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ORIGINAL ARTICLE

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Characterization by antagonists of P2-receptors mediating endothelium-dependent relaxation in the rat aorta

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Abstract The receptors through which 2-methylthio ATP (MeSATP), adenosine 5′-O-(2-thiodiphosphate) (ADPβS), UTP and ATP elicit endothelium-dependent relaxation of noradrenaline-precontracted rings of the rat aorta were characterized by means of a series of antagonists. The acetylcholine-induced relaxation and the degradation of MeSATP, UTP and ATP were also studied.

The potency of the nucleotides at producing relaxation decreased in the order MeSATP (EC_{50} 0.24 μM) > ADPβS (0.43 μM) > UTP (1.09 μM) > ATP (3.53 μM). MeSATP, ADPβS and UTP did not cause relaxation when the endothelium had been destroyed; high concentrations of ATP still caused some relaxation. The relaxation by MeSATP, ADPβS and UTP became very small after treatment of the rings with N^G-nitro-L-arginine methyl ester; the relaxation by ATP was less affected. Pre-exposure to MeSATP (100 μM) abolished or almost abolished the relaxation normally elicited by MeSATP and ADPβS, did not change that elicited by UTP and slightly enhanced the relaxation elicited by ATP. Of nine compounds examined as antagonists, six attenuated selectively the effect of some or all of the nucleotides (as compared to acetylcholine): suramin, reactive blue 2, pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate (*iso*-PPADS), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate (PPADS), reactive red 2 and 5,5′-(1,1′-biphenyl-4,4′-diylbisazo)-*bis*-7-amino-6-hydroxy-naphthalene-1,4-disulphonate (NH05). Decreases of maximal relaxations and slopes different from unity in Schild plots often indicated non-competitive kinetics of the antagonism. For each of the six 'selective' antagonist, the apparent K_d values against MeSATP and against ADPβS were similar: none of the six differentiated between MeSATP and ADPβS. Also, for each of four 'selective' antagonists, the apparent K_d values against UTP and against ATP were similar: none of the four differentiated between these two nucleotides (two antagonists did not act against UTP and ATP in the 'selective' concentration range). On the other hand, for five of the six 'selective' antagonists (the exception being NH05), the apparent K_d values against MeSATP and ADPβS were considerably lower than those against UTP and ATP. At the highest concentrations tested against agonist-evoked relaxations, the antagonists did not alter the removal from the incubation medium, by pieces of rat aorta, of MeSATP, UTP and ATP.

It is concluded that nucleotides cause endothelium-dependent relaxation of the rat aorta through two sites: a P2Y-receptor and a P2U-receptor. The receptors may be pharmacologically similar to a bovine endothelial P2Y ($P2Y_1$) and a cloned rat P2U ($P2Y_2$) receptor, respectively. ATP acts mainly through the P2U-receptor. Suramin, reactive blue 2, *iso*-PPADS, PPADS and reactive red 2 are more potent at the P2Y- than the P2U-receptor. NH05 does not discriminate between the two receptors but is the most potent P2U antagonist so far described.

Key words Rat aorta · Endothelium · P2Y-receptor · P2U-receptor · P2-receptor antagonists · Suramin · Reactive blue 2 · NH05

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Introduction

Extracellular nucleotides contribute to the local regulation of vascular tone (for review see Ralevic and Burnstock 1991). They can cause both vasoconstriction, mainly by activation of P2-receptors located on the smooth muscle cells, and vasodilation, mainly by activation of P2-receptors located on the endothelium.

The endothelial relaxation-mediating P2-receptors are not homogeneous. In some vessels, two receptors with different ligand sensitivities and transduction pathways can be distinguished: a P2Y-receptor selectively activated by 2-methylthio ATP (MeSATP) and adenosine 5′-O-(2-thiodiphosphate) (ADPβS), and a P2U-receptor selectively activated by UTP; ATP can act on either receptor (for review see Pirotton et al. 1993; Boarder et al. 1995). The evidence consists mainly of agonist cross-desensitization

phenomena (Dainty et al. 1991; Motte et al. 1993; Wilkinson et al. 1993, 1994a; Chen et al. 1996a; Mateo et al. 1996). In addition, the P2-receptor antagonist suramin potently blocks endothelial responses mediated by the P2Y subtype, but is considerably less potent (Chen et al. 1996a; Mateo et al. 1996; Ralevic and Burnstock 1996a), or even inactive at up to 100 μM (Wilkinson et al. 1993, 1994b), against responses mediated by the P2U subtype. The P2 antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate (PPADS) also blocks selectively the P2Y-receptor (Windscheif et al. 1994; Brown et al. 1995; Mateo et al. 1996; Ralevic and Burnstock 1996a,b).

Endothelium-dependent relaxation by ATP and other nucleotides has also been demonstrated in the rat aorta (Rapoport et al. 1984; White et al. 1985; Rose'Meyer and Hope 1990; Dainty et al. 1991, 1994; Dominiczak et al. 1991; Frew et al. 1993; Mombouli and Vanhoutte 1993; García-Velasco et al. 1995; Saïag et al. 1996). The rank order of agonist potency MeSATP > UTP = ATP (Dainty et al. 1991; García-Velasco et al. 1995) is consistent with a mixed P2Y and P2U population (O'Connor et al. 1991). In support of this view, pre-exposure to a high concentration of MeSATP did not alter relaxations caused by ATP or UTP (Dainty et al. 1991). An alternative receptor pattern was recently suggested on the basis of differential blockade by the P2 antagonist reactive blue 2: that ATP, MeSATP and UTP each acted on a different endothelial receptor in rat aorta, giving a total number of three receptors, the site of action of UTP being a 'pyrimidinoceptor' activated by UTP but not by ATP (García-Velasco et al. 1995; see also Yang et al. 1996).

The present experiments were carried out in order to investigate the endothelial P2-receptors of the rat aorta by means of a series of antagonists. Antagonists have been tested at these receptors three times: reactive blue 2 in the study of García-Velasco et al. (1995) and in a report demonstrating that it attenuated the effect of ADPβS (Saïag et al. 1996), suramin in a report demonstrating that it attenuated the effects of both ATP and UTP (Dainty et al. 1994). We used in addition the small suramin analogue 8-(3,5-dinitro-phenylenecarbonylimino)-1,3,5-naphthalenetrisulphonate (XAMR0721; van Rhee et al. 1994); PPADS (Lambrecht et al. 1992) and its isomer pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate (*iso*-PPADS; Connolly 1995); reactive red 2 (Bültmann and Starke 1995); the bisnormethyl analogue of Evans blue 6,6′-(1,1′-biphenyl-4,4′-diylbisazo)-*bis*-4-amino-5-hydroxy-naphthalene-1,3-disulphonate (NH01) and the bisnormethyl analogue of trypan blue 5,5′-(1,1′-biphenyl-4,4′-diylbisazo)-*bis*-7-amino-6-hydroxy-naphthalene-1,4-disulphonate (NH05; Wittenburg et al. 1996); and 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS; McMillian et al. 1988; Bültmann and Starke 1994). Effects of the antagonists were tested on relaxations caused by MeSATP, ADPβS, UTP, ATP and, for comparison, acetylcholine. The effect on the degradation of MeSATP, UTP and ATP was also studied. Some results have been presented in abstract form (Bültmann et al. 1996b).

