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Pharmacological characterization of nucleotide P2Y receptors on endothelial cells of the mouse aorta

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1 Nucleotides regulate various effects including vascular tone. This study was aimed to characterize P2Y receptors on endothelial cells of the aorta of C57BL6 mice. Five adjacent segments (width 2 mm) of the thoracic aorta were mounted in organ baths to measure isometric force development.

2 Nucleotides evoked complete (adenosine 5′ triphosphate (ATP), uridine 5′ triphosphate (UTP), uridine 5′ diphosphate (UDP); >90%) or partial (adenosine 5′ diphosphate (ADP)) relaxation of phenylephrine precontracted thoracic aortic rings of C57BL6 mice. Relaxation was abolished by removal of the endothelium and was strongly suppressed (>90%) by inhibitors of nitric oxide synthesis.

3 The rank order of potency was: UDP ~ UTP ~ ADP > adenosine 5′-[γ-thio] triphosphate (ATPγS) > ATP, with respective pD_2 values of 6.31, 6.24, 6.22, 5.82 and 5.40. These results are compatible with the presence of $P2Y_1$ (ADP > ATP), $P2Y_2$ or $P2Y_4$ (ATP and UTP) and $P2Y_6$ (UDP) receptors.

4 $P2Y_4$ receptors were not involved, since $P2Y_4$-deficient mice displayed unaltered responses to ATP and UTP.

5 The purinergic receptor antagonist suramin exerted surmountable antagonism for all agonists. Its apparent pK_b for ATP (4.53 ± 0.07) was compatible with literature, but the pK_b for UTP (5.19 ± 0.03) was significantly higher. This discrepancy suggests that UTP activates supplementary non-$P2Y_2$ receptor subtype(s).

6 Further, pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS) showed surmountable (UTP, UDP), nonsurmountable (ADP) or no antagonism (ATP).

7 Finally, 2′-deoxy-N^6-methyladenosine3′,5′-bisphosphate (MRS2179) inhibited ADP-evoked relaxation only.

8 Taken together, these results point to the presence of functional $P2Y_1$ (ADP), $P2Y_2$ (ATP, UTP) and $P2Y_6$ (UDP) receptors on murine aorta endothelial cells. The identity of the receptor(s) mediating the action of UTP is not fully clear and other P2Y subtypes might be involved in UTP-evoked vasodilatation.
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Abbreviations: ACh, acetylcholine; ADP, adenosine 5′ diphosphate; ATP, adenosine 5′ triphosphate; ATPγS, adenosine 5′-[γ-thio] triphosphate; MRS2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid; UDP, uridine 5′ diphosphate; UTP, uridine 5′ triphosphate

Introduction

Purine and pyrimidine nucleotides are released under a variety of stress circumstances. Platelet degranulation and shear stress or damage of the endothelial lining cause extracellular release of adenosine triphosphate (ATP) and uridine triphosphate (UTP) (John & Barakat, 2001; Lazarowski & Boucher, 2001). Extracellular nucleotides act through cell surface receptors which can be divided in the P2Y and the P2X receptor families (Ralevic & Burnstock, 1998). P2X receptors are membrane ion channels made by the assembly of subunits of the same (homo-oligomers) or different (hetero-oligomers) subtypes. The P2X receptor family consists of seven subtypes ($P2X_{1-7}$) that are mainly activated by ATP (North, 2002). P2Y receptors consist of seven membrane spanning domains and eight subtypes have been identified: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Van Der Giet *et al.*, 2002; Abbracchio *et al.*, 2003). P2Y receptors are coupled *via* G-protein to phospholipase C, resulting in IP_3 generation and Ca^{2+} release from intracellular stores, or to stimulation/inhibition of adenylate cyclase (Boarder & Hourani, 1998).

$P2Y_1$ and $P2Y_2$ are the major nucleotide receptors on human vascular endothelial cells (Wang *et al.*, 2002) and mediate vasodilatation by releasing nitric oxide (NO),

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prostanoids or endothelium-derived hyperpolarizing factor (EDHF) (Wihlborg *et al.*, 2003). Rat vascular endothelial cells express $P2Y_1$ and $P2Y_2$ receptors (Hansmann *et al.*, 1997; Kumari *et al.*, 2003) that mediate relaxation mainly *via* the NO pathway (Buvinic *et al.*, 2002). In contrast, vascular smooth muscle cells express multiple P2Y and P2X receptors (Kunapuli & Daniel, 1998) that cause contraction. As a consequence, nucleotides are important regulators of the arterial tone.

