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Involvement of ATP in the non-adrenergic non-cholinergic inhibitory neurotransmission of lamb isolated coronary small arteries

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1 The involvement of non-adrenergic non-cholinergic (NANC) transmitters, such as nitric oxide (NO) and adenosine 5′-triphosphate (ATP), in the neurogenic relaxation of lamb coronary small arteries was investigated in vessel segments with an internal lumen diameter of 200–550 μm, isolated from the left ventricle of the heart, and suspended for isometric tension recording in microvascular myographs.

2 In both endothelium-intact and -denuded coronary small arteries treated with phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M), and atropine (10^{-6} M) and contracted to 3×10^{-7} M of the thromboxane analogue U46619, electrical field stimulation (EFS) evoked frequency-dependent relaxations, which were markedly reduced in the presence of tetrodotoxin (10^{-6} M).

3 Exogenous NO added as acidified sodium nitrite (10^{-6}–10^{-3} M) and L-nitrosocysteine induced potent relaxations of lamb coronary small arteries. However, both inhibition of NO synthase with N^G-nitro-L-arginine (L-NOARG, 3×10^{-5} M), and mechanical endothelial cell removal increased rather than inhibited relaxations to EFS. In small arteries processed for NADPH-diaphorase histochemistry, activity was only observed within endothelial cells.

4 In arteries contracted to U46619, exogenously added ATP caused concentration-dependent relaxations with pD_2 and maximum responses of 4.72 ± 0.12 and $89.6\pm3.8\%$ ($n=12$), respectively. ADP and the P_{2Y}-agonist, 2-methylthio-ATP, induced relaxations equipotent to ATP, while the P_{2X}-agonist, α, β-methylene ATP (10^{-9}–10^{-4} M), and the P_{2U}-agonist, UTP (10^{-9}–10^{-4} M) only caused small transient relaxations at the highest concentrations (10^{-4} and 10^{-3} M).

5 ATP and EFS-induced relaxations were unchanged in the presence of the P_1-purinoceptor antagonist, 8-phenyltheophylline (10^{-5} M), while this antagonist inhibited the concentration-dependent relaxations to adenosine. In contrast, the P_2-purinoceptor antagonist, suramin (3×10^{-5} M), markedly reduced the relaxations to EFS.

6 After desensitization of P_{2X}-purinoceptors with α, β-methylene ATP (2×10^{-5} M), the relaxations to exogenous added ATP were enhanced, but this procedure did not influence the relaxations to EFS. In contrast, the P_{2Y}-purinoceptor antagonist, basilen blue E-3G (3×10^{-5} M, earlier named reactive blue 2) significantly inhibited the concentration-relaxation curves to ATP and almost abolished the EFS-induced relaxations.

7 Mechanical removal of the endothelium significantly inhibited ATP-induced maximal relaxations without affecting sensitivity, pD_2 and maximum relaxations being 4.72 ± 0.12 and $89.7\pm3.8\%$ ($n=10$), and 5.45 ± 0.38 and $48.0\pm8.6\%$ ($P<0.05$, paired t test, $n=10$) in endothelium-intact and -denuded coronary small arteries, respectively. However, incubation with L-NOARG did not change relaxations elicited by ATP.

8 The present study suggests that in NANC conditions neurogenic relaxations of coronary small arteries are mediated by ATP, which relaxes coronary small arteries through P_{2Y}-purinoceptors. A prejunctional modulation of these relaxations by endothelial-derived NO cannot be excluded.

Keywords: ATP; coronary small arteries; electrical field stimulation; endothelium; nitric oxide

Introduction

Besides metabolic and humoral factors, neural mechanisms play a role in the control of coronary blood flow through the release of noradrenaline and acetylcholine from sympathetic and parasympathetic nerve terminals, respectively (Young *et al*., 1987; Bassenge & Heusch, 1990). In addition to these classical transmitters, several peptides such as neuropeptide Y, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and tachykinins have been demonstrated to be present in nerve fibres around coronary arteries and to influence coronary blood flow (Franco-Cereceda, 1988; Gulbenkian *et al*., 1993; Yoita *et al*., 1994). Despite the fact that non-adrenergic non-cholinergic (NANC) nerves containing NO or ATP have been proposed to regulate the blood flow in certain vascular beds (Burnstock, 1990; Burnett *et al*., 1992), little information is available concerning the role of such nerves in the coronary circulation. NO was first identified as endothelium-derived relaxing factor (EDRF) and later proposed as a putative NANC inhibitory neurotransmitter in both central and peripheral neurones including those involved in the control of cardiovascular function (Bredt *et al*., 1990; Bredt & Snyder, 1992). Nitrergic perivascular nerves have been demonstrated by either immunohistochemical localization of NO synthase (NOS) or NADPH-diaphorase (NADPH-d) histochemistry (Bredt *et al*., 1990; Burnett *et al*., 1992), and blockers of NOS reduced inhibitory neurotransmission in cerebral (Toda & Okarmura, 1992), mesenteric (Ahlner *et al*., 1991) and penile arteries (Simonsen *et al*., 1995), thus providing evidence for a functional nitrergic innervation of these

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vascular beds. However, such experiments have not been performed yet in coronary small arteries.

Adenosine 5′-triphosphate (ATP) is now considered to be a neurotransmitter in both neural (Evans *et al*., 1993; Edwards *et al*., 1993) and vascular preparations (White, 1988; Burnstock, 1990). Both neurally released and exogenously applied ATP elicits vasoconstriction through activation of P_{2X}-purinoceptors in systemic arteries (Evans & Cunnane, 1992; Evans & Kennedy, 1994), but it induces vasodilatation through P_{2Y}-purinoceptors in the portal vein and pulmonary arteries (Liu *et al*., 1992; Brizzolara *et al*., 1993). Exogenously added ATP relaxes isolated proximal coronary arteries from guinea-pig and rabbits (Corr & Burnstock, 1991; 1994; Keef *et al*., 1992) and induces vasodilatation in rat perfused hearts (Hopwood & Burnstock, 1987) through activation of P_{2Y}-purinoceptors. A less potent vasoconstriction to ATP mediated by P_{2X}-purinoceptors has also been observed in the coronary circulation (Hopwood & Burnstock, 1987; Keef *et al*., 1992; Corr & Burnstock, 1994). Moreover, ATP has been detected in the effluent perfusate of isolated hearts (Paddle & Burnstock, 1974; Vial *et al*., 1987; Borst & Schrader, 1991) and is increased during nerve and β-adrenoceptor stimulation (Fredholm *et al*., 1982; Borst & Schrader, 1991), ischaemia and hypoxia (Paddle & Burnstock, 1974; Borst & Schrader, 1991), and in response to increased flow (Vials & Burnstock, 1996). ATP-release has also been measured in cell cultures of cardiomyocytes (Forrester & Williams, 1977), vascular smooth muscle and endothelial cells (Pearson & Gordon, 1979; Yang *et al*., 1994). However, the origin of ATP in whole hearts is difficult to evaluate due to the several sources, and despite the fact that vascular purinoceptors mediating responses to ATP have been characterized in the coronary circulation. Hence, a role for ATP in the neural control of coronary arteries has not been assessed.

