine, at a concentration shown to be effective in canine coronary arteries (Satoh et al., 1991). This indicates that ATP-sensitive K^+ channels are not involved in these responses.

The blocker of large-conductance Ca^{2+}-activated K^+ channels, charybdotoxin, has been shown to inhibit part of the relaxation and hyperpolarization caused by exogenously added NO and acetylcholine in rabbit aortic rings (Bolotina et al., 1994), and NO donors were shown to increase the conductance of Ca^{2+}-activated K^+ channels in rabbit coronary artery myocytes (George and Shibata, 1995). In lamb coronary small arteries, charybdotoxin did not alter the responses to either acetylcholine or A23187, excluding a role of large Ca^{2+}-activated K^+ channels in these responses. In contrast, the blocker of small-conductance Ca^{2+}-activated K^+ channels, apamin, caused leftward shifts in the concentration–response curves for acetylcholine and significantly inhibited the relaxations with A23187. The K^+-channel blockade by apamin could depolarize the endothelial cells, leading to reduced formation and/or release of the L-NOARG-sensitive factor (Groschner et al., 1992). However, in the present study, apamin also inhibited the relaxations in response to the two NO donors, NO added as acidified sodium nitrite and L-nitrosocysteine, indicating that small-conductance Ca^{2+}-activated K^+ channels in the smooth muscle cells may mediate the relaxations in response to NO. The concentration of apamin applied was above the concentration required to block small Ca^{2+}-activated K^+ channels (Gebremedhin et al., 1996). However, elimination of the K^+ gradient by an increase in the external K^+ concentra-

tion to 80 mM also inhibited the relaxations caused by the NO donors, and under these conditions apamin caused no further inhibition. This supports the possibility that apamin inhibits the relaxations induced by the NO donors through a selective action on K^+ channels. Therefore, our results suggest that the L-NOARG-sensitive factor released by either A23187 or by stimulation of the endothelial muscarinic M_1 receptors produces hyperpolarization through the opening of apamin-sensitive K^+ channels in the smooth muscle and relaxation or inhibition of the contractile response to acetylcholine, respectively, in lamb coronary resistance arteries. Moreover, the fact that combined treatment with apamin and the inhibitor of guanylate cyclase, ODQ, did not additionally enhance contractions induced by acetylcholine nor additionally inhibited the relaxations with A23187 or NO, compared to those in the presence of either blocker alone, suggests that activation of small-conductance K^+ channels is exerted through a guanylate cyclase-dependent mechanism.

It has been suggested that, in guinea-pig coronary arteries, where acetylcholine-induced endothelium-dependent relaxation is only partially sensitive to inhibition by L-arginine analogues, activation of muscarinic M_3 receptors releases both EDNO and EDHF (Eckman et al., 1994; Hammarström et al., 1995). In contrast, in the rabbit saphenous artery, activation of muscarinic M_1 receptors was shown to induce hyperpolarization, while stimulation of muscarinic M_3 receptors results in release of EDNO (Komori and Suzuki, 1987). Muscarinic M_1 receptors also mediate the relaxation in response to acetylcholine in rat coronary small arteries (Simonsen et al., 1996) and vasodilation in the dog coronary circulation (Pelc et al., 1988). The present results further support the possibility of heterogeneity in both muscarinic receptor subtypes and endothelial mediators involved in the response to acetylcholine along the coronary vascular tree.

In summary, the present results indicate that basally released NO, through activation of guanylate cyclase, reduces resting tension, while muscarinic M_1 receptor-released endothelial NO or a NO-related compound inhibits the contractile responses to acetylcholine in lamb coronary small arteries through activation of guanylate cyclase, followed by an increase in the apamin-sensitive K^+ conductance of the smooth muscle.

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