Methods

Male Wistar rats (250 to 300 g) were decapitated. The thoracic aorta was cleaned of adherent tissue and cut into rings of about 4 mm length. In some rings the endothelium was removed by gently rubbing the intimal surface, a procedure that did not compromise the ability of the smooth muscle to relax as indicated by an unchanged relaxation effect of sodium nitroprusside. The incubation medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Tension measurement. Aortic rings were mounted in a 5.9-ml organ bath. Unless stated otherwise the bath fluid was replaced every 15 min. Two stainless steel hooks were inserted through the lumen; the lower hook was fixed and the upper one attached to an isometric force transducer (K30, Hugo Sachs Elektronik, Hugstetten, Germany). During a 60-min equilibration period, the resting tension was twice adjusted to 9.8 mN (Graphtec thermal pen recorder, Ettlingen, Germany). Noradrenaline (1 μM) was then added to the medium twice, 60 and 80 min after the beginning of the experiment. During the plateau of the second noradrenaline contraction, acetylcholine (1 μM) was added in order to examine the condition of the endothelium. The endothelium was considered intact when acetylcholine caused at least 40% relaxation; it was considered removed when acetylcholine failed to elicit relaxation. Rings that did not satisfy these criteria were discarded.

In order to determine concentration-response curves for agonist- (nucleotide- or acetylcholine-) induced relaxations, noradrenaline (1 μM usually) was again added to the medium either twice, 105 and 165 min, or three times, 105, 165 and 225 min, after the beginning of the experiment ('precontraction'). Nucleotides or acetylcholine were administered in a cumulative fashion during the plateau of the noradrenaline response, i.e. from 5 to 7 min after the addition of noradrenaline onwards. They were washed out together with noradrenaline when the relaxation elicited by the highest concentration was maximal. It took 5 to 10 min to determine a concentration-relaxation curve. Unless stated otherwise, only one agonist was studied per preparation. Relaxations were measured at their maximum and expressed as a percentage of the respective noradrenaline precontraction. P2-Receptor antagonists were added to the medium at two increasing concentrations immediately after the first and second concentration-response curve to acetylcholine or the nucleotides.

In order to determine concentration-response curves for nucleotide-induced contractions, the nucleotides were administered in a cumulative fashion three times, 110, 170 and 230 min after the beginning of the experiment, to endothelium-intact, non-precontracted rings (after the two initial – 60 and 80 min – responses to noradrenaline). They were washed out when the contraction elicited by the highest concentration was maximal. It took about 10 min to determine a concentration-contraction curve. Three different nucleotides were studied in each preparation in varying order of application. Contractions were measured at their maximum and expressed as a percentage of the second initial noradrenaline contraction.

For the computation of maximal effects and EC_{50} values of agonists (concentrations producing 50% of the respective maximum), logistic curves were fitted to weighted mean relaxation or contraction values by means of Eq. 25 of Waud (1976) and nonlinear regression. Differences between fitted curves were tested according to p. 371 of Motulsky and Ransnas (1987). Apparent antagonist K_d values were derived by one of two procedures. If, in a pair of antagonist and agonist, the antagonist did not change the maximum of the agonist concentration-response curve, the apparent K_d was derived from the shift of the curve to the right at the level of the EC_{50}, using Eq. 4 of Furchgott (1972). If, in an antagonist-agonist pair, the antagonist at any concentration depressed the maximum of the agonist concentration-response curve, the apparent K_d value was derived from a double reciprocal plot according to pp. 335 and 342 of Kenakin (1993); this was done in such cases also for antagonist concentrations that left the maximum of

the curve unchanged; a prerequisite was that the maximum was not decreased by more than 50%. The procedure described by Kenakin (1993) holds true for pseudo-irreversible antagonism; for antagonist concentrations causing only a shift to the right without a change of the maximum, the procedure yields apparent K_d values identical to those derived according to Furchgott (1972). If an antagonist did not change the maximum and at least 3 antagonist concentrations had been tested, data were also subjected to analysis according to Arunlakshana and Schild (1959), based on curve shifts at the level of the EC_{50}.

Removal of nucleotides from the medium. Single aortic rings were split open longitudinally and placed in 0.6 ml medium (37°C). MeSATP, ATP or UTP stock solution, 25 µl, was added to give an initial concentration of 10 µM. For the determination of the time course of nucleotide removal, two aliquots of 100 µl were taken, the first immediately after addition of the nucleotides, without replacement, the second 2 to 60 min later. For the study of the influence of P2 antagonists, 30 min of incubation with the antagonists or solvent (water) preceded the addition of the nucleotides, and the incubation with the nucleotides was allowed to proceed for 30 min. Aliquots containing MeSATP or ATP were diluted 1:100, and their nucleotide content was assayed by means of luciferin-luciferase (see Driessen et al. 1993). Aliquots containing UTP were diluted 1:10, then incubated with nucleoside diphosphate kinase (1 U per sample) and ADP (10 µM) for 10 min at room temperature, again diluted 1:10, and the ATP formed by the enzymatic reaction was determined. Blank values from diluted (1:10) samples containing nucleoside diphosphate kinase and ADP only were subtracted from experimental values. Under the conditions indicated, UTP was quantitatively converted to ATP. The antagonists did not interfere with the bioluminescence assay. The percentage degradation of the nucleotides was calculated as "100 · (1–content of second aliquot/content of first aliquot)" and corrected for spontaneous loss in the absence of tissue (up to 32% within 60 min).

Materials. 8-(3,5-Dinitro-phenylenecarbonylimino)-1,3,5-naphthalenetrisulphonate trisodium (XAMR0721) and reactive red 2 disodium were synthesized in our laboratory as previously described (van Rhee et al. 1994; Bültmann and Starke 1995). The identity and purity of the products was confirmed by TLC and ^{1}H-NMR and IR spectroscopy. Other compounds were 6,6′-(1,1′-biphenyl-4,4′-diylbisazo)-*bis*-4-amino-5-hydroxy-naphthalene-1,3-disulphonate tetrasodium (NH01; Aldrich, Steinheim); suramin hexasodium (Bayer, Wuppertal, Germany); 2-methylthio ATP tetrasodium (MeSATP), reactive blue 2 (Biotrend, Köln, Germany); pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate tetrasodium (PPADS), pyridoxalphosphate-6-azophenyl-2′,5′-disulphonate tetrasodium (*iso*-PPADS; Cookson, Southampton, UK); acetylcholine chloride, adenosine 5′-O-(2-thiodiphosphate) trilithium (ADPβS), L-arginine hydrochloride, ATP disodium, 4,4′-diisothiocyanatostilbene-2,2′-disulphonate disodium (DIDS), Evans blue tetrasodium, N^G-nitro-L-arginine methyl ester (L-NAME), (–)-noradrenaline bi-(+)-tartrate, UTP trisodium (Sigma, Deisenhofen, Germany); and 5,5′-(1,1′-biphenyl-4,4′-diylbisazo)-*bis*-7-amino-6-hydroxy-naphthalene-1,4-disulphonate tetrasodium (NH05; Syntec, Wolfen, Germany). Antagonists and noradrenaline were dissolved in distilled water. Acetylcholine and nucleotides were dissolved in medium.