In the present study, we have first revealed a NANC relaxation to electrical field stimulation (EFS) in coronary small arteries isolated from the lamb. The involvement of NO in this NANC inhibitory neurotransmission was evaluated by means of the NOS inhibitor, N^G-nitro-L-arginine (L-NOARG), and the histochemical NADPH-d reaction to detect NOS activity (Hope & Vincent, 1989). Second, a role for ATP in the NANC relaxation of the coronary small arteries, was assessed by the use of selective ligands for the P_1-purinoceptors, 8-phenyltheophylline (8-PT), P_{2X}, α, β-methylene ATP and P_{2Y}-purinoceptors, basilen blue E-3 (earlier reactive blue 2, Burnstock & Warland, 1987). Their effects on responses to both EFS and purinoceptor agonists were investigated.

Methods

Tissue preparation

Hearts from 3–6 month old male lambs were obtained at the local slaughterhouse. They were excised immediately after death 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 (mM): 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 pH at 7.4.

Dissection and mounting

Third to fourth order small subepicardial arteries of the left anterior descending coronary artery (LAD) of the left ventricle were dissected as previously described (Simonsen *et al*., 1993). Segments (ca. 2 mm long) of the coronary small vessels were mounted as ring preparations on two 40 μm wires on an isometric double myograph (JP Trading, Aarhus, Denmark) by fixing one of the wires to a force transducer and the second wire to a length displacement device. 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 the internal circumference, L_{100}, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ* was calculated (Mulvany & Halpern, 1977). The vessels were set to the internal circumference L_1, given by $L_1 = 0.9 \times L_{100}$. The force development is close to maximal at this internal circumference (Simonsen *et al*., 1993). The effective internal lumen diameter was determined as $1_1 = L_1/\pi$.

The presence or absence of the endothelial cell layer was evaluated by inducing a stable contraction with 3×10^{-7} M U46619 and then adding either 3×10^{-6} M of the calcium-ionophore, A23187, or 3×10^{-8} M bradykinin. Relaxation greater than 50% was taken as evidence of endothelial integrity, while total inhibition of relaxation to the calcium-ionophore or bradykinin was indicative of successful mechanical removal of the endothelial cells. Vessels supposed to have intact endothelium, but which relaxed less than 50% were discarded.

Electrical field stimulation

For electrical field stimulation (EFS), segments of coronary small arteries were mounted in a myograph as described previously, between two platinum electrodes approximately 2 mm apart from each other. The preparations were stimulated transmurally by trains with 0.3 ms square pulses applied at frequencies of 0.5–32 Hz for a period of 20 s with a Cibertec CS20 stimulator (Letica, Barcelona, Spain), with constant current output adjusted to 35 mA.

In order to study relaxations to EFS, a contraction was induced with 3×10^{-7} M U46619 and when a stable plateau was reached, a first control frequency-response curve was obtained, the preparations were washed and equilibrated for at least 45 min. In some experiments the myograph chamber was separated to obtain parallel control frequency-response curves. Phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M), and atropine (10^{-6} M) were kept present throughout the experiment. A first control curve to EFS was obtained, and after wash and equilibration the vessels were treated with either L-NOARG, tetrodotoxin (10^{-6} M), 8-phenyltheophylline (8-PT; 10^{-5} M), the P_2-purinoceptor antagonist, suramin (3×10^{-5} M) for 30 min, or basilen blue E-3G (3×10^{-5} M, earlier named reactive blue 2) for 20 min, before a second frequency-response curve was performed. The desensitization with α, β-methylene ATP (α,β-meATP) was performed as described previously (Corr & Burnstock, 1994), by stimulating for 10 min with 10^{-5} M α, β-methylene ATP and then repeating the stimulation until the vessel did not respond to it; this was usually obtained with the second stimulation. The effect of different blockers on the relaxations to exogenously added ATP (10^{-8}–10^{-3} M) was examined by constructing concentration-response curves in a similar manner. In addition, the effect of suramin (3×10^{-5} M) on the relaxations to ATP in vessels which had been desensitized with α,β-meATP was examined.

Histochemistry

Coronary small arteries either isolated from the heart by dissection or blocks of myocardium were immersed in ice-cold (4°C) 4% paraformaldehyde with 0.1% glutaraldehyde in phosphate buffer (PB) 0.1 M, pH 7.2. After a 24 h fixation period, samples were kept in a cryoprotective solution of 15% sucrose in 0.1 M PB overnight. Cross and longitudinal sections (30 μm) were obtained with a cryostat and processed for NADPH diaphorase histochemistry following the protocol by Hope and Vincent (1989). The preparations were incubated in 0.1 M PB, p.H 8.0, containing 1 mM β-NADPH, 0.5 mM nitroblue tetrazolium and 0.3% Triton X-100 for 30–45 min at 37°C and protected from light. Sections were rinsed in PB, dehydrated and mounted on to poly-L-lysine covered slides for light microscopic examination.

Drugs

Adenosine and ATP (sodium salt) were purchased from ICN Biochemicals (Barcelona, Spain); atropine α, β-methylene ATP, ADP, basilen blue E-3G (earlier reactive blue 2, bradykinin, calcium-ionophore (A23187) 9,11-dideoxy-11α, 9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), N^G-nitro-L-arginine (L-NOARG), 8-phenyltheophylline (8-PT), phentolamine HCl, (±)-propranolol HCl and uridine 5′-triphosphate (UTP) were from Sigma (St. Louis, Mo, U.S.A.); 2-methylthioadenosine 5′ triphosphate (2-MeSATP) and suramin were from Research Biochemicals Incorporated (MA, USA.). Most drugs were prepared daily in distilled water, and the purinoceptor agonists were kept on ice. Dilutions of the calcium-ionophore, A23187, were prepared as a 10^{-2} M stock solution in dimethylsulphoxide and further diluted in PSS. Previous experiments showed that the solvents had no effect on the preparations at the concentrations applied. Concentrations of drugs were expressed as the final organ chamber concentrations (M).

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 (Mulvany & Halpern, 1977). By use of a computer programme (GraphPad, Institute for Scientific Information, San Diego, California, U.S.A.), the concentration-response curves to ATP were fitted to the classical 'Hill-equation': $R/R_{max} = A(M)^{n_H}/A(M)^{n_H} + EC_{50}(M)^{n_H})$, 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 half maximal vessel response (R_{max}), when A(M), and $EC_{50}(M)$ are given in molar concentration; n_H is a curve fitting parameter or Hill-coefficient. Similarly, the frequency-response curves were fitted with the same programme and the EF_{50} values, which are the stimulus strength producing a 50% relaxation of the maximum relaxation reached, were determined. The relaxation responses were normalized to the initial tone in the vessel induced with U46619, and presented on a semi-logarithmic scale.

The results are expressed as means ± s.e.mean, where *n* represents the number of animals studied in each experiment. The frequency – or concentration-response curves before and after treatment were compared by analysis of variance (ANOVA) for repeated measures, and by paired *t* test for comparisons of the individual concentrations or frequencies. Probability levels under 5% were considered significant. NS indicates no significant difference.