Little is known about nucleotide receptors mediating vasodilatation of murine blood vessels. We reported that ATP induces relaxation in the murine aorta (Crauwels *et al.*, 2003). It has already been proposed that this ATP response is mediated by $P2Y_1$ and $P2Y_2$ receptors (Beny, 2004). However, no rank order of potency has been determined. The present study was aimed to further characterize endothelial P2Y receptors. To this end, different nucleotides (ATP, adenosine 5′ diphosphate (ADP), uridine 5′ triphosphate (UTP), uridine 5′ diphosphate (UDP), adenosine 5′-[γ-thio] triphosphate (ATPγS)) were tested to determine their rank order of relaxation potency. Further, the functionality of the $P2Y_4$ receptor subtype was determined in $P2Y_4^{-/0}$-mice. Finally, in antagonist studies with selective (2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179) (Kaiser & Buxton, 2002)) and nonselective (suramin and pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS) (Boarder & Hourani, 1998; Bowler *et al.*, 2003)) P2Y antagonists, apparent pK_b values were calculated. Since pK_b values of competitive receptor antagonists are independent of the type and concentration of the agonist, apparent pK_b values are important tools for the identification of the different P2Y subtypes.

Methods

Mice

The studies were approved by the Ethical Committee of the University of Antwerp, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. C57BL6 mice (age 25 ± 7 weeks, body weight 26.3 ± 3.6 g) were used. $P2Y_4^{-/0}$ and the corresponding wild-type (WT) mice (CD1/129SV strain, age 12–18 weeks, body weight 31.3 ± 4.5 g) were used to test the role of the $P2Y_4$ subtype. Genotyping was carried out using two primer sets as previously described (Robaye *et al.*, 2003).

Isolating and mounting of blood vessels

After anaesthesia (sodium pentobarbital, 75 mg kg^{-1}, i.p.), the aorta was carefully removed, stripped of adherent tissue and dissected systematically. The root was defined as the first 1 mm of the ascending aorta outside the heart. The thoracic aorta was divided in five sequential 2 mm wide segments (TA1 → TA5) starting 3 mm from the origin of the left subclavian artery (where the azygos vein crosses the aorta) down to the diaphragm (Crauwels *et al.*, 2003). Segments were mounted between two parallel tungsten wire hooks in 10 ml organ baths. Tension was measured isometrically with a Statham UC2 force transducer (Gould) connected to a data acquisition system (Moise 3, EMKA Technologies). Vessels were immersed in Krebs–Ringer solution (37°C, and continuously aerated with 95% O_2/5% CO_2, pH 7.4) with (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025 and glucose 11.1. Segments of the thoracic aorta and the aortic root were gradually stretched until a stable loading tension of 20 and 10 mN, respectively, was attained. Between each concentration–response curve, the Krebs–Ringer solution was replaced three times to wash out the agents.

Removal of the endothelial cells

Endothelial denudation was performed by injecting 5 ml of 0.01% Triton X 100 in Krebs–Ringer solution directly into the left ventricle, followed by washing with Krebs–Ringer solution. The effectiveness of the method for endothelial denudation was checked by the absence of responses to acetylcholine (ACh). Further immunohistochemical staining of the endothelial cells was carried out using a specific monoclonal antibody (CD31) and sections were developed with the ABC IgG method (Crauwels *et al.*, 2003).

Vasomotor studies

Indomethacin 10 μM was present in all studies to avoid any vasomotor interference due to contractile prostanoids (Vanhoutte *et al.*, 2005). Rings were first contracted with a depolarizing potassium solution (50 mM). After three washing steps, a cumulative concentration–response curve was made for phenylephrine (3 nM–30 μM) and the concentration (EC_{50}) resulting in 50% of the maximal contraction (E_{max}) was assessed for each vessel segment. At the top phenylephrine concentration segments were exposed for 15 min to the purinergic agonist (7×10^{-5} M) followed by three washing steps. This pretreatment was used to minimize the shift between the first and second curve seen with all purinergic agonists. In relaxation studies, vessels were precontracted with their individual EC_{50} of phenylephrine, followed by cumulative concentration–response curves for ATP, UTP, ADP, UDP, ATPγS or adenosine. To evaluate the contribution of the endothelium, the agonist experiments were repeated in vessels without endothelium. The role of eNOS was assessed with the combination of the NOS inhibitors N^{ϖ}-nitro-L-arginine (LNA) (300 μM) and N^{ϖ}-nitro-L-arginine methyl ester (L-NAME) (300 μM), both incubated for 20 min.