Statistics. Data are expressed as either the arithmetic mean ± SEM or, in the case of fitted curves, the EC_{50} and maximal effect with the SE as defined by Waud (1976). Differences between means

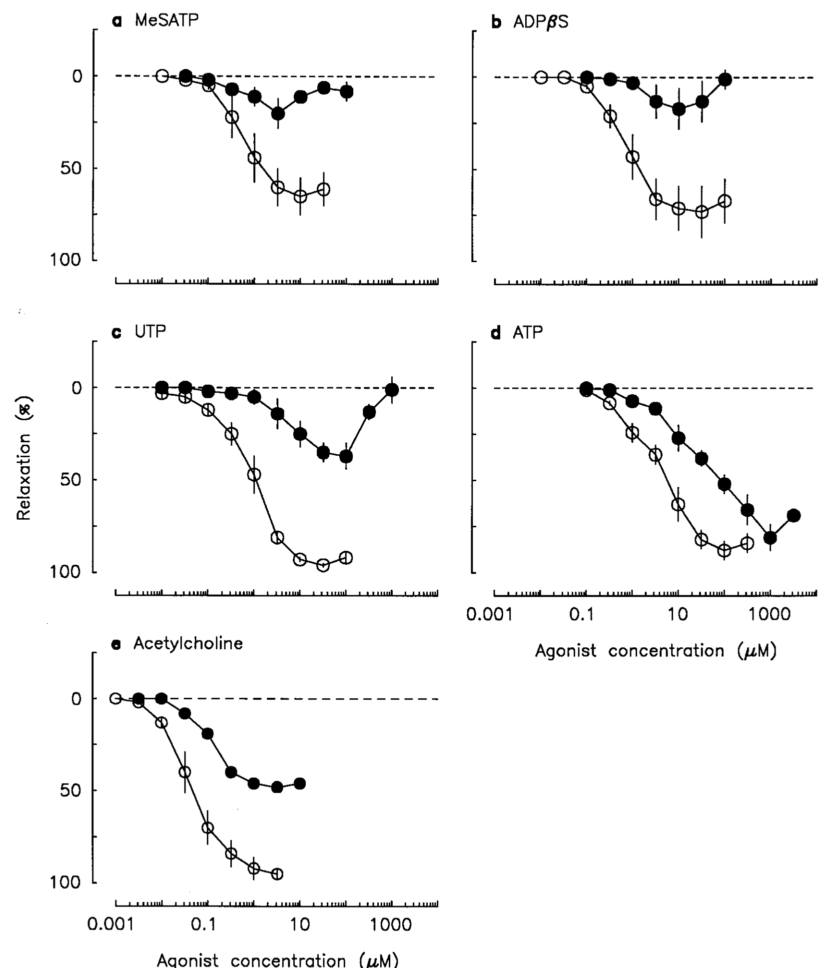

Fig. 1 a–e Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on nucleotide- and acetylcholine-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline was added to the medium twice, interval 60 min, concentration 1 µM upon first and 0.01 µM upon second addition. MeSATP (**a**), ADPβS (**b**), UTP (**c**), ATP (**d**) or acetylcholine (**e**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. L-NAME (30 µM) was added 30 min before the second addition of noradrenaline, i.e. about 35 min before the second nucleotide or acetylcholine concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second curves in the presence of L-NAME (●), as a percentage of the respective response to noradrenaline. Means ± SEM from 4 experiments each

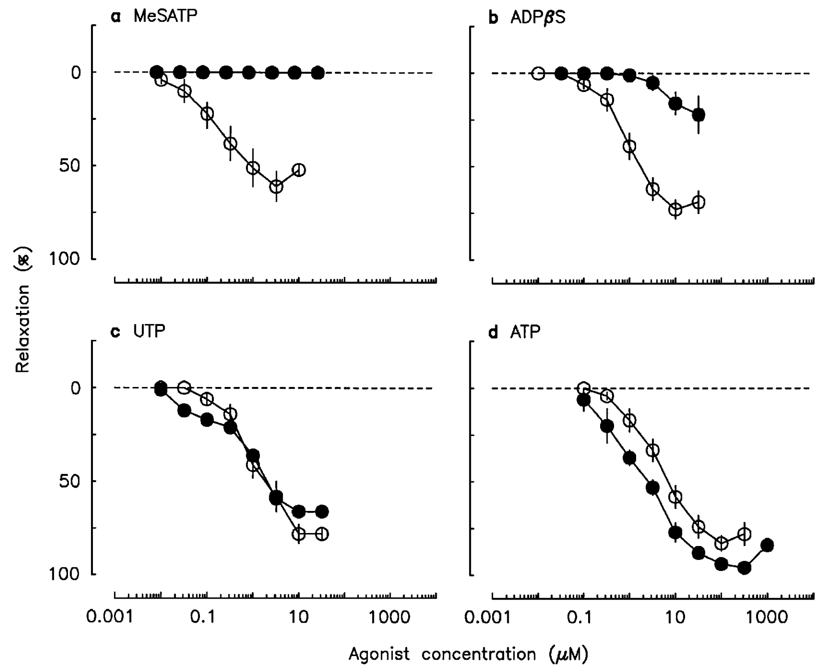

Fig. 2a–d Effect of a high concentration of MeSATP on nucleotide-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline (1 μM) was added to the medium twice, interval 60 min. MeSATP (**a**), ADPβS (**b**), UTP (**c**) or ATP (**d**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. A high concentration of MeSATP (100 μM) was added immediately after the second addition of noradrenaline, i.e. about 5 min before the second nucleotide concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second curves in the presence of MeSATP (100 μM; ●), as a percentage of the respective response to noradrenaline. Means ± SEM from 4 to 5 experiments

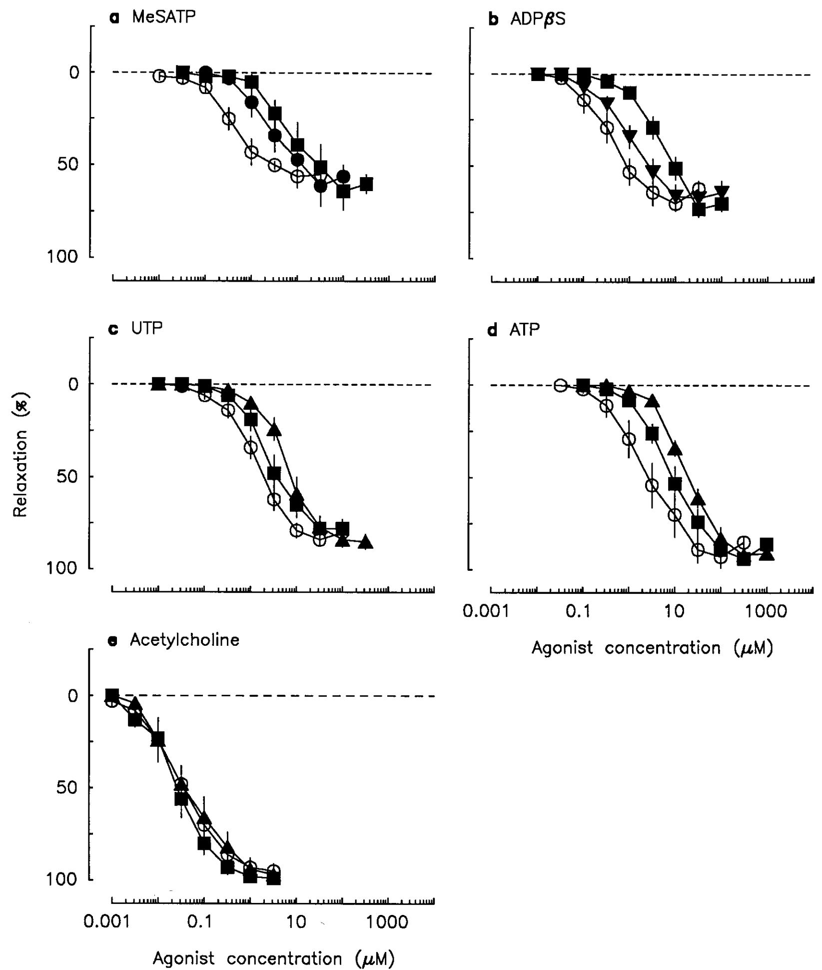

Fig. 3a–e Effect of suramin on nucleotide- and acetylcholine-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline (1 μM) was added to the medium three times, interval 60 min. MeSATP (**a**), ADPβS (**b**), UTP (**c**), ATP (**d**) or acetylcholine (**e**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. Suramin was added at two increasing concentrations immediately after the first and second nucleotide or acetylcholine concentration-relaxation curve, i.e. about 50 min before the second and third concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second and third curves in the presence of suramin (▼ 3.2; ● 10; ■ 32; ▲ 100 μM), as a percentage of the respective response to noradrenaline. Means ± SEM from 4 to 8 experiments