Results

Responses to electrical field stimulation

U46619 (3×10^{-7} M) induced sustained contractions, and in the presence of atropine (10^{-6} M), phentolamine (3×10^{-6} M) and propranolol (3×10^{-6} M) to inhibit muscarinic receptors and adrenoceptors, respectively, EFS (0.3 ms, 20 second trains, supramaximal current) caused frequency-dependent (0.5 – 32 Hz) relaxations of lamb coronary small arteries which reached a maximum in 20 – 23 s with an $EF_{50} = 2.5 \pm 0.3$ Hz and maximum relaxations 55.6 ± 3.5% of the precontraction ($n = 35$, Figure 1a). The EFS-elicited relaxations were reproducible, EF_{50} and maximum relaxations being 2.5 ± 0.4 Hz and 59.9 ± 8.9%, and 2.5 ± 0.6 Hz and 54.7 ± 9.1% ($n = 6$) in a first and second frequency-response curve, respectively. The frequency-dependent relaxations to EFS were markedly reduced in the presence of 10^{-6} M tetrodotoxin, although relaxations still persisted at 16 and 32 Hz, the maximum relaxation being 28.3 ± 3.6% ($n = 7$) (Figure 1b and 2). In fresh preparations contracted to 1.6 ± 0.2 Nm^{-1} by U46619, EFS induced relaxations with EF_{50} and maximum of 0.8 ± 0.2 Hz and 79.5 ± 8.8% ($n = 4$), respectively, while after 8 h cold storage (4°C) U46619 contracted the preparations from the same animals to 1.3 ± 0.4 Nm^{-1}, but the maximum relaxations to EFS were reduced to 15.0 ± 1.2% ($P < 0.05$, Student's *t* test, $n = 4$). Papaverine (10^{-4} M) relaxed the latter preparations to 75.0 ± 5.9% ($n = 4$).

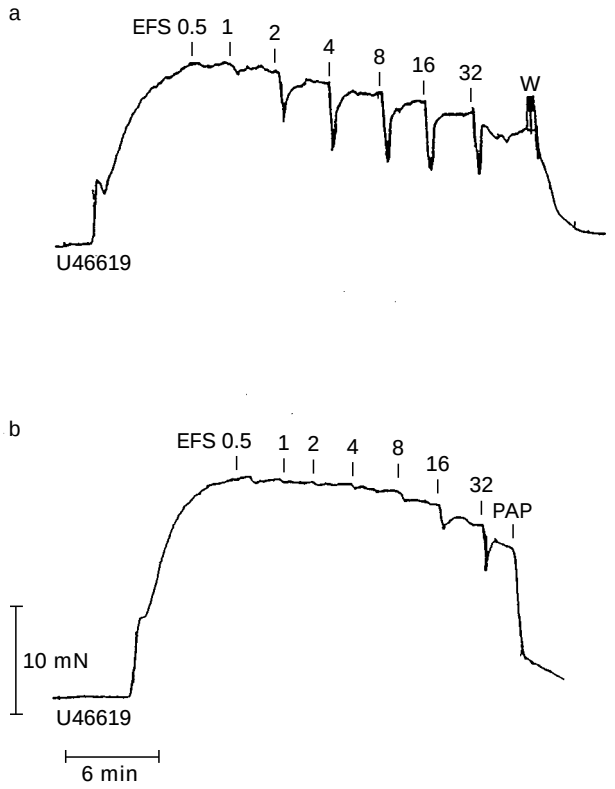

Figure 1 Isometric tension recordings of lamb coronary small arteries contracted to the thromboxane analogue, U46619 (10^{-7} M) in the presence of phentolamine (3×10^{-5} M), propranolol (3×10^{-6} M) and atropine (10^{-6} M) to block adrenergic and muscarinic neurotransmission, respectively, and stimulated with increasing frequencies (0.5 – 32 Hz) of electrical field stimulation (EFS, 0.3 ms, 20 second trains, constant current adjusted to 35 mA) in (a) control conditions, and (b) in the presence of tetrodotoxin (10^{-6} M). W: wash out, PAP: addition of papaverine (10^{-4} M). Vertical bar represents force (mN) and horizontal bar time (min).

Effects of endothelial cell removal and L-NOARG on responses to EFS

The contractions to U46619 were matched to reach the same level, and were 2.5 ± 0.7 Nm^{-1} and 3.0 ± 0.4 Nm^{-1} ($n = 5$) in endothelium-intact and denuded coronary small arteries, respectively. Endothelial cell removal reduced relaxations to bradykinin (3×10^{-8} M) which relaxed by 73.8 ± 6.9 and 4.8 ± 2.8% ($n = 5$, $P < 0.05$, paired *t* test) endothelium-intact and denuded lamb coronary small arteries, respectively, while papaverine (10^{-4} M) was still able to relax endothelium-denuded preparations by 98.6 ± 5.2% ($n = 5$). Mechanical endothelial cell removal increased, rather than inhibited, the relaxations to EFS especially at low frequencies (0.5 – 8 HZ) (Figure 2a, Table 1). The maximum relaxations elicited by EFS were also increased in the presence of L-NOARG (3×10^{-5} M) (Figure 2a, Table 1).

Exogenous NO, added either as acidified sodium nitrite ($10^{-6} - 10^{-3}$ M) or L-nitrosocysteine ($10^{-8} - 3 \times 10^{-5}$ M) induced potent relaxations with pD_2-values and maximum relaxations of 4.61 ± 0.17 and 88.0 ± 3.0% ($n = 5$), and 6.48 ± 0.14 and 87.6 ± 4.0% ($n = 6$), respectively, in lamb coronary small arteries (Figure 2b).

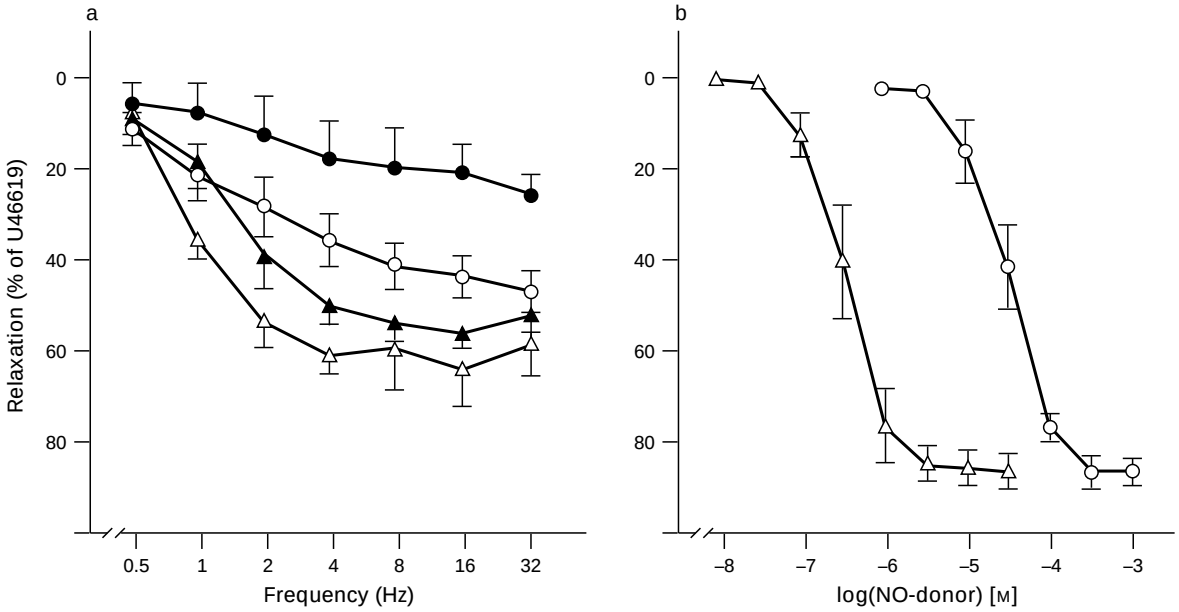

Figure 2 Average relaxations in lamb coronary small arteries contracted to the thromboxane analogue U46619. (a) Relaxations to electrical field stimulation (EFS) with increasing frequency in endothelium-intact (○) and denuded arteries (△), and in endothelium-intact segments in the presence of 3×10^{-5} M N^G-nitro-L-arginine (L-NOARG, ▲) or 10^{-6} M tetrodotoxin (●). (b) Relaxations to nitric oxide (NO) added as acidified sodium nitrite (○) or L-nitrosocysteine (△). The experiments were performed in the presence of 10^{-6} M atropine, 3×10^{-6} M phentolamine and 3×10^{-6} M propranolol. Relaxations are expressed as percentage of U46619-induced contractions. Points represent means and vertical lines s.e.mean from 5–6 vessel segments.