In antagonist studies, the first cumulative concentration–response curve was performed in the absence of antagonists and was repeated three times in the presence of increasing concentrations of suramin (10–30–100 μM), PPADS (1–3–10 μM) or MRS2179 (1–3–10 μM), added to the organ bath 20 min prior to the nucleotide cumulative concentration–response curve. To minimize time effects, all results were compared to a ring that was not treated with the antagonist. In antagonist studies with UTP and UDP, only the top antagonist concentrations were used.

Calculation of pD_2 *and apparent* K_b *values*

To normalize the cumulative concentration–response curves, the precontraction level was set to 100%. All results are shown as % relaxation, which is 100% − % contraction. EC_{50} values (p$D_2 = -\log EC_{50}$) were obtained from individual concentration–response curves by curve fitting to a four-parameter logistic function (Prism GraphPad Software; version 4.0). To

classify the P2Y receptors, the apparent K_b values of each antagonist were calculated using the EC_{50} values of each individual ring (Furchgott, 1972) with correction for possible time effects:

$$K_b = X_b/(R/Rt - 1)$$

X_b is the antagonist concentration, R is the ratio of the EC_{50} with antagonist and the first EC_{50} without antagonist, R_t is the ratio of the EC_{50} of the second, third or fourth EC_{50} and the first EC_{50} in the segments not exposed to the antagonist.

When different concentrations of antagonist were used, the Schild plot analysis was performed to determine the pA_2 and the slope of the curve.

Statistical analysis

All results are expressed as mean $\pm$ s.e.m.; n represents the number of mice. For statistical analysis, the SPSS for Windows package was used. A 5% level of significance was selected. Results were compared to the corresponding time control and evaluation was done using one-way analysis of variance (ANOVA) or Student's t-test.

Materials

Sodium pentobarbital (Nembutal®) was obtained from Sanofi (Brussels, Belgium), indomethacin from Federa (Belgium), antibody against CD31 from BD (Erembodegem, Belgium), ABC kit from Vektor Laboratories (Burlingame, CA, U.S.A.) and OCT from Klinipath (The Netherlands). Phenylephrine hydrochloride, LNA, L-NAME, all nucleotides and all antagonists were obtained from Sigma (Bornem, Belgium).

Results

Agonist studies

After precontraction of the thoracic aorta segments (to 12.24 ± 3.28 mN, $n = 15$) with the EC_{50} of phenylephrine (256 ± 97 nM, $n = 15$), nucleotides evoked concentration-dependent complete (ATP, ATPγS, UTP and UDP) or partial (ADP) relaxation (Figures 1 and 2). In the thoracic aorta, EC_{50} values displayed the following rank order of potency: UDP $\sim$ UTP $\sim$ ADP $>$ ATPγS $>$ ATP (Table 1). Adenosine, a potential degradation product of adenosine nucleotides, induced small relaxations but only at high concentrations (30 μM or more, Figure 2c).

The nucleotide-evoked relaxations disappeared after endothelial cell removal, while the phenylephrine contraction remained. In contrast, adenosine-evoked relaxation remained after removal of the endothelial cells (not shown). The efficacy of endothelial denudation was confirmed by the disappearance of CD31 staining (Figure 1) and the absence of relaxation after ACh administration (not shown).

There were no regional differences in sensitivity or maximal amplitude among the five adjacent segments within the thoracic aorta (e.g. pD_2 of ATP: TA1 5.38 ± 0.05, TA2 5.29 ± 0.06, TA3 5.50 ± 0.04, TA4 5.41 ± 0.08, TA5 5.47 ± 0.05, one-way ANOVA, $P = 0.135$). Therefore, the data of the five thoracic aorta segments were used to calculate the

