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Endothelium-dependent relaxation evoked by ATP and UTP in the aorta of $P2Y_2$-deficient mice

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1 Based on pharmacological criteria, we previously suggested that in the mouse aorta, endothelium-dependent relaxation by nucleotides is mediated by $P2Y_1$ (adenosine diphosphate (ADP)), $P2Y_2$ (adenosine triphosphate (ATP)) and $P2Y_6$ (uridine diphosphate (UDP)) receptors. For UTP, it was unclear whether $P2Y_2$, $P2Y_6$ or yet another subtype was involved. Therefore, in view of the lack of selective purinergic agonists and antagonists, we used $P2Y_2$-deficient mice to clarify the action of UTP.

2 Thoracic aorta segments (width 2 mm) of $P2Y_2$-deficient and wild-type (WT) mice were mounted in organ baths to measure isometric force development and intracellular calcium signalling.

3 Relaxations evoked by ADP, UDP and acetylcholine were identical in knockout and WT mice, indicating that the receptors for these agonists function normally.

4 $P2Y_2$-deficient mice showed impaired ATP- and adenosine 5′[γ-thio] triphosphate (ATPγS)-evoked relaxation, suggesting that in WT mice, ATP and ATPγS activate predominantly the $P2Y_2$ subtype.

5 The ATP/ATPγS-evoked relaxation and calcium signals in the knockout mice were partially rescued by $P2Y_1$, as they were sensitive to 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179), a $P2Y_1$-selective antagonist.

6 In contrast to ATP, the UTP-evoked relaxation was not different between knockout and WT mice. Moreover, the action of UTP was not sensitive to MRS2179. Therefore, the action of UTP is probably mediated mainly by a $P2Y_6$(like) receptor subtype.

7 In conclusion, we demonstrated that ATP-evoked relaxation of the murine aorta is mainly mediated by $P2Y_2$. But this $P2Y_2$ receptor has apparently no major role in UTP-evoked relaxation. The vasodilator effect of UTP is probably mediated mainly by a $P2Y_6$(like) receptor.
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Abbreviations: ACh, acetylcholine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPγS, adenosine 5′[γ-thio] triphosphate; MRS2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate; NO, nitric oxide; UDP, uridine diphosphate; Up_3U, P^1,P^4-di(uridine 5′-) triphosphate; Up_4U (INS365), P^1,P^4-di(uridine 5′-) tetraphosphate; UTP, uridine triphosphate; WT, wild type

Introduction

Nucleotides are important regulators of various biological functions. They are released in physiological and pathophysiological circumstances. Hypoxia, damage of the endothelial lining and platelet degranulation give rise to massive release of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and uridine triphosphate (UTP) into the circulation (Lazarowski *et al*., 2000; Lazarowski & Boucher, 2001; Di Virgilio & Solini, 2002). These nucleotides act through cell-surface receptors, which can be divided into 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 has seven subtypes ($P2X_{1-7}$) that are all 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-proteins to phospholipase C, resulting in inositol 1,4,5-trisphosphate generation and Ca^{2+} release from intracellular stores, or in stimulation/inhibition of adenylate cyclase (Boarder & Hourani, 1998).

In humans, endothelial P2Y receptors mediate vasodilatation by releasing nitric oxide (NO), prostanoids or endothelium-derived hyperpolarizing factor (Wihlborg *et al*., 2003), whereas in rat and mouse, P2Y receptors mediate endothelium-dependent relaxation mainly *via* the NO pathway (Buvinic *et al*., 2002; Guns *et al*., 2005). In contrast, vascular smooth muscle cells express multiple P2Y and P2X receptors

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(Kunapuli & Daniel, 1998) that cause contraction. As a consequence, nucleotides are important regulators of the arterial tone.