Table 1 Relaxations to electrical field stimulation in lamb coronary small arteries contracted to U46619 (10^{-7} M) in a first control curve, and a second curve obtained in the presence of, N^G-nitro-L-arginine (3×10^{-5} M, L-NOARG) or after mechanical endothelial cell removal (−E)

	n	*U46619* (Nm^{-1})	EF_{50} (Hz)	*Maximum relax.* (%)	l_0 (μm)
+E	5	2.5±0.7	3.1±0.7	50.8±6.6	440±58
−E	5	3.0±0.4	0.9±0.1*	65.4±8.0	440±58
Control	6	2.5±0.7	3.1±0.6	35.9±7.6	344±28
L-NOARG	6	2.8±0.4	1.8±0.4	58.0±2.5*	344±28

Values are mean±s.e.mean; *n*, indicates number of vessels examined. EF_{50} is the frequency required to produce half maximal relaxation. The maximum relaxations are expressed as percentage of the contractions induced by U46619. l_0, is the normalized internal diameter of the coronary arterial segment at which the experiments were performed. The experiments were performed in the presence of phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M) and atropine (10^{-6} M). Significantly different from same parameter obtained in a first control curve (paired *t* test): $^*P<0.05$.

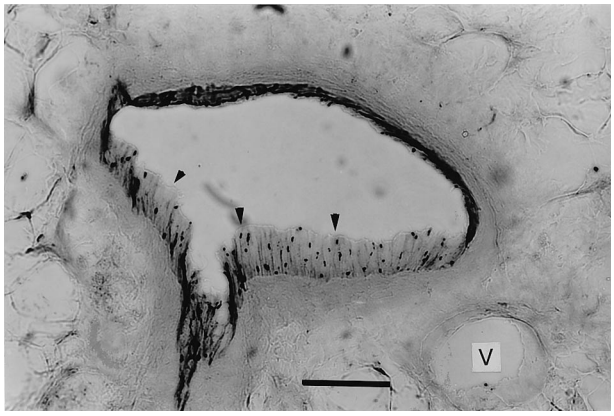

Figure 3 NADPH-d histochemistry performed in a section of lamb coronary small artery from the left ventricle. NADPH-diaphorase (NADPH-d) activity is observed within endothelial cells as particulate precipitations (arrows) in the artery, but not in the venule (V). No reaction is seen in the adventitia or media of the artery. Bar = 100 μm.

NADPH-d histochemistry

NADPH-diaphorase (NADPH-d) activity was observed as a blue reaction product within endothelial cells of both large and small coronary arteries, while in the endothelium of venules there was no positive reaction (Figure 3). In contrast to the strong activity observed in the intima, no reaction was found in either the adventitia or media of the coronary small arteries. Penile small arteries were used as positive controls for NADPH-d activity in nerve fibres (Simonsen *et al*., 1995).

Effect of ATP and purinoceptor agonists

ATP (10^{-7}–10^{-3} M) induced reproducible concentration-dependent relaxations of lamb coronary small arteries with $pD_2=4.72\pm0.12$ ($n=12$) and maximum relaxations of 89.6±3.8% ($n=12$) of the U46619-induced contraction. Transient contractile responses to ATP were observed only at the highest concentrations (10^{-4}–10^{-3} M). ADP and 2-me-SATP induced concentration-dependent relaxations of lamb coronary arteries contracted to U46619 with pD_2 values of 4.44±0.12 ($n=3$) and 4.57±0.19 ($n=6$), respectively, and maximum relaxations of 85±3.7 ($n=3$) and 70.6±5.4 ($n=6$), respectively. In contrast, the P_{2X}-selective agonist, α,β-meATP (10^{-9}–10^{-4} M, $n=4$), and the P_{2U}-selective agonist, UTP (10^{-9}–10^{-3} M, $n=4$) caused only small transient relaxations at the highest concentrations (10^{-4} M and 10^{-3} M).

Effect of 8-phenyltheophylline and suramin on the relaxations to EFS

The P_1-purinoceptor blocker 8-PT (10^{-5} M) did not influence the relaxations elicited by either EFS or exogenously added ATP, but this treatment inhibited relaxations to adenosine

(Figure 4). Thus, adenosine relaxed coronary small arteries contracted to U46619 with pD_2-values and maximum relaxations of 5.36 ± 0.19 and $81.5 \pm 4.3\%$ and 4.61 ± 0.32 ($P < 0.05$, $n = 6$, paired t test) and $63.5 \pm 11.8\%$ (NS, $n = 6$) in the absence and presence of 8-PT, respectively. In contrast, the P_2-purinoceptor antagonist, suramin (3×10^{-5} M), significantly reduced the relaxations to EFS (Table 2). The effect of suramin (3×10^{-5} M) on the relaxations of lamb coronary arteries by exogenously added ATP was variable ($n = 5$), but when the vessels were first desensitized with α, β-meATP (2×10^{-5} M, see below), suramin inhibited both the sensitivity and maximum relaxations obtained with ATP. Thus, coronary small arteries were relaxed with $pD_2 = 5.75 \pm 0.13$ ($n = 6$) and maximum relaxations of $91.0 \pm 4.4\%$ ($n = 6$) after treatment with α, β-meATP, and with $pD_2 = 5.34 \pm 0.05$ ($P < 0.05$, $n = 7$, Student's t test) and maximum relaxations of $53.3 \pm 6.5\%$ ($P < 0.05$, $n = 7$, Student's t test) after treatment with α, β-meATP and in the presence of suramin (3×10^{-5} M).