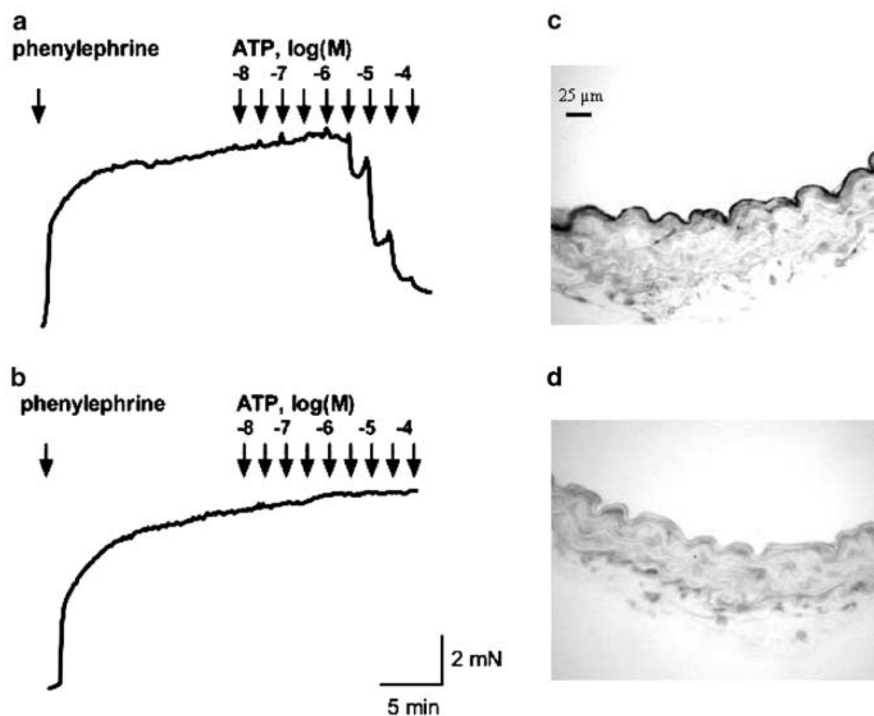

Figure 1 Representative tracings of concentration–response curves of purinergic agonists. ATP induced relaxation in phenylephrine precontracted blood vessels with endothelium (a), but not in endothelium-denuded rings (b). Staining with an antibody against CD31 confirmed the effectiveness of the endothelial denudation. Micrographs of a vessel with endothelium (c) and an endothelial-denuded vessel (d).

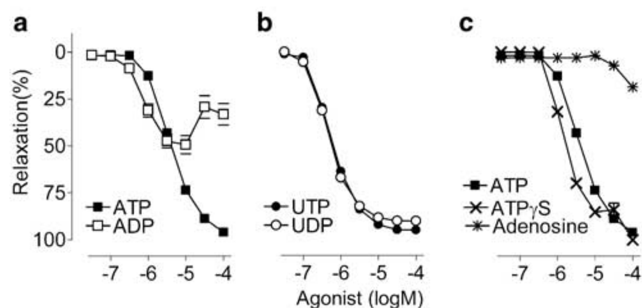

Figure 2 Cumulative concentration–response curves of phenylephrine precontracted thoracic aorta segments. (a) ATP evoked complete relaxation, whereas ADP evoked partial relaxation of the thoracic aorta. (b) UTP and UDP both evoked potent and complete relaxation. (c) ATPγS was more potent than ATP, while adenosine caused a small relaxation. Mean ± s.e.m.; $n = 5$.

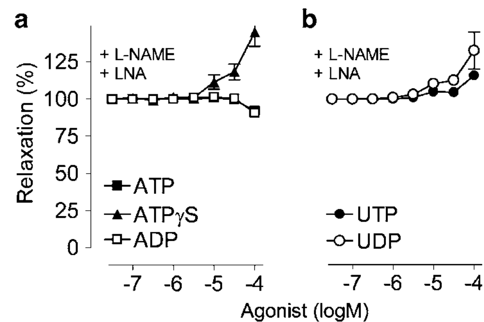

Figure 3 Cumulative concentration–response curves for (a) ATP, ADP and ATPγS. (b) UTP and UDP of phenylephrine precontracted thoracic aorta segments in the presence of the combination of the NOS inhibitors LNA (300 μM) and L-NAME (300 μM). Mean ± s.e.m.; $n = 5$.

Table 1 pD_2 values of nucleotides inducing endothelium-dependent relaxations in phenylephrine precontracted segments of the thoracic aorta and the aortic root

	Thoracic aorta −log (M)	*Aortic root −log (M)*	P-*value*[a]
ATP	5.40 ± 0.01	5.52 ± 0.07	0.1281
ATPγS	5.82 ± 0.02	6.12 ± 0.08	0.0066
ADP	6.22 ± 0.07	6.56 ± 0.12	0.0401
UTP	6.24 ± 0.02	6.91 ± 0.09	< 0.0001
UDP	6.31 ± 0.02	6.69 ± 0.05	0.0001

Values represent mean ± s.e.m. of five mice (ATPγS, $n = 3$).
[a]Aortic root *versus* thoracic aorta (Student's *t*-test).