In the past, we demonstrated that nucleotides evoked complete (ATP, UTP, uridine diphosphate (UDP); >90%) or partial (ADP) relaxation of phenylephrine precontracted thoracic aortic rings of wild-type (WT; C57Bl6) mice. These results pointed to the presence of functional $P2Y_1$ (ADP> ATP), $P2Y_2$ (ATP and UTP) and $P2Y_6$ (UDP) receptors. However, apparent pK_b values of purinergic antagonists such as suramin and pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid suggested that the action of UTP was not exclusively due to the $P2Y_2$ receptor subtype (Guns *et al*., 2005). Therefore, we used $P2Y_2$-deficient mice to investigate further the action of UTP. In addition, the vasodilator effects of the nucleotides ATP, adenosine 5′[γ-thio] triphosphate (ATPγS), ADP, UDP and of acetylcholine (ACh) were tested on the thoracic aorta segments. Further, we determined the effect of the dinucleotides P^1,P^4-di(uridine 5′-) triphosphate (Up_3U) and P^1,P^4-di(uridine 5′-) tetraphosphate (Up_4U (INS365)), which are less rapidly degraded by ectonucleotidases than UTP and UDP, and exhibit relative $P2Y_6$ and $P2Y_2$ selectivity respectively (Pendergast *et al*., 2001; Yerxa *et al*., 2002). Finally, we evaluated the effect of the $P2Y_1$-selective antagonist 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179) on the relaxation responses as well as on the intracellular calcium signalling in intact aortic rings upon stimulation with ATP, ATPγS, ADP and UTP.

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. $P2Y_2^{-/-}$ and the corresponding WT mice (both B6D2/SV129 strain, age 12–18 weeks and body weight 21.9 ± 2.8 and 22.0 ± 1.7 g respectively) were used. Genotyping was carried out using two primer sets as previously described (Homolya *et al*., 1999). $P2Y_2^{-/-}$ mice, on a B6D2 genetic background (Homolya *et al*., 1999), were provided as breeder pairs by B.H. Koller. They were crossed with the SV129 strain by J. Leipziger (Matos *et al*., 2005).

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 thoracic aorta was divided into 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 and 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 (in 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 were gradually stretched until a stable loading tension of 20 mN was attained (Crauwels *et al*., 2003; Guns *et al*., 2005).

Vasomotor studies

Indomethacin (10 μM) was present in all studies to avoid any vasomotor interference due to contractile prostanoids (Vanhoutte *et al*., 2005). Between each concentration–response curve, the Krebs–Ringer solution was replaced three times to wash out the agents. Rings were first contracted with a depolarizing potassium solution (50 mM). After three washing steps, a cumulative concentration–response curve was drawn for phenylephrine (3 nM–30 μM) and the concentration (EC_{50}) resulting in 50% of the maximal contraction was assessed for each vessel segment. At the highest phenylephrine concentration, segments were exposed for 4 min to ATP (7×10^{-5} M) followed by three washing steps. This ATP pretreatment was used to minimize the shift between the first and second curve seen with all purinergic agonists (Guns *et al*., 2005). In relaxation studies, vessels were precontracted with their individual EC_{50} of phenylephrine, followed by cumulative concentration–response curves.

To assess the function of the $P2Y_2$ subtype, two different protocols were used. In the first protocol, consecutive concentration–response curves were performed for ATP, UTP, ADP/ACh, UDP/ATPγS and ATP/ADP in the presence of MRS2179. In these experiments, we observed a decrease of maximal relaxation capacity during the experiment; therefore, in a second experiment, ATPγS, UTP, UDP, Up_3U and Up_4U were tested simultaneously on a particular thoracic aorta segment followed by a second cumulative concentration–response curve in the presence of MRS2179 (10 μM). MRS2179 (10 μM) was added to the organ bath 20 min before the nucleotide cumulative concentration–response curve. Interference of regional differences among the different segments was excluded by using a rotation system. In this manner, each agonist was tested once on each of the five thoracic aorta segments.