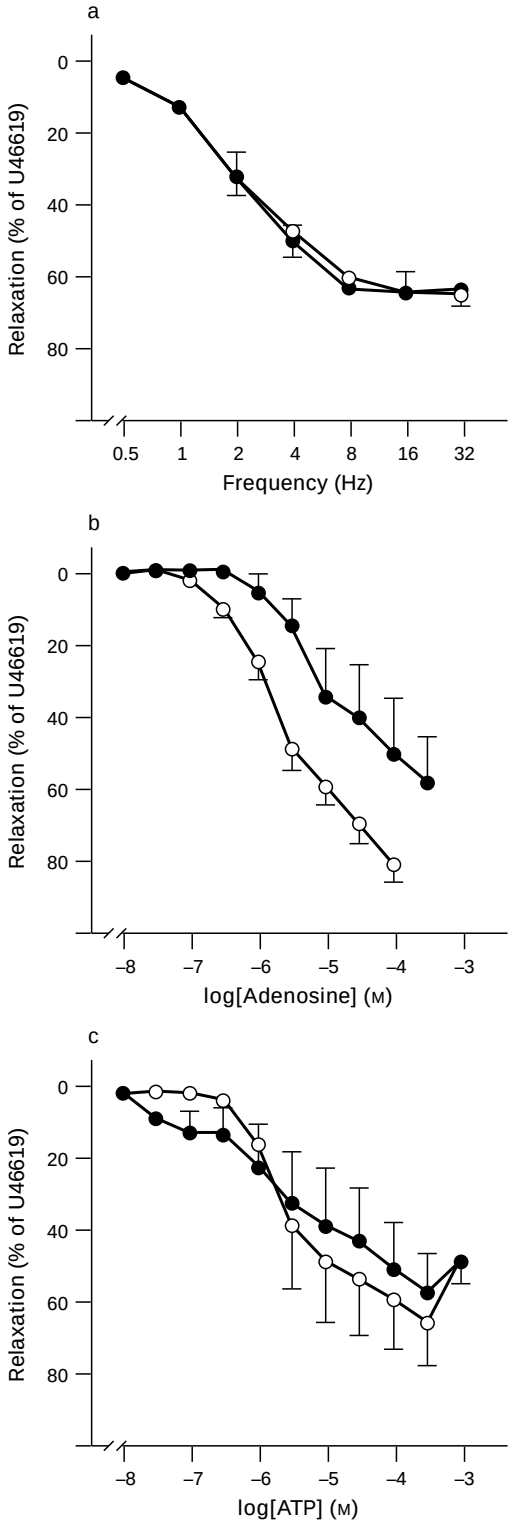

Figure 4 Average effect of the P_1-receptor antagonist, 8-phenyltheophylline, on the relaxations to (a) electrical field stimulation, (b) adenosine, and (c) ATP in lamb coronary small arteries. Responses obtained in the absence (○) and presence (●) of 10^{-5} M 8-phenyltheophylline. The experiments were performed in the presence of 10^{-6} M atropine, 3×10^{-6} M phentolamine and 3×10^{-6} M propranolol. Points represent means and vertical lines s.e.mean from 5–6 vessel segments.

Effects of α, β-methylene ATP and basilen blue on the responses to EFS and ATP

The P_{2X}-ligand, α, β-meATP (10^{-5} M) induced a small contraction (0.05 ± 0.02 Nm^{-1}) when added at resting tension. After 10 min no contractile or relaxant effect to α, β-methylene ATP was observed, when a second dose giving a final concentration of 2×10^{-5} M was added to an artery preconstricted with U46619. Desensitization with 2×10^{-5} M α, β-meATP did not influence the relaxations elicited by EFS (Figures 5 and 6), but it caused significant leftwards shifts of the relaxation curves to exogenous ATP, pD_2-values and maximum responses being 4.67 ± 0.17 and $88.0 \pm 6.8\%$ before, and 5.53 ± 0.22 ($P < 0.05$, $n = 6$, paired t test) and $93.4 \pm 4.6\%$ (NS, $n = 6$) after treatment with α, β-meATP.

The P_{2Y}-purinoceptor antagonist, basilen blue E-3G (earlier named reactive blue 2) at a concentration of 3×10^{-5} M did not alter either the resting tension of lamb coronary small arteries or the contractile responses to U46619 (3×10^{-7} M) (Table 2). Treatment of lamb coronary small arteries with 3×10^{-5} M basilen blue abolished the relaxations to EFS at low frequencies (0.5–8 Hz), and caused a $77.3 \pm 4.9\%$ ($n = 6$) inhibition of the maximum response (Figure 6a, Table 2). In addition, the relaxations to exogenously added ATP were reduced by basilen blue (Figure 6b), pD_2-values and maximum relaxations being 4.77 ± 0.19 and 91.3 ± 3.3 before, and 3.09 ± 0.09 ($P < 0.01$, $n = 6$, paired t test) and $49.3 \pm 5.0\%$ ($P < 0.05$, $n = 6$, paired t test) after incubation, respectively, with basilen blue. Basilen blue also inhibited the relaxations to the P_{2Y}-agonist, 2-MeSATP. Thus, 2-MeSATP relaxed coronary small arteries with $pD_2 = 4.63 \pm 0.20$ and maximum re-

Table 2 Effect of the purinoceptor blockers 8-phenyltheophylline (8-PT, 10^{-5} M), suramin (3×10^{-5} M), α, β-methylene ATP (α, β-meATP, 2×10^{-5} M) and basilen blue (BB, 3×10^{-5} M) on relaxations to electrical field stimulation (EFS) in U46619-contracted lamb coronary small arteries

	n	*U46619* (Nm^{-1})	EF_{50} (Hz)	*Maximum relax.* (%)	l_0 (μm)
Control	5	1.8 ± 0.7	2.4 ± 0.6	66.4 ± 2.9	379 ± 33
8-PT	5	2.0 ± 0.7	2.1 ± 0.3	65.3 ± 5.8	379 ± 33
Control	5	1.6 ± 0.2	1.9 ± 1.0	61.9 ± 11.0	372 ± 21
Suramin	5	1.3 ± 0.2	16.6 ± 5.3*	22.9 ± 4.8*	372 ± 21
Control	6	1.8 ± 0.3	2.1 ± 0.3	53.0 ± 8.3	495 ± 38
α, β-meATP	6	2.2 ± 0.5	1.9 ± 0.3	56.8 ± 4.7	495 ± 38
Control	6	1.7 ± 0.3	2.4 ± 0.5	66.9 ± 6.3	437 ± 32
BB	6	1.8 ± 0.4	–	15.0 ± 3.3*	437 ± 32

Values are mean ± s.e.mean; n, indicates number of vessels examined. EF_{50} is the frequency required to produce half maximal relaxation. The maximal relaxations are expressed as percentage of the contractions induced by U46619. l_0, is normalized internal diameter of the coronary arterial segment at which the experiments were performed. The experiments were performed in the presence of phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M) and atropine (10^{-6} M). Significantly different from same parameter obtained in a first control curve (paired t test): *$P < 0.05$.

laxations of 75.0±4.7% in the absence, and relaxations obtained to the highest concentration of 2-MeSATP applied (3×10^{-4} M) were 7.5±4.7% ($P<0.05$, $n=3$, paired t test) in the presence of 3×10^{-5} M basilen blue. The endothelium-dependent relaxations to 10^{-7} M bradykinin (73.1±7.93%, $n=6$) were unchanged by basilen blue.