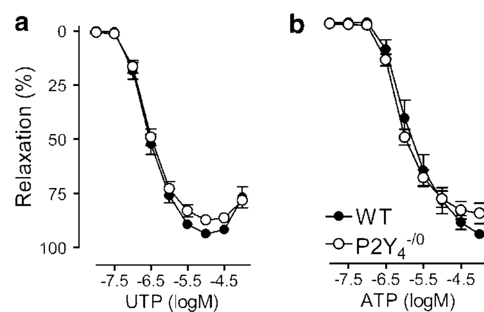

Figure 4 Cumulative dose–response curves in the thoracic aorta segments of $P2Y_4^{-/0}$ and WT mice for (a) UTP and (b) ATP. Mean ± s.e.m.; $n = 6$.

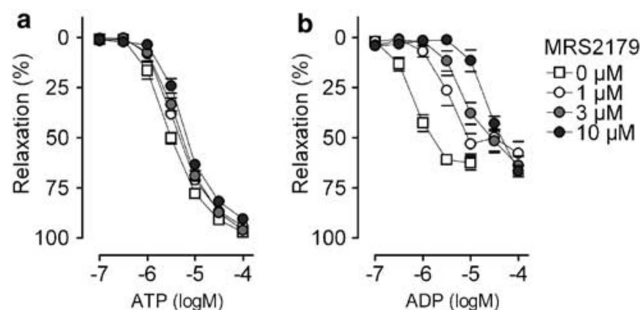

Figure 5 Cumulative dose–response curves in segments of the thoracic aorta for (a) ATP and (b) ADP in the presence of MRS2179, a selective $P2Y_1$ receptor antagonist. Mean ± s.e.m.; $n = 5$.

average of each thoracic aorta. Segments of the aortic root were more sensitive to ADP, UTP, UDP and ATPγS than those of the thoracic aorta (Table 1). Furthermore, ADP evoked complete relaxation of the aortic root in contrast to partial relaxation of the thoracic aorta (not shown).

In the presence of the combination of LNA and L-NAME, the relaxation was almost completely abolished (ATP and ADP) or converted to a weak contraction (ATPγS, UTP and UDP) (Figure 3).

$P2Y_4$-deficient mice

$P2Y_4^{-/0}$ and WT mice displayed identical relaxation curves for ATP as well as for UTP. For ATP- and UTP-evoked relaxation, $P2Y_4^{-/0}$ mice did not differ from WT mice, both with respect to the maximal amplitude, the slope of the curve and the sensitivity (Figure 4).

Antagonist studies

MRS2179, a selective $P2Y_1$ receptor antagonist, caused a rightward shift of the ADP concentration–response curves (Figure 5), without affecting ATP, UTP or UDP responses (Table 2).

Suramin caused concentration-dependent shifts of all agonist concentration–response curves, without suppressing the maximum (Figure 6). Apparent pK_b values were highest for ADP, intermediate for UTP and UDP and lowest for ATP.

In contrast, PPADS displayed surmountable (UTP and UDP) and nonsurmountable (ADP) antagonism. Relaxation evoked by ATP was not affected by PPADS (Figure 7). The apparent pK_b for UDP was higher ($P = 0.0039$) than that for UTP (Table 2).

Schild plot analysis of antagonists studies resulted in a pA_2 of suramin of 4.48 (ATP) (confidence limits: 4.34–4.81) and of 5.64 (ADP) (confidence limits: 5.10–8.40). The pA_2 of MRS2179 for ADP was 7.29 (confidence limits: 6.59–9.77). All slopes did not differ from unity. The pA_2's of PPADS could not be determined since the antagonism was not surmountable.

Table 2 Apparent pK_b values of different antagonists in isolated thoracic aorta and aortic root

Tissue *Agonist*	*Suramin* *−log(M)*	*PPADS* *−log(M)*	*MRS2179* *−log(M)*
Thoracic aorta			
ATP	4.53±0.07	<5	<5
ADP	5.55±0.11	n.s.	6.62±0.09
UTP	5.19±0.03	5.22±0.10	<5
UDP	5.26±0.06	5.73±0.06	<5
Aortic root			
ADP	5.61±0.22	6.26±0.53	6.75±0.26
UDP	5.24±0.08	5.71±0.10	<5

Values represent mean±s.e.m. of five mice, except for the aortic root: $n=4$. n.s. = nonsurmountable antagonism.