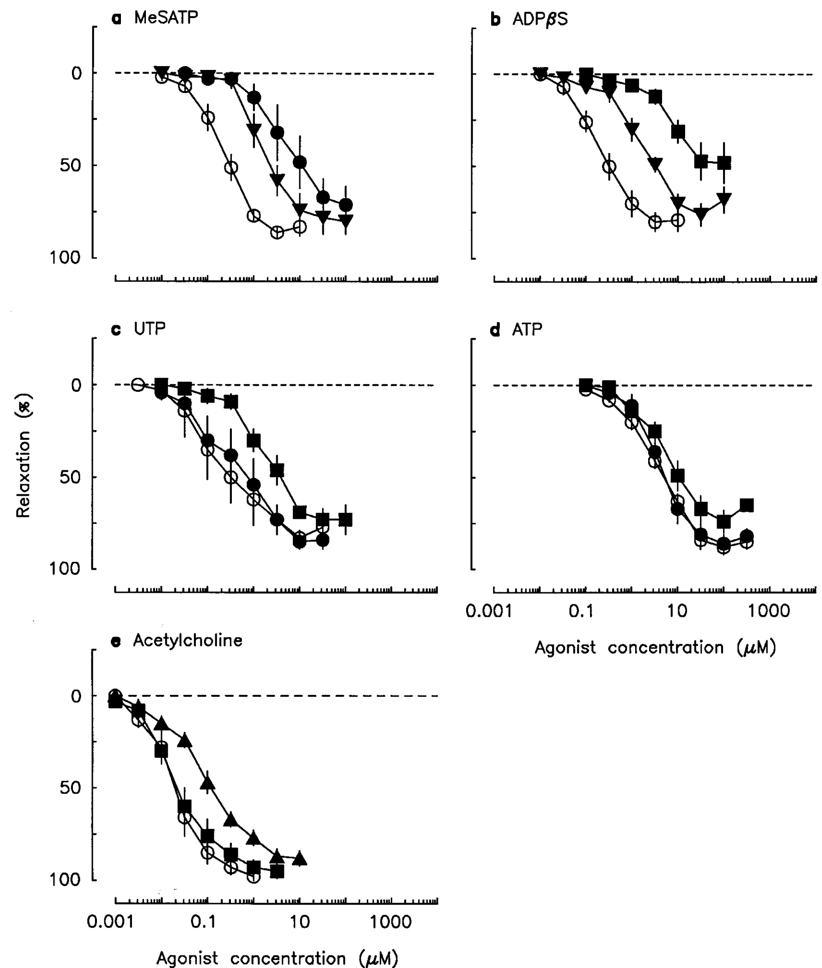

Fig. 4 a–e Effect of reactive blue 2 on nucleotide- and acetylcholine-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline (1 μM) was added to the medium three times, interval 60 min. MeSATP (**a**), ADPβS (**b**), UTP (**c**), ATP (**d**) or acetylcholine (**e**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. Reactive blue 2 was added at two increasing concentrations immediately after the first and second nucleotide or acetylcholine concentration-relaxation curve, i.e. about 50 min before the second and third concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second and third curves in the presence of reactive blue 2 (▼ 3.2; ● 10; ■ 32; ▲ 100 μM), as a percentage of the respective response to noradrenaline. Means ± SEM from 4 to 6 experiments

were tested for significance by the Mann-Whitney test, with Bonferroni correction if applicable. $P < 0.05$ was taken as the limit of statistical significance.

Results

Relaxation: general

Aortic rings were precontracted two or three times with noradrenaline (1 μM usually), and one concentration-relaxation curve was determined during each precontraction.

In endothelium-intact rings, the first precontraction by noradrenaline (1 μM) averaged 6.3 ± 0.1 mN (n = 401). When added during the plateau of this contraction, increasing concentrations of MeSATP, ADPβS, UTP, ATP and acetylcholine caused increasing relaxation (open symbols in Figs. 1–6). The relaxations were rapid and did not differ in time course between the five agonists. The EC_{50} values (and maximal percentage relaxations) were 0.24 ± 0.04 μM (61 ± 2%) for MeSATP (n = 67), 0.43 ± 0.07 μM (71 ± 3%) for ADPβS (n = 105), 1.09 ± 0.31 μM (77 ± 5%) for UTP (n = 83), 3.53 ± 0.26 μM (95 ± 2%) for ATP (n = 83) and 30 ± 5 nM (94 ± 1%) for acetylcholine (n = 65). In the absence of other drugs, the second and third precontraction elicited by noradrenaline (1 μM) slightly exceeded the first one, amounting to 7.0 ± 0.2 and 7.4 ± 0.2 mN, respectively (n = 64), and second and third nucleotide and acetylcholine concentration-relaxation curves could practically be superimposed upon the first (n = 10–21; not shown).

A single series of experiments was carried out in endothelium-denuded aortic rings. Three concentration-relaxation curves for different agonists were determined in each ring. The first precontraction to noradrenaline (1 μM) was 5.6 ± 0.5 mN (n = 8; not significantly different from endothelium-intact rings). MeSATP (0.1–320 μM), ADPβS (0.1–320 μM), UTP (1–1000 μM) and acetylcholine (0.001–10 μM) failed to elicit relaxation in endothelium-denuded aortic rings (n = 4–5). High concentrations of ATP (320–3200 μM) caused a slowly developing relaxation, with an EC_{50} value of 498 ± 26 μM and a maximal relaxation by 80 ± 1% (n = 4; not shown).

Concentration-relaxation curves for nucleotides and acetylcholine were then determined in endothelium-intact preparations after blockade of nitric oxide synthase by N^G-nitro-L-arginine methyl ester (L-NAME; 30 μM). L-NAME was added 30 min before the second noradrenaline precontraction. It increased the second precontraction caused by noradrenaline (1 μM) to 11.5 ± 0.7 mN (n = 6; $P < 0.001$ vs. experiments without L-NAME), and for this