Effects of endothelial cell removal and L-NOARG on relaxations to ATP

In order to determine whether ATP-elicited relaxations are endothelium-dependent the effect of mechanical endothelial cell removal and NOS blockade were examined on ATP-elicited relaxations of lamb coronary small arteries. The contractions to U46619 were matched to reach the same level, and were 2.2±0.2 Nm^{-1} and 1.7±0.2 Nm^{-1} ($n=10$) in endothelium-intact and denuded coronary small arteries, respectively. In preparations where mechanical endothelial cell removal reduced relaxations to 3×10^{-8} M bradykinin from 82.5±7.3% to 3.6±1.3% ($n=10$, $P<0.05$, paired t test), the relaxations to ATP were also significantly reduced (Figure 7a). Thus, pD_2-values and maximum relaxations for ATP were 4.70±0.16 and 81.4±5.6% ($n=10$) and 5.45±0.38 (NS, $n=10$) and 48.0±8.6% ($P<0.05$, $n=10$, paired t test) in en-

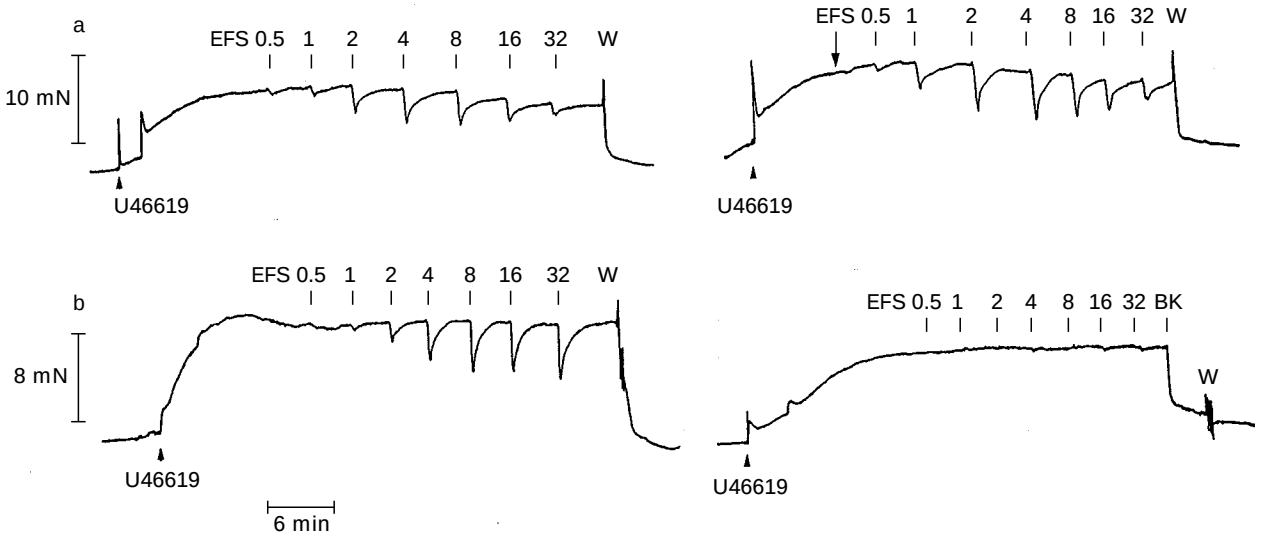

Figure 5 Isometric force recordings showing the effects of (a) the P_{2X}-receptor antagonist, α, β-methylene ATP and (b) the P_{2Y}-receptor antagonist, basilen blue E-3G, on the relaxant responses elicited by electrical field stimulation (EFS) in lamb coronary small arteries. After a first control stimulus-response curve (left panel), the arteries were thoroughly washed in physiological salt solution (PSS) for 45 min, and were (a) desensitized by stimulation twice with 10^{-5} M α, β-methylene ATP (second time, ↓), or (b) incubated for 30 min with 3×10^{-5} M basilen blue E-3G before the construction of a second curve. The experiments were performed in the presence of 10^{-6} M atropine, 3×10^{-6} M phentolamine and 3×10^{-6} M propranolol. W: wash out, BK: 10^{-7} M bradykinin. Vertical bar represents force (mN) and horizontal bar time (min).

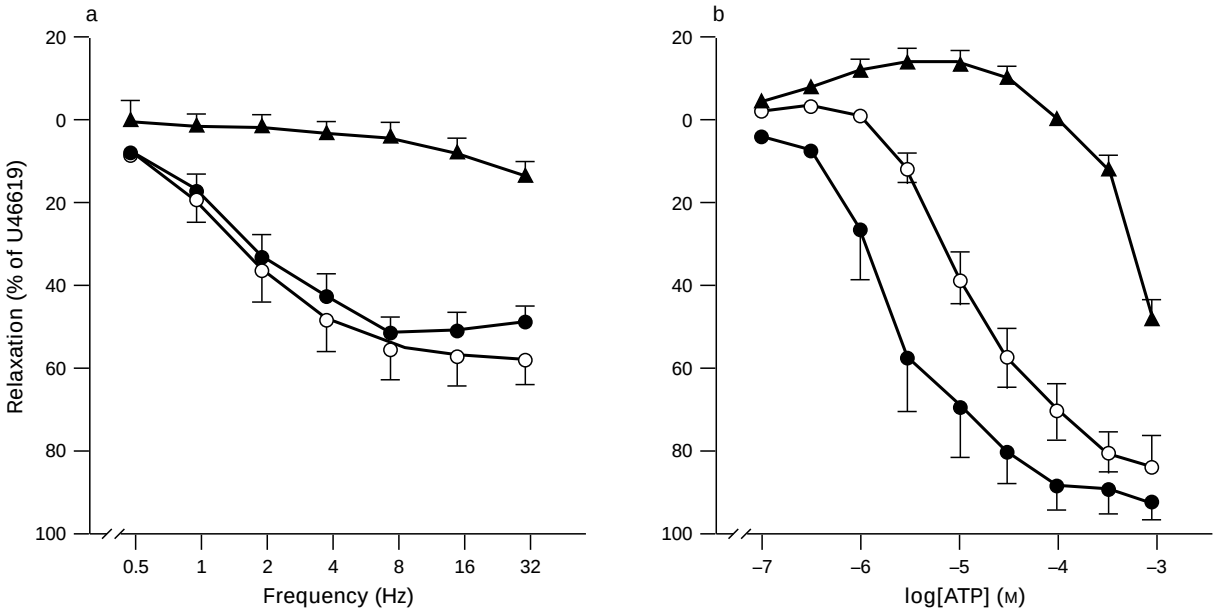

Figure 6 Effects of α, β-methylene ATP and basilen blue on (a) frequency-response curves to electrical field stimulation, and (b) concentration-response curves to exogenously added ATP in lamb coronary small arteries. Control stimulus-response curves (○) and after treatment with either (2×10^{-5} M) α, β-methylene ATP (●) or (3×10^{-5} M) basilen blue (▲). The experiments were performed in the presence of atropine (10^{-6} M), phentolamine (3×10^{-6} M) and propranolol (3×10^{-6} M). Responses are expressed as percentage of the preconstriction elicited by 3×10^{-7} M U46619. Points represent means and vertical lines s.e.mean of 6–12 arteries.