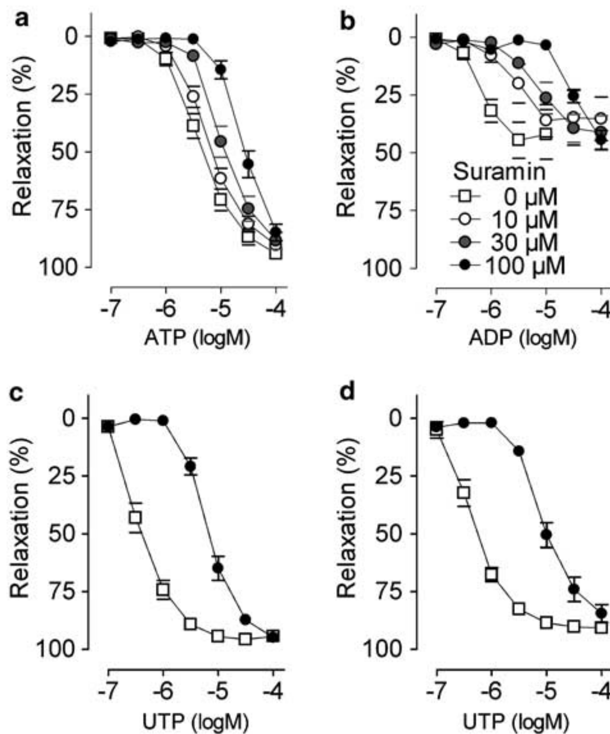

Figure 6 Cumulative dose–response curves in segments of the thoracic aorta for (a) ATP, (b) ADP, (c) UTP and (d) UDP in the presence of suramin, a nonselective purinergic receptor antagonist. Curves displayed concentration-dependent rightward shifts in the presence of suramin. Mean±s.e.m.; $n=5$.

Furthermore, apparent pK_b values for ADP and UDP antagonism were not different between root and thoracic aorta (Table 2).

Discussion

Agonist studies

Nucleotides evoked relaxation of murine aorta segments. These relaxations were endothelium-dependent as removal of the endothelial cells abolished all relaxation. Three mediators are known to evoke endothelium-dependent relaxations in humans: NO, prostanoids and endothelium-derived hyperpolarizing factor (EDHF) (Wihlborg *et al.*, 2003). The involvement of prostanoids can be excluded since indomethacin (10 μM) was always present in the Krebs–Ringer solution. The contribution of the NO pathway to the relaxation evoked by nucleotides was studied with NOS inhibitors. In the presence of the NOS inhibitors LNA and L-NAME, endothelium-dependent relaxation evoked by ATPγS, UTP and UDP was converted to a contraction while administration of ATP and ADP resulted in a small relaxation, most likely due to their degradation product adenosine. Therefore, the relaxation caused by nucleotides as seen in our experiments is solely mediated by NO derived from eNOS and neither prostanoids (indomethacin) nor endothelium-derived hyperpolarizing factor are involved. This conclusion is consistent with earlier observations of vasodilatation in mouse aortas (Crauwels *et al.*, 2000; 2003) and is fully compatible with results of P2Y$_{1/2}$-mediated vasodilatation of rat mesenteric arteries (Buvinic *et al.*, 2002).

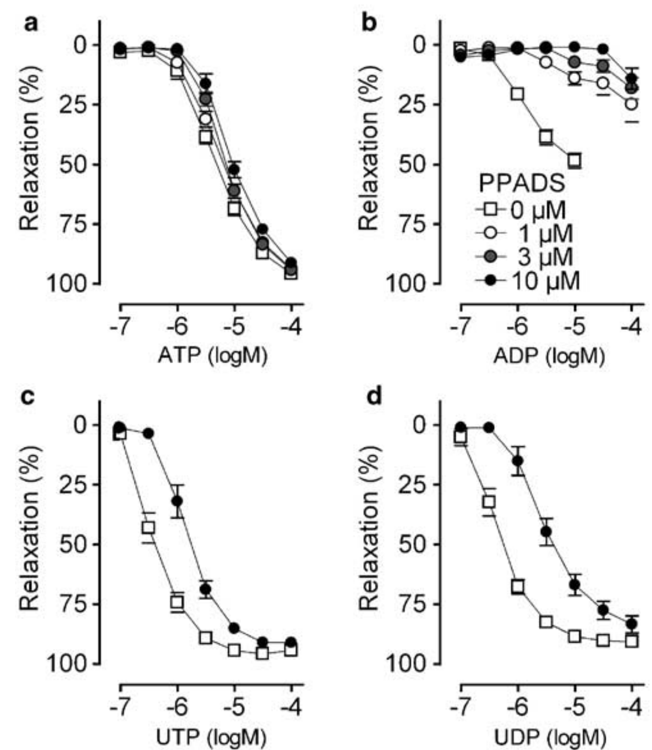

Figure 7 Cumulative dose–response curves in segments of the thoracic aorta for (a) ATP, (b) ADP, (c) UTP and (d) UDP in the presence of PPADS, a nonselective purinergic receptor antagonist. PPADS showed surmountable (UTP and UDP) or nonsurmountable (ADP) antagonism, while ATP-evoked vasodilatation was not affected. Mean±s.e.m.; $n=5$.