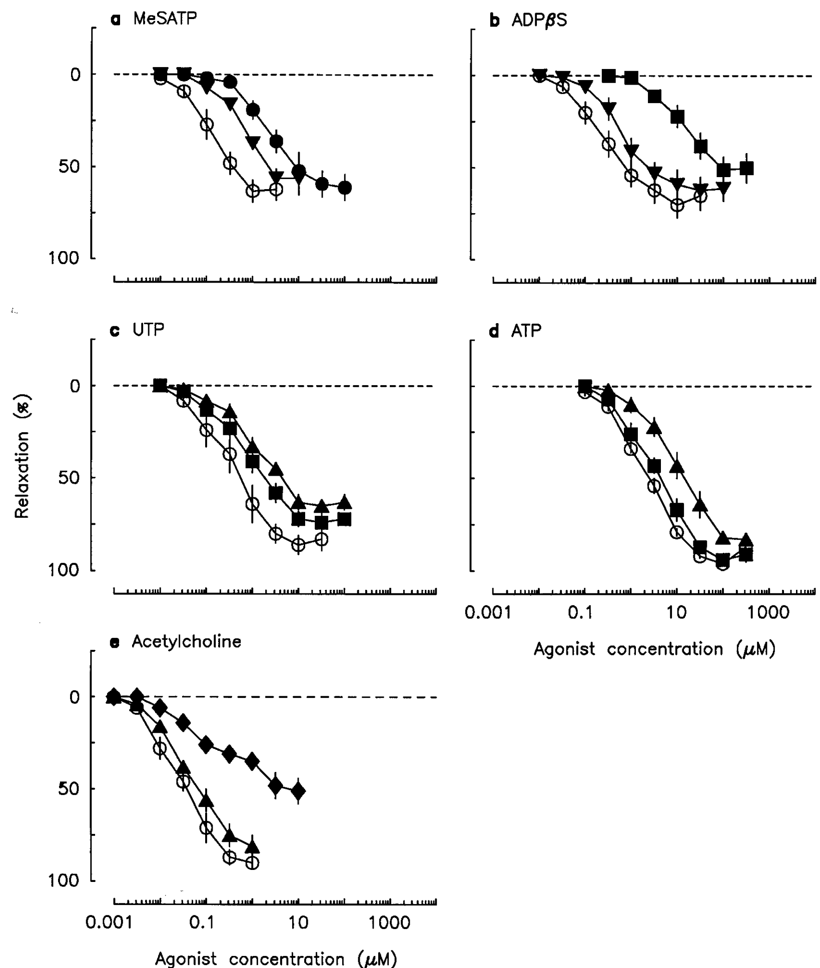

Fig. 5 a–e Effect of *iso*-PPADS on nucleotide- and acetylcholine-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline (1 μM) was added to the medium three times, interval 60 min. MeSATP (**a**), ADPβS (**b**), UTP (**c**), ATP (**d**) or acetylcholine (**e**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. *iso*-PPADS was added at two increasing concentrations immediately after the first and second nucleotide or acetylcholine concentration-relaxation curve, i.e. about 50 min before the second and third concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second and third curves in the presence of *iso*-PPADS (▼ 0.32; ● 1; ■ 3.2; ▲ 10; ◆ 32 μM), as a percentage of the respective response to noradrenaline. Means ± SEM from 5 to 6 experiments

reason 0.01 μM noradrenaline was used in subsequent experiments when L-NAME was present. Even noradrenaline (0.01 μM) elicited a (second) precontraction in the presence of L-NAME which was slightly higher (8.5 ± 0.5 mN; $n = 20$) than the (second) precontraction produced by noradrenaline (1 μM) in the absence of L-NAME (7.0 ± 0.2 mN; see above; $P < 0.05$). L-NAME markedly reduced the maximal relaxation produced by MeSATP, ADPβS and UTP, and the concentration-response curves of these three agonists became decidedly bell-shaped (Fig. 1a–c). The concentration-response curve of ATP was shifted to the right (Fig. 1d). L-NAME also reduced the maximum of the concentration-relaxation curve of acetylcholine (Fig. 1e; cf. Hatake et al. 1995). In additional experiments, the substrate of nitric oxide synthase, L-arginine (10 mM), was added 45 min before the second precontraction by noradrenaline (0.01 μM), followed by L-NAME (30 μM) 30 min before the second precontraction. Under these conditions, the concentration-relaxation curve of acetylcholine was not changed as compared to the absence of L-arginine and L-NAME ($n = 4$). The maxima of the concentration-relaxation curves of ADPβS and UTP still were slightly reduced, but much less so than in the presence of L-NAME alone ($n = 7$ and 4, respectively; not shown).

Nucleotide interactions were studied as follows. Immediately after the application of noradrenaline (1 μM; second precontraction), a high concentration of MeSATP (100 μM) was added and this was followed, when the contraction plateaued and without washout, by the usual MeSATP, ADPβS, UTP or ATP concentration-relaxation curves. The initial high concentration of MeSATP reduced the noradrenaline response to 4.8 ± 0.4 mN ($n = 15$; $P < 0.001$ vs. second precontraction without MeSATP). Preexposure to MeSATP (100 μM) abolished or greatly attenuated the relaxations normally produced by increasing concentrations of MeSATP and ADPβS (Fig. 2a,b). The relaxations caused by UTP were not changed (Fig. 2c) and the responses to ATP even slightly enhanced (Fig. 2d).

Relaxation: effect of P2-receptor antagonists

Effects of nine putative P2 antagonists – suramin, its congener XAMR0721, reactive blue 2, PPADS, *iso*-PPADS, reactive red 2, the Evans blue analogue NH01, the trypan blue analogue NH05, and DIDS - on concentration-relaxation curves of ADPβS, UTP, acetylcholine, and in most cases also MeSATP and ATP, were determined.

XAMR0721 (0.32 and 1 μM), NH01 (10 μM) and DIDS (3.2 μM) affected the concentration-relaxation curves of ADPβS, UTP and acetylcholine to a similar degree, causing shifts to the right or decreases of the maxi-