Calcium measurements

After mounting in a wire myograph (Danish Myo Technology A/S, Denmark), the relaxed aorta segments (1 mm) were incubated for 2.5 h in a Krebs–Ringer solution with 0.02% pluronic F-127, 0.01% bovine serum albumin and 10 μM Fura2-acetoxymethyl ester (Fura2-AM). After incubation, the tissue was rinsed with regular Krebs–Ringer solution for 0.5 h (5 ml min^{-1}). Excitation wavelengths (340 and 380 nm) were delivered at a frequency of 1 Hz with a DeltaRam Multiwavelengths Illuminator (Photon Technology International, PTI, U.S.A.). Emission (dichroic mirror, emission filter XF2031, XF3007, Omega optical Inc., Germany) was measured with a microscope photometer (D-104, PTI, U.S.A.). The emission ratio, measured at dual excitation wavelengths (340/380 ratio), was calculated with Felix software (PTI, U.S.A.) and used as a relative measure of free $[Ca^{2+}]_i$. Before starting an experiment, the mean background emission values for excitation at 340 and 380 nm were determined before loading with Fura2-AM over a period of 60 s. These background values were real-time subtracted from the emission values during the experiment. Intracellular calcium signals

were measured upon stimulation of aorta segments from WT or $P2Y_2$-deficient mice with ATP and UTP (both 10 μM) for 5 min in the absence or presence of MRS2179 (10 μM). After each stimulation, the agonist was washed out by perfusion with Krebs–Ringer solution (5 ml min^{-1}) for 10 min.

Statistical analysis

All results are expressed as mean ± s.e.m.; *n* represents the number of mice. Statistical analysis was performed by SPSS package for windows (version 11.5). The knockout and WT mice were compared by using a Student's *t*-test.

Materials

Sodium pentobarbital (Nembutal®) was purchased from Sanofi (Brussels, Belgium) and indomethacin was from Federa (Brussels, Belgium). Further, phenylephrine hydrochloride, all nucleotides and MRS2179 were obtained from Sigma (Bornem, Belgium). Up_3U and Up_4U were provided by Inspire Pharmaceuticals (Durham, NC, U.S.A.).

Results

Agonists

The relaxation capacity of the different nucleotides was tested in the phenylephrine precontracted thoracic aorta of $P2Y_2$-knockout mice and the corresponding WT mice. ADP evoked in both strains transient relaxations. At higher ADP concentrations, ADP became less active and contractile effects were sometimes observed. ATP and ATPγS, on the other hand, evoked sustained relaxation in WT mice, but transient responses in $P2Y_2$-deficient mice (Figure 1).

Furthermore, in cumulative concentration–response curves, ATP displayed a greater sensitivity and effectiveness in WT mice in comparison to the $P2Y_2$-knockout mice (Figure 2a; Table 1). The relaxation evoked by ATPγS was severely impaired in the $P2Y_2$-knockout mice, whereas the WT mice showed complete relaxation.

The cumulative concentration–response curves of $P2Y_2$-knockout mice for UTP, ADP, UDP and ACh (not shown) were not different from those of WT mice with respect to potency and maximal vasodilator capacity (Figures 2 and 3; Table 1). Finally, the vasodilator potency of the dinucleotides Up_3U and Up_4U was evaluated. Both compounds were less potent and less effective than UTP and UDP, and their action did not differ between the $P2Y_2$-knockout mice and WT mice (Figure 4).

Antagonist

MRS2179 was used to determine the contribution of the $P2Y_1$ receptor. In the presence of MRS2179, the concentration–response curve of ATP showed a larger rightward shift in the $P2Y_2$-knockout mice than in the WT mice (Figure 5).