We found regional differences between the thoracic aorta and the aortic root. The aortic root was more sensitive to most nucleotides. Moreover, ADP evoked only partial relaxation in the thoracic aorta, whereas the aortic root relaxed completely. This could point to a smaller number of P2Y$_1$ receptors, increased activity of nucleotide degrading enzymes, greater effectiveness of contractile purinergic receptors on the SMC or lower efficacy of the NO–cyclic GMP vasodilator system in the thoracic aorta. Since ATPγS also displayed

greater sensitivity in the aortic root, differences in the activity of ATP-degrading enzymes seem unlikely. Moreover, ACh also showed a 0.5 log unit greater sensitivity in the aortic root (Crauwels *et al*., 2003), indicating that the effect is not nucleotide receptor-specific. It rather points to a greater efficacy of the NO–cyclic GMP vasodilator pathway in the aortic root.

The agonist studies provided part of the information necessary for identification of functional P2Y receptors on murine aortic endothelial cells. ADP preferentially activates $P2Y_1$ receptors (Palmer *et al*., 1998). Hence, the relaxation evoked by ADP suggests the presence of $P2Y_1$ receptors in murine aorta. However, we cannot fully exclude $P2Y_{12}$ and $P2Y_{13}$ receptors. These are mainly present on platelets, but recent evidence suggests that $P2Y_{12}$ is also expressed in human vascular smooth muscle cells, where it induces contractions (Wihlborg *et al*., 2004), and in rat brain capillary endothelial cells (Simon *et al*., 2002).

The relaxation evoked by ATP suggests the presence of functional $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors (Boarder & Hourani, 1998). ATP is very susceptible to degradation by ecto-enzymes present on the endothelial cells (Harden *et al*., 1997). Therefore, we tested the stable analogue ATPγS and adenosine, a potential degradation product of ATP and ADP. Aorta rings were slightly more sensitive to ATPγS than to ATP, suggesting a moderate degradation of ATP during the experiment. Adenosine induced only weak, endothelium-independent relaxations at very high concentrations, confirming previous experiments in mouse aorta (Talukder *et al*., 2002). Hence, a major contribution of adenosine in relaxation induced by adenosine nucleotides seems unlikely.

The relaxing effect of UTP has been attributed to $P2Y_2$ receptors (Kumari *et al*., 2003), but the $P2Y_4$ receptor might also be involved. Furthermore, an UTP-selective response has been identified in guinea pig cardiac endothelial cells (Yang *et al*., 1996): it is unclear if this response involves the $P2Y_4$ receptor, since in mouse and rat that subtype is responsive to both ATP and UTP, but the properties of the guinea pig are unknown. On the other hand, the only UDP-selective receptor is the $P2Y_6$ subtype (Communi *et al*., 1996; Lazarowski *et al*., 2001). In contrast to rat aorta (Kumari *et al*., 2003) and human arteries (Wihlborg *et al*., 2003) with a small effect of UDP in vasodilatation, the murine aortic $P2Y_6$ receptor subtype appears to play a more prominent role. Hence, the results of the agonist studies are compatible with the presence of $P2Y_1$ (ADP > ATP), $P2Y_2$ or $P2Y_4$ (ATP, UTP) and $P2Y_6$ (UDP) receptor subtypes.

$P2Y_4$-deficient mice

At present there are no clear reports of $P2Y_4$ receptors mediating vasodilatation of blood vessels. However, in rat arteries, the $P2Y_4$ subtype is associated with contractile effects (Rubino *et al*., 1999; Horiuchi *et al*., 2001) and the involvement of $P2Y_4$ subtypes in vasodilatation of blood vessels has never been excluded. The lack of distinction between $P2Y_4^{-/0}$ and WT mice with respect to both ATP- and UTP-induced relaxation responses indicates that this subtype is not involved in the endothelial-dependent responses to these agonists. This finding is compatible with the observation that mRNA of the $P2Y_4$ subtype was not detectable in murine mesenteric arteries (Vial & Evans, 2002) or murine aorta (unpublished results). Furthermore, the murine $P2Y_4$ receptor is little sensitive to suramin inhibition (Suarez-Huerta *et al*., 2001), whereas suramin displaced the curves of all agonists (vide infra).