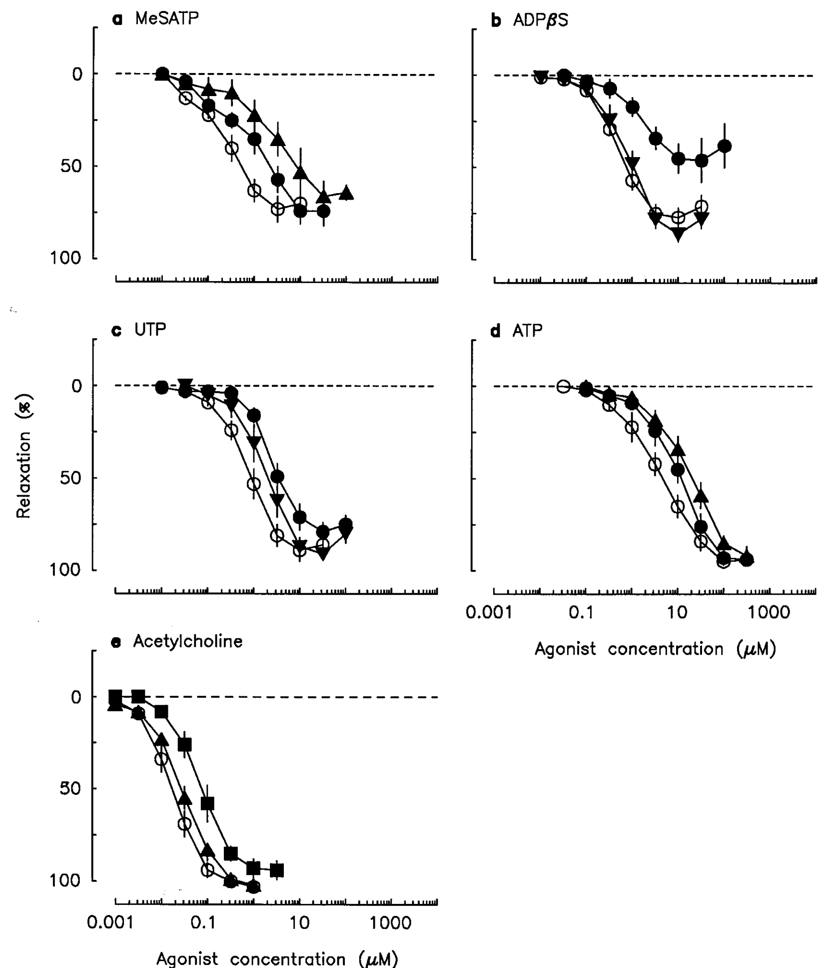

Fig. 6 a–e Effect of NH05 on nucleotide- and acetylcholine-evoked relaxations of rat aortic rings precontracted with noradrenaline. Noradrenaline (1 μM) was added to the medium three times, interval 60 min. MeSATP (**a**), ADPβS (**b**), UTP (**c**), ATP (**d**) or acetylcholine (**e**) was administered in a cumulative fashion during the plateau of each response to noradrenaline. NH05 was added at two increasing concentrations immediately after the first and second nucleotide or acetylcholine concentration-relaxation curve, i.e. about 50 min before the second and third concentration-relaxation curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (○), and second or third curves in the presence of NH05 (▼ 0.32; ● 1; ▲ 3.2; ■ 10 μM), as a percentage of the respective response to noradrenaline. Means ± SEM from 4 to 7 experiments

mal relaxation or both; NH01 (3.2 μM) and DIDS (1 μM) were inactive against all three agonists (n = 4–6). These compounds, in other words, lacked selectivity against the nucleotides as compared to acetylcholine, and their effects are not documented further.

The other six compounds displayed at least some selectivity against the nucleotides as compared to acetylcholine, as shown for suramin, reactive blue 2, *iso*-PPADS and NH05 in Figs. 3–6. Results with the six antagonists are summarized in Table 1. The selectivity against nucleotides (as compared to acetylcholine) was greatest for suramin (Fig. 3), reactive blue 2 (Fig. 4), *iso*-PPADS (Fig. 5) and NH05 (Fig. 6) which, in a 10- to at least 100-fold concentration range, antagonized exclusively the effect of one or several of the nucleotides (Table 1). The selectivity against nucleotides (as compared to acetylcholine) was less for PPADS and reactive red 2: PPADS antagonized exclusively the effects of MeSATP and ADPβS but not acetylcholine at a single concentration only (1 μM), and reactive red 2 did so at an only 3.2-fold concentration range (1–3.2 μM; Table 1).

Comparison shows that suramin, reactive blue 2, *iso*-PPADS, PPADS and reactive red 2 all were more potent against MeSATP and ADPβS than against UTP and ATP. Only NH05 was similarly potent against all four nucleotides; it was also the most potent antagonist against UTP and ATP (Fig. 6). None of the compounds differentiated clearly between MeSATP and ADPβS, or between UTP and ATP (Table 1).

Decreases by the antagonists of the maximal relaxation were often observed (Figs. 4b, 5b, 5c, 6b and indices a and b in Table 1). Moreover, there was sometimes an increase in apparent K_d with increasing antagonist concentration, and as expected the slopes of plots according to Arunlakshana and Schild (1959) differed from unity in these cases (suramin against MeSATP and ADPβS, reactive blue 2 against MeSATP; Table 1). The only case in which there was no depression of the maximal relaxation, in which the apparent K_d values were concentration-independent over a 10-fold concentration range, and in which the slope of the Schild plot therefore did not differ from unity, was *iso*-PPADS against MeSATP (Table 1).

None of the antagonists altered the resting tension of the aortic rings or the response to noradrenaline.

Contraction

In non-precontracted aortic rings with intact endothelium, increasing concentrations of MeSATP, ADPβS, UTP and ATP caused increasing contraction (Fig. 7). In the case of MeSATP, ADPβS and ATP, the threshold concentrations

Table 1 Apparent K_d values of P2-receptor antagonists and effects on the concentration-relaxation curve of acetylcholine. K_d values were calculated from the experiments of Figs. 3–6 and additional experiments using the same protocol. From means of 3 to 8 experiments; *n.e.*, no effect at concentration indicated

Antagonist, μM		Apparent K_d value (μM) against				Change of acetylcholine concentration-relaxation curve
		MeSATP	ADPβS	UTP	ATP	
Suramin	3.2	*n.e.*	1.5			*n.e.*
	10	2.4	2.6	*n.e.*	*n.e.*	*n.e.*
	32	3.6	2.6	25.6	12.0	*n.e.*
	100	4.7	5.7	36.5	26.1	*n.e.*
	320					*n.e.*
Reactive blue 2	1	0.51	0.82			
	3.2	0.54	0.47			*n.e.*
	10	0.84	1.57[a]	*n.e.*	*n.e.*	*n.e.*
	32		0.69[a]	6.5	21.0	*n.e.*
	100					shifted to right
iso-PPADS	0.32	0.08	0.18			*n.e.*
	1	0.11	0.19	*n.e.*		*n.e.*
	3.2	0.08	0.05[a]	1.7[a]	*n.e.*	*n.e.*
	10		–[b]	3.6[a]	3.3	*n.e.*
	32					shifted to right and maximum decreased
PPADS	1	0.43	0.18	*n.e.*	*n.e.*	*n.e.*
	3.2					shifted to right
Reactive red 2	1	0.82[a]	0.33[a]			*n.e.*
	3.2	–[b]	–[b]	*n.e.*	*n.e.*	*n.e.*
	10					shifted to right
NH05	0.32		*n.e.*	0.19		*n.e.*
	1	0.44	0.34[a]	0.21	1.0	*n.e.*
	3.2	0.35	–[b]	0.33[a]	1.8	*n.e.*
	10					shifted to right

[a] maximum of concentration-relaxation curve reduced, but by less than 50%; apparent K_d value determined according to Kenakin (1993)
[b] maximum of concentration-relaxation curve reduced by more than 50%; K_d determination according to Kenakin (1993) not feasible

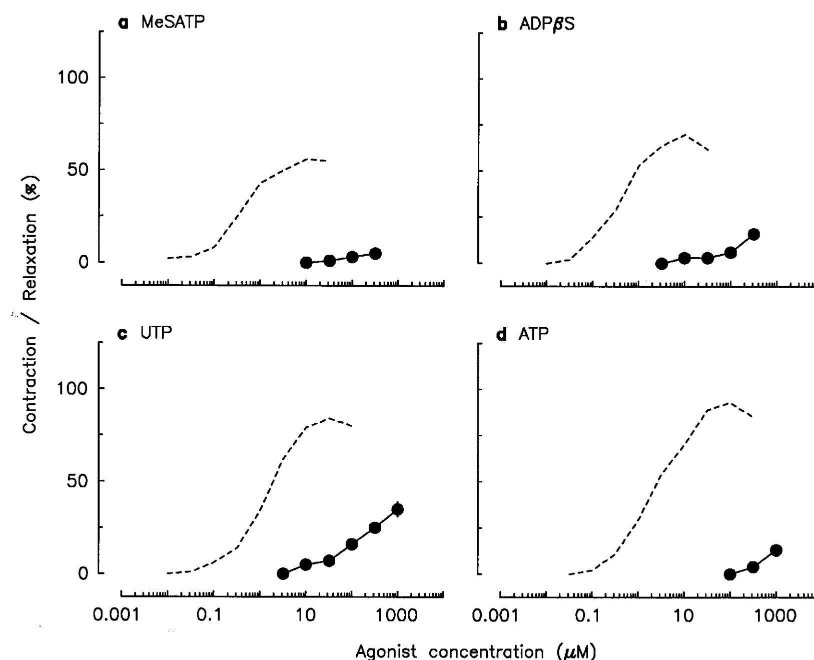

Fig. 7 a–d Nucleotide-evoked contractions of non-precontracted rat aortic rings. Three concentration-contraction curves were determined in each ring, interval 60 min, each for a different agonist. MeSATP (**a**), ADPβS (**b**), UTP (**c**) or ATP (**d**) was administered in a cumulative fashion. *Abscissae*, agonist concentration. *Ordinates* show contraction (●) as a percentage of an initial contraction caused by noradrenaline (1 μM). Means ± SEM from 3 to 5 experiments. Concentration-relaxation curves are shown for comparison (*dashed lines*; first curves from Fig. 3)

for contraction were similar to, or even higher than, the concentrations causing maximal relaxation. In the case of UTP, the concentration-response curves for relaxation and contraction overlapped to a greater extent (Fig. 7).