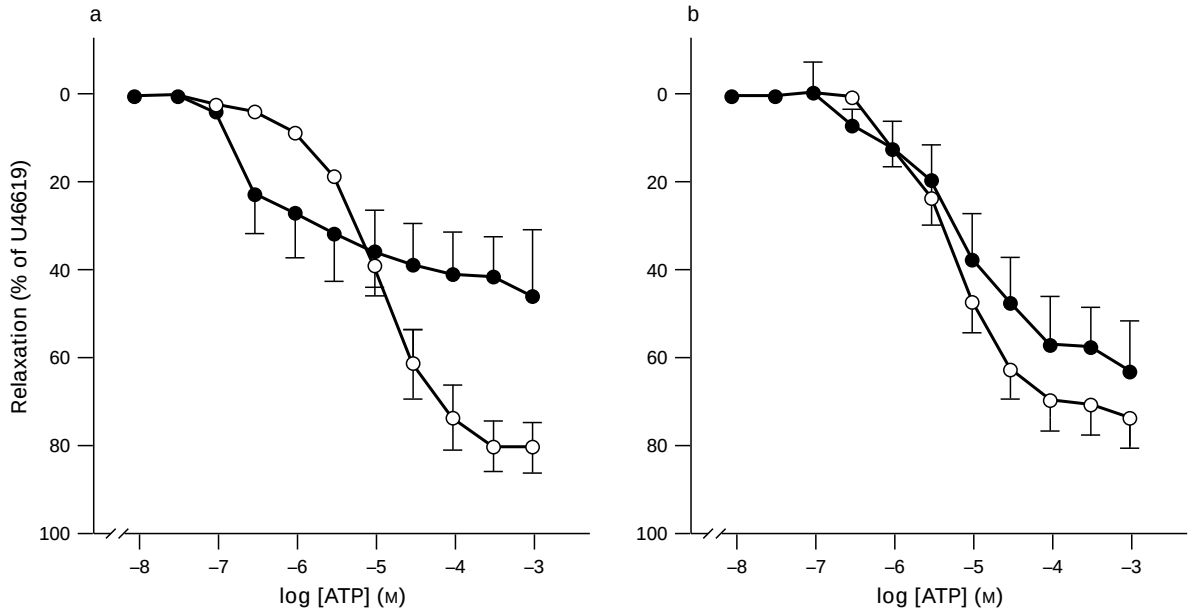

Figure 7 Concentration-response curves to ATP in lamb coronary small arteries precontracted with U46619 (3×10^{-7} M). (a) In endothelium-intact (○) and endothelium-denuded (●) arteries, and (b) endothelium-intact preparations in the absence (○) and presence (●) of N^G-nitro-L-arginine (L-NOARG, 3×10^{-5} M). Relaxations are expressed as percentage of U46619-induced contractions. Points represent means and vertical lines s.e.mean of 9–10 vessel segments.

dothelium-intact and denuded preparations, respectively. Incubation with the NOS inhibitor, L-NOARG (3×10^{-5} M), reduced the relaxations to 3×10^{-8} M bradykinin from 98.6 ± 1.2 to $84.1\pm4.9\%$ ($n=6$, $P<0.05$, paired t test). However, L-NOARG did not alter the ATP-elicited relaxations in coronary small arteries contracted to U46619. Thus, the pD_2-values and maximum relaxations to ATP were 5.19 ± 0.14 and 74.8 ± 6.1 ($n=9$), and 5.09 ± 0.22 and 65.0 ± 9.0 ($n=9$) in the absence and presence of 3×10^{-5} M L-NOARG, respectively (Figure 7b).

Discussion

The present results demonstrate neurogenic relaxations to EFS in lamb coronary small arteries, which were markedly reduced by the antagonist of P_2-purinoceptors, suramin, and also by the P_{2Y}-receptor antagonist, basilen blue, thus providing evidence for a functional purinergic innervation in this vascular bed.

The persistence of relaxant responses to EFS after inhibition of adrenergic and cholinergic neurotransmission, and the marked inhibition of these relaxations by tetrodotoxin and after cold storage, indicate the presence of an inhibitory NANC neurogenic component in lamb coronary small arteries.

Endothelial cells have been described either to mediate, to inhibit or not to play any role in the neurogenic responses elicited by EFS in arterial preparations. Thus, endothelium-dependent neurogenic relaxations probably mediated by tachykinins have been described in aortic rings and rabbit hindlimb (Perisco *et al*., 1993; Gustafsson *et al*., 1994), whereas inhibitory purinergic neurotransmission has been suggested to be endothelium-dependent in pulmonary arteries (Liu *et al*., 1992), but endothelium-independent in rabbit portal vein (Brizzolara *et al*., 1993). In addition, endothelial cells can reduce EFS-elicited adrenergic contractions in systemic arteries (Tesfamarian *et al*., 1989; Bucher *et al*., 1992), but they do not affect either the neurogenic cholinergic contractions of bovine isolated proximal coronary arteries (Kalsner & Quillan, 1989), or the noradrenaline outflow from either large canine coronary arteries (Cohen *et al*., 1984) or stimulated sympathetic nerves of the rat perfused heart (Schwarz *et al*., 1995). In lamb coronary arteries, endothelial cell removal, which abolished the relaxations to bradykinin, did not inhibit the relaxations to EFS indicating that the NANC neurogenic relaxations do not involve a mediator released from the endothelium. However, endothelial cell removal tended to increase EFS-elicited relaxations, which suggests an inhibitory role of the endothelium on the neurogenic NANC relaxations of lamb coronary small arteries.

In the heart, Tanaka and colleagues (1993) have found that intracardiac neurones in ganglia adjacent to small blood vessels of the guinea-pig left atrium, display NADPH-d activity, which indicates the presence of neural NO. Moreover, inhibition of NO synthesis has been shown to augment centrally induced sympathetic coronary vasoconstriction in cats (Goodson *et al*., 1994), and both endogenous and exogenously added NO inhibit noradrenaline outflow from sympathetic nerves in perfused rat hearts (Schwarz *et al*., 1995), which suggests that NO from sources other than the endothelium might either influence or be involved in the neurotransmission of the coronary circulation (Schwarz *et al*., 1995). However, neither the histochemical nor the functional findings of the present study support a role for neural NO as mediator of the NANC inhibitory neurotransmission in lamb isolated coronary small arteries. Firstly, inhibition of NOS by L-NOARG enhanced rather than decreased the relaxations to EFS. Secondly, the histochemical NADPH-d reaction, considered as a specific marker for NOS (Hope & Vincent, 1989) under appropriate fixation conditions with paraformaldehyde (Matsumoto *et al*., 1993), was only found within the endothelial cells of the small coronary arteries, but not in the adventitia or media. The histochemical findings are consistent with those obtained for the coronary arteries of other species (Ursell & Mayes, 1993; Dikranian *et al*., 1994) and indicate that, despite some neurones in intracardiac ganglia containing NOS (Tanaka *et al*., 1993), the coronary circulation does not receive a direct nitrergic innervation, in contrast to the cerebral, mesenteric and penile arterial circulations (Ahlner *et al*., 1991; Toda & Okamura, 1992; Burnett *et al*., 1992; Simonsen *et al*., 1995). On the other hand, the present results suggest that en-

dothelial cells through NO might modulate prejunctionally the NANC inhibitory neurotransmission, since endothelial cell removal, like treatment with L-NOARG, tended to increase the relaxations to EFS in lamb coronary small arteries.

In the present study, ATP was considered as a putative neurotransmitter in lamb coronary arteries, since exogenously added ATP had been earlier shown to relax isolated proximal coronary arteries from both guinea-pig and rabbits even in the absence of endothelial cells (Corr & Burnstock, 1991; 1994; Keef *et al*., 1992). Moreover, the magnitude of the relaxations to ATP in endothelium-denuded preparations was similar to those elicited by electrical stimulation in lamb coronary small arteries.