Antagonist studies

Additional information was acquired by performing antagonist studies. Relaxation of murine aorta by ADP and ATP has been already described previously (Crauwels *et al*., 2003; Beny, 2004). However in the present study, we used MRS2179, a selective $P2Y_1$ receptor antagonist, to investigate more thoroughly the role of $P2Y_1$ receptors in ADP- and ATP-evoked vasodilatation. MRS2179 antagonized ADP-evoked relaxation, but not that elicited by ATP, UTP or UDP. This indicates that $P2Y_{12}$ (Wihlborg *et al*., 2004) or $P2Y_{13}$ (Marteau *et al*., 2003) receptors were presumably not involved. Moreover, the fact that MRS2179 did not affect ATP-evoked relaxations to any extent points out that ATP mainly activates $P2Y_2$ receptors in contrast to the suggestion made by Beny (2004).

Suramin, a nonselective purinergic antagonist exerted competitive and surmountable antagonism for all nucleotides and displayed three different classes of apparent pK_b values, ATP, ADP and UTP/UDP, indicating that at least three different P2Y subtypes are involved. The apparent pK_b values for ADP were in the same range as reported for the $P2Y_1$ (ADPβS, pK_b of 5.24) receptor of rat aorta endothelial cells (Hansmann *et al*., 1997).

The discrepancy between the apparent pK_b values of suramin for ATP and UTP indicates that ATP and/or UTP do not solely act on $P2Y_2$ receptor subtypes. Since ATP did not react with $P2Y_1$ (MRS2179 data, vide supra), plausible explanations are that ATP activates (besides $P2Y_2$ receptor subtypes) also contractile $P2X_4$ (Yamamoto *et al*., 2000) or vasodilator $P2Y_{11}$ receptor subtypes (Wang *et al*., 2002). However, to our knowledge, there is no orthologue of the $P2Y_{11}$ receptor in the murine genome. Furthermore, the apparent pK_b values of suramin for ATP found in this study (Table 2) are consistent with previous studies in cultured rat aortic smooth muscle cells (pA_2 of 4.48) (Kumari *et al*., 2003) and in rat aorta endothelial cells (apparent pK_b: 4.58). In contrast, the apparent pK_b for UTP (Table 2) of the mouse aorta is different from those of the rat, (4.45) and (4.44) (Hansmann *et al*., 1997; Kumari *et al*., 2003). This suggests that in the murine aorta, UTP activates a supplementary P2Y subtype. The $P2Y_4$ receptor is a potential candidate, but the experiments with $P2Y_4^{-/0}$ mice exclude the involvement of the $P2Y_4$ subtype. Another candidate for UTP is the $P2Y_6$ receptor subtype which is generally considered to be UDP-selective (Lazarowski *et al*., 2001). However, since UTP and UDP displayed identical apparent pK_b values for suramin and had very similar concentration–response curves in the agonist studies, the presence of a UTP- and UDP-sensitive $P2Y_6$-like receptor seems a possibility, as already proposed previously (Vial & Evans, 2002). In this hypothesis, UTP may act directly or indirectly (after breakdown to UDP) on the $P2Y_6$ receptor. Yet, this $P2Y_6$-like receptor hypothesis does not fully stroke with the different apparent pK_b values for UTP and UDP of PPADS. Hence, the identity of the UTP receptor remains unclear and requires further investigation.

Conclusion

In murine aorta nucleotides induce an endothelium-dependent relaxation that is mediated by NO. ADP ($P2Y_1$) induced partial relaxation that was antagonized by suramin, PPADS and MRS2179. ATP ($P2Y_2$) caused complete relaxation that was antagonized by suramin, but not by the $P2Y_1$-selective antagonist MRS2179. UDP ($P2Y_6$) and UTP evoked complete relaxation that was inhibited by suramin and PPADS. Further, the results of the $P2Y_4^{-/0}$ mice clearly showed that the $P2Y_4$ subtype is not involved in UTP-evoked vasodilatation. Hence, the action of UTP is mediated probably partially *via* $P2Y_2$ receptors, and partially *via* $P2Y_6$ receptors either directly or as a result of degradation into UDP. Taken together, these results point to the presence of functional $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors on murine aorta endothelial cells.

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