Removal of nucleotides from the medium

Pieces of rat aorta removed added MeSATP, UTP and ATP from the medium. MeSATP and ATP were removed at similar rates and more rapidly than UTP (Fig. 8).

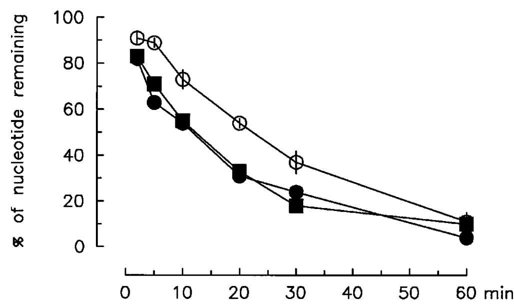

Fig. 8 Removal of exogenous nucleotides from the medium. Pieces of rat aorta were incubated in medium containing MeSATP (■), UTP (○) or ATP (●), initial concentration 10 μM. *Abscissae*, incubation time. *Ordinates* show the percentage of nucleotide remaining in the medium, corrected for spontaneous loss in the absence of tissue. Means ± SEM from 5 to 6 experiments

Table 2 Effect of P2-receptor antagonists on the removal of MeSATP, UTP and ATP from the medium by pieces of rat aorta. Nucleotides (initial concentration 10 μM) were incubated with the tissue for 30 min. Nucleotide removal was corrected for spontaneous decay. Means ± SEM from 5 to 12 experiments

Antagonist, μM		Percentage removal of		
		MeSATP	UTP	ATP
–		72 ± 4	65 ± 2	76 ± 2
Suramin	100	75 ± 2	59 ± 4	69 ± 2
Reactive blue 2	32	73 ± 1	58 ± 3	66 ± 3
iso-PPADS	10	71 ± 3	59 ± 2	70 ± 3
PPADS	3.2	75 ± 3	59 ± 7	72 ± 5
Reactive red 2	3.2	67 ± 4	63 ± 5	67 ± 3
NH05	3.2	72 ± 3	73 ± 3	77 ± 3
Evans blue	100	44 ± 2*	8 ± 7**	38 ± 3**

* and ** denote significant differences ($P < 0.05$ and $P < 0.01$, respectively) from control (no antagonist)

The effect of P2-receptor antagonists on the removal of the nucleotides was assessed using a nucleotide incubation period of 30 min. At the highest concentration tested against nucleotide-evoked relaxation (Table 1; in the case of PPADS even at a higher concentration), none of the antagonists caused any change (Table 2). Evans blue (100 μM), a potent ecto-nucleotidase inhibitor (Bültmann et al. 1995), reduced the removal of MeSATP, UTP and ATP (Table 2).

Discussion

Nucleotide-evoked relaxation

As shown previously (see Introduction), exposure to MeSATP, ADPβS, UTP and ATP led to relaxation of noradrenaline-precontracted rings of the rat aorta. Removal of the endothelium abolished, and the NO-synthase inhibitor L-NAME greatly attenuated, the response to MeSATP, ADPβS and UTP (Fig. 1a–c); an excess of L-arginine overcam the attenuation by L-NAME, at least to a great extent. The relaxation elicited by these nucleotides, therefore, was entirely due to activation of endothelial receptors, and nitric oxide was the main mediator (see Saïag et al. 1996 for a possible contribution of prostacyclin). ATP, on the other hand, relaxed also endothelium-denuded preparations, although only at high concentrations (cf. Rapoport et al. 1984; White et al. 1985; Rose' Meyer and Hope 1990; Dominiczak et al. 1991; García-Velasco et al. 1995), and its effect was attenuated much less by L-NAME (Fig. 1d; cf. Dominiczak et al. 1991; Frew et al. 1993; but see Mombouli and Vanhoutte 1993). It has been suggested that the endothelium-independent relaxation caused by ATP is mediated – presumably after conversion of ATP to adenosine – by an adenosine A_2-receptor located on the smooth muscle cells (Rose'Meyer and Hope 1990; see also Prentice and Hourani 1996).

Our rank order of agonist potency MeSATP > UTP > ATP for the endothelium-dependent relaxation is similar to previous studies (Dainty et al. 1991; García-Velasco et al. 1995) and suggests that more than a single P2-receptor is involved (see Introduction). This view is supported by the agonist interactions (Fig. 2). They confirm previous findings (Dainty et al. 1991) and indicate, firstly, that MeSATP and ADPβS act on a common receptor, and secondly, that this receptor is distinct from the receptor (or receptors; see below) for UTP and ATP.

Effects of the antagonists

Difficulties are commonly encountered when P2-receptor antagonists are studied in intact tissues (cf. Bültmann et al. 1996a,c). For example, the antagonists often produce non-P2-receptor effects. In the present study, non-P2 effects showed up as an attenuation of the response to acetylcholine. In order to avoid erroneous interpretation, the three antagonists that were not selective for nucleotides at all were excluded from any further evaluation (XAMR0721, NH01 and DIDS); as to the remaining six 'selective' antagonists, conclusions will be based solely on concentrations that did not alter the relaxation caused by acetylcholine (Table 1). Secondly, the antagonists frequently act in a non-competitive manner. In the present study, depressions of maximal responses to nucleotides and slopes of Schild regressions different from unity indicated non-competitive kinetics. Thirdly, antagonists may block the breakdown of nucleotides (see Crack et al. 1994; Bültmann et al. 1995). Pieces of rat aorta in fact removed MeSATP, UTP and ATP from the medium, presumably by ecto-nucleotidase-catalyzed breakdown. However, with the exception of Evans blue (Bültmann et al. 1995), which was not used for receptor characterization, this breakdown was not attenuated by the antagonists (Table 2). Apparently the nucleotide-degrading enzymes of rat aorta differ from those of other tissues which are inhibited by the same concentrations of at least some of the antagonists (e.g., Bültmann et al. 1996a; Ziganshin et al. 1996). Fourthly, antagonist effects become difficult to interpret when agonists activate more than a single P2-re-

ceptor. In the present work, all nucleotides also activated a contraction-mediating receptor (Fig. 7; cf. White et al. 1985; Dominiczak et al. 1991; Mombouli and Vanhoutte 1993; García-Velasco et al. 1995). However, in the case of MeSATP, ADPβS and ATP contractions were minimal and concentration-contraction curves far to the right of concentration-relaxation curves. UTP was the exception, with more prominent contraction and a smaller distance of contraction and relaxation curves (Fig. 7). When an agonist (such as UTP) causes relaxation at low and contraction at moderately higher concentrations, and when an antagonist selectively blocks the relaxation-mediating receptor, then the superimposed contraction component may lead to overestimation of the apparent receptor affinity of the antagonist. The concomitant activation of a contraction mechanism by UTP, therefore, does not explain one of our decisive findings, namely the lower potency of five of the six 'selective' antagonists against UTP than against MeSATP and ADPβS (see below).