Burnstock & Kennedy (1985) discriminated between two major subclasses of purinoceptors on the basis of response profiles to a number of ATP analogues. Thus, the purinoceptors designated as P_1 and P_2 are characterized by their selectivity for adenosine and ATP, respectively, and P_2-receptors were further subdividied into P_{2X}, where the rank order is α, β-meATP > ATP = 2-MeSATP, P_{2Y} receptors, where 2-MeSATP is most potent and α, β-meATP has no effect, and the P_{2U}-purinoceptor where UTP is the most potent agonist, while P_{2T}, P_{2Z} and P_{2D}-purinoceptors have not so far been described in blood vessels (Fredholm *et al*., 1994; Harden *et al*., 1995). The order of potency for the relaxant effects of the purinergic agonists in lamb coronary small arteries (ATP = 2-MeATP = ADP > > α, β-meATP, UTP) is consistent with that obtained in guinea-pig perfused hearts and in isolated proximal coronary arteries from rabbits and guinea-pigs (Hopwood *et al*., 1987; Keef *et al*., 1992; Corr & Burnstock, 1994), and suggests that P_{2Y}-receptors mediate the relaxations elicited by ATP in lamb coronary small arteries. However, the characterization of purinoceptors only by the use of agonists requires some caution, since the rank order of agonist potency may be substantially influenced by the different susceptibilities of purine analogues to degradation by ectonucleotidases (Evans & Kennedy, 1994; Crack *et al*., 1995).

8-Phenyltheophylline (8-PT) is considered to be a selective P_1-purinoceptor blocker (Fredholm *et al*., 1994; Harden *et al*., 1995) and inhibited the relaxations to exogenously added adenosine in the present study. However, 8-PT did not change the relaxations to EFS or exogenously added ATP, which excludes an involvement of P_1-purinoceptors in the neurogenic relaxations. In contrast, the selective P_2-purinoceptor antagonist, suramin (Hoyle *et al*., 1990; Fredholm *et al*., 1994), did significantly reduce the relaxations to EFS suggesting that P_2-purinoceptors mediate the NANC relaxations in lamb coronary arteries.

P_{2X}-purinoceptors mediating transient vasoconstriction to purinoceptor agonists have been described in the guinea-pig perfused heart (Hopwood *et al*., 1987) and in proximal coronary arteries from guinea-pigs and rabbits (Keef *et al*., 1992; Corr & Burnstock, 1994). Also in the present study, high concentrations of ATP and the P_{2X}-purinoceptor agonist, α, β-meATP, elicited transient contractions in lamb coronary small arteries. α, β-MeATP acts as a selective antagonist at the P_{2X}-receptors by desensitizing the receptor and has no effect on the P_{2Y}-purinoceptors. Accordingly, the contractions in lamb coronary small arteries disappeared after exposure to the ligand. In addition, α, β-meATP induced significant leftwards shifts in the concentration-relaxation curves to exogenously added ATP, but did not influence the relaxations to EFS indicating that P_{2X}-receptors might mediate contractions to exogenously added ATP, but they are not involved in relaxations to either ATP or EFS in coronary small arteries. Moreover, the finding that the general P_2-purinoceptor blocker suramin markedly inhibited relaxations to EFS, whereas its inhibitory effect on exogenous ATP was only consistently observed after desensitization of the contractile P_{2X}-purinoceptors, gives further support to the contention that exogenous ATP but not nerve-released ATP activate the P_{2X}-receptors in lamb coronary small arteries. Further investigations might show whether this can be ascribed to either differential access to the P_{2X}-purinoceptors, or different metabolism by the tissue of the exogenous and nerve-released ATP (Crack *et al*., 1995).

The present results agree with earlier studies in peripheral small arteries which have shown that P_{2X}-purinoceptors involved in neurogenic responses mediate contractions and are not involved in the relaxations to ATP (Evans & Cunnane, 1992; Ralevic & Burnstock, 1996).

ATP has been previously shown to mediate NANC relaxations to EFS in pulmonary arteries (Liu *et al*., 1992) and portal vein (Brizzolara *et al*., 1993). The P_{2Y}-receptor antagonist, basilen blue (earlier named reactive blue 2, Burnstock & Warland, 1987; Fredholm *et al*., 1994), antagonized the relaxations to the agonist selective for P_{2Y}-receptors, 2-me-SATP, confirming the presence of P_{2Y}- receptors in lamb coronary small arteries. Moreover, the relaxant responses elicited by exogenously added ATP and EFS were significantly inhibited by basilen blue, thus, indicating that the NANC neurogenic responses might be mediated by ATP activating P_{2Y}-purinoceptors and causing relaxations of lamb coronary small arteries. Thus, in addition to pulmonary arteries and the portal vein, the present results suggest ATP mediates the neurogenic NANC relaxations to EFS in lamb coronary small arteries.

The location of vascular P_{2Y}-purinoceptors on either smooth muscle or endothelial cells, as well as the mediators involved in the vasodilatation elicited by purinoceptor-activation have been shown to be variable depending on the vascular bed and animal species. Thus, P_{2Y}-purinoceptors are located on the endothelium in large canine coronary arteries (White & Angus, 1987; Houston *et al*., 1987), but P_{2Y}-purinoceptors are present on the smooth muscle and to a lesser degree on the endothelium in guinea-pig and rabbit large coronary arteries (Keef *et al*., 1992; Corr & Burnstock, 1994). In the present study, mechanical endothelial cell removal, but not inhibition of NOS partially reduced the relaxations elicited by exogenously added ATP, thus indicating that ATP relaxes lamb coronary small arteries through receptors located on both smooth muscle and endothelial cells. Furthermore, the present results exclude the possibility that NO is the mediator of the endothelial component of the relaxations to ATP.

In addition to a role for ATP as neurotransmitter or co-transmitter in the cardiovascular system (White, 1988; Burnstock, 1990), there are studies providing evidence for ATP release from extraneuronal sites. Thus, ATP has been detected in the superfusate from cell cultures of vascular smooth muscle and endothelial cells (Pearson & Gordon, 1979; Yang *et al*., 1994), and 15% of the adenine nucleotides released from the perfused heart are derived from endothelial cells (Borst & Schrader, 1991). In addition, ATP has been shown to be released from endothelial cells by stimulation of endothelial P_{2Y}-receptors by ATP (Yang *et al*., 1994) and ATP release elicited by adrenoceptor agonists has been observed in aorta and pulmonary arteries (Sedaa *et al*., 1990; Takeuchi *et al*., 1994). However, the reduction of the relaxations to ATP found in endothelium-denuded lamb coronary small arteries is probably due to release of endothelium-derived relaxing factors other than ATP. Furthermore, EFS-induced release of ATP from endothelial cells is unlikely, since these relaxations were not reduced after mechanical endothelial cell removal in lamb coronary small arteries. However, in the present study basilen blue was more effective in inhibiting the relaxations to EFS than tetrodotoxin and, in addition to release of ATP from nerves, EFS might induce release of ATP from non-endothelial non-neural sources in lamb coronary small arteries.

In summary, the present study suggests that in NANC conditions neurogenic relaxations of coronary small arteries are not mediated by NO, but the parameters of EFS applied induce release of ATP, which relaxes lamb coronary small arteries through P_{2Y}-purinoceptors. A prejunctional modulation of these relaxations by endothelial-derived NO cannot be excluded.

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