Assuming then that most of the common difficulties with P2 antagonists were minimized in the present study, the observations with the six 'selective' antagonists permit three conclusions. First, since none of the six differentiated clearly between MeSATP and ADPβS, these agonists in fact share a common endothelial receptor in rat aorta. Second, since five of the six (the exception being NH05) were considerably less potent against UTP than against MeSATP and ADPβS, UTP in fact acts on a different receptor. Third, since ATP behaved like UTP in terms of antagonism, two of the six 'selective' antagonists being ineffective and the other four similarly potent against either, there is no reason to postulate different sites of action of UTP and ATP: both seem to act at the same site. (Reactive blue 2 and NH05 tended to be more potent against UTP than against ATP; one possible reason is the concomitant activation by UTP, but not by ATP, of a contraction mechanism as discussed in the preceding paragraph.)

The first two conclusions agree with those from the agonist interaction experiments discussed above. The third conclusion goes beyond in that it indicates identity of the sites of action of UTP and ATP. The endothelial receptors thus dissected by means of antagonists doubtlessly are the P2Y-receptor (for MeSATP and ADPβS) and the P2U-receptor (for UTP and ATP) that are assumed to exist in a number of endothelial cell preparations (see Introduction). ATP can act in principle on both (Pirotton et al. 1993; Boarder et al. 1995). However, as in rat aorta it acts mainly at P2U-receptors in the endothelium of the bovine aorta (Motte et al. 1993; Wilkinson et al. 1994b) and of the golden hamster mesenteric vascular bed (Ralevic and Burnstock 1996a).

An alternative receptor pattern in rat aortic endothelium was suggested by García-Velasco et al. (1995): viz., that MeSATP, UTP and ATP each acted at a separate site, the receptor for UTP being a 'pyrimidinoceptor' insensitive to ATP (Häussinger et al. 1987; von Kügelgen et al. 1987). The suggestion was based on the observation that reactive blue 2 (100 μM) attenuated the endothelium-dependent relaxation produced by ATP but did not alter responses to MeSATP and UTP. This finding stands in contrast to our study: 100 μM, the concentration inactive against MeSATP and UTP in the hands of García-Velasco et al. (1995), is about 100 times the apparent K_d of reactive blue 2 against MeSATP and ADPβS and about 10 times its apparent K_d against UTP in the present experiments (Table 1). We have no explanation for the discrepancy. Cibacron blue 3GA is an isomer that is often used as 'reactive blue 2' (see footnote in von Kügelgen et al. 1994). However, 1 μM cibacron blue 3GA also antagonized the relaxant effect of both MeSATP and ATP in rat aorta (Bültmann and Tuluc, unpublished).

Do the endothelial P2Y- and P2U-receptors of rat aorta resemble other P2Y- and P2U-receptors in their antagonist sensitivity profiles?

Some P2Y-receptors differ clearly from those of the rat aorta. For example, at the P2Y-receptor of the guinea-pig taenia coli, reactive blue 2 is considerably less potent (K_d value 7.8–18.2 μM; Bültmann et al. 1996a) than in rat aorta while reactive red 2 is considerably more potent (K_d 0.028 μM; Bültmann and Starke 1995). The P2Y-receptor in bovine aortic and pulmonary endothelial cells, on the other hand, is blocked by suramin and reactive blue 2 with K_d values (2.2–3.2 and 0.48 μM, respectively; Wilkinson et al. 1993; Chen et al. 1996a) close to those in rat aorta and, like the rat aortic P2Y-receptor, is sensitive to 1 μM of PPADS (Brown et al. 1995), suggesting pharmacological similarity. A P2Y-receptor has been cloned from bovine endothelial cells and shown to be the bovine homologue of the $P2Y_1$-receptor (Henderson et al. 1995; see Burnstock and King 1996 for nomenclature). A rat $P2Y_1$-receptor has also been cloned (Tokuyama et al. 1995). Data on its pharmacological properties are sparse and contradictory (K_d of PPADS about 0.1 μM at rat ileal $P2Y_1$-receptors vs. > 100 μM at rat brain microvascular endothelial $P2Y_1$-receptors; Pacaud et al. 1996; Webb et al. 1996; see also Boyer et al. 1994).

The existence of subtypes of the P2U-receptor has been suggested on the basis of their sensitivity to suramin (Dainty et al. 1994): P2U-receptors were blocked by suramin (100 μM) in rat aortic endothelium (Dainty et al. 1994) and several other tissues (e.g. PC12 cells: Murrin and Boarder 1992; bovine adrenal medullary endothelial cells: Mateo et al. 1996) but not in canine tracheal epithelium (Dainty et al. 1994) and bovine aortic endothelium (Wilkinson et al. 1993, 1994b). Our results confirm the suramin sensitivity of the rat aortic endothelial receptors. A P2U-receptor has recently been cloned from rat alveolar cells and the pituitary gland and shown to be the rat homologue of the $P2Y_2$-receptor (Rice et al. 1995; Chen et al. 1996b; see Burnstock and King 1996). The cloned pituitary P2U-receptor was blocked by suramin (100 μM; Chen et al. 1996b) and, hence, may be pharmacologically similar to that in rat aorta.

P2-receptor-mediated endothelium-dependent vasodilation has been demonstrated in rat blood vessels other than the aorta. P2Y-receptors mediate vasodilator effects of MeSATP or ADPβS in the coronary (Fleetwood and Gordon 1987; Hopwood and Burnstock 1987; Vials and

Burnstock 1994), mesenteric (Ralevic and Burnstock 1988, 1996b; Windscheif et al. 1994), pancreatic (Saïag et al. 1996), renal (Churchill and Ellis 1993; Eltze and Ullrich 1996) and pulmonary (Liu et al. 1989) vasculature. Suramin and reactive blue 2 blocked the coronary, renal and pulmonary, and PPADS blocked the mesenteric, receptors at concentrations similar to those found effective against MeSATP and ADPβS in the present work. P2U-receptors insensitive to PPADS mediate the relaxant effect of UTP and ATP in the mesenteric arterial bed (Ralevic and Burnstock 1996b; Windscheif et al. 1994). Taken together with the present results, these observations suggest that endothelial P2-receptors are identical throughout the rat vasculature (but see the PPADS-insensitive $P2Y_1$-receptor in rat brain microvascular endothelial cells mentioned above: Webb et al. 1996).

Conclusion

The approach of this study is new for endothelial P2-receptors: comparison by means of a series of antagonists. The results indicate that nucleotides cause endothelium-dependent relaxation of the rat aorta via two sites: a P2Y-receptor activated by MeSATP and ADPβS and pharmacologically similar to the P2Y ($P2Y_1$) subtype of bovine vascular endothelial cells; and a P2U-receptor which is the common site of action of UTP and ATP and possibly pharmacologically similar to the cloned rat P2U ($P2Y_2$) subtype. The relaxation mediated by either subtype is primarily due to release of nitric oxide. Suramin, reactive blue 2, *iso*-PPADS, PPADS and reactive red 2 are all considerably more potent antagonists at the P2Y- than the P2U-receptor. NH05 does not discriminate between the two receptors but is the most potent antagonist of a P2U-receptor-mediated effect so far described.

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