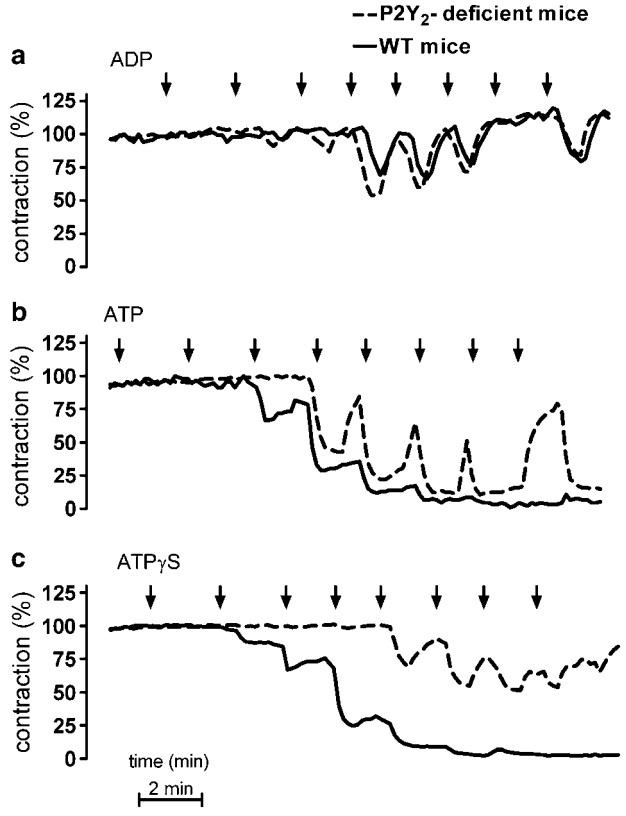

Figure 1 Representative tracings of cumulative concentration–response curves (3×10^{-8}–1×10^{-4} M) of (a) ADP, (b) ATP and (c) ATPγS of phenylephrine-precontracted aortic rings from $P2Y_2$-deficient or WT mice.

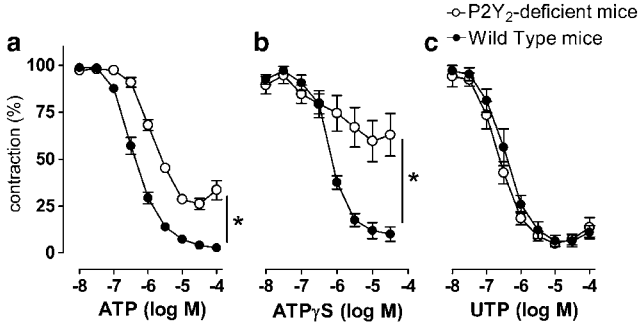

Figure 2 Cumulative concentration–response curves of $P2Y_2$-deficient *versus* WT mice for ATP (a), ATPγS (b) and UTP (c). Results are expressed as mean ± s.e.m.; $n = 5$. *$P2Y_2$-deficient mice different from WT mice ($P < 0.005$).

Table 1 The pD_2 values (−log M) of nucleotide agonists inducing relaxation of the thoracic aorta of WT and $P2Y_2$-deficient mice

	WT	*$P2Y_2$-deficient mice*	P-*value*[a]
ATP	6.41 ± 0.04	5.90 ± 0.05	< 0.0001
ATPγS	6.17 ± 0.05	—[b]	
ADP	6.44 ± 0.13	6.32 ± 0.17	0.573
UTP	6.46 ± 0.07	6.65 ± 0.07	0.109
UDP	6.78 ± 0.14	6.80 ± 0.13	0.914
Up_3U	5.88 ± 0.14	6.12 ± 0.11	0.227
Up_4U	5.88 ± 0.17	5.79 ± 0.12	0.692

Values represent mean ± s.e.m. of five mice.
[a]WT *versus* $P2Y_2$-deficient mice (Student's *t*-test).
[b]Curve fitting was not appropriate.

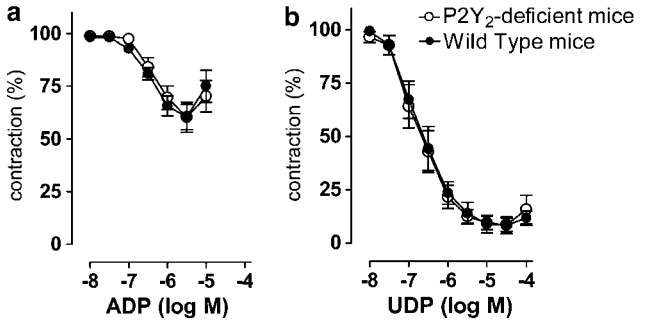

Figure 3 Cumulative concentration–response curves of $P2Y_2$-deficient and WT mice for ADP (a) and UDP (b). Results are expressed as mean ± s.e.m.; $n = 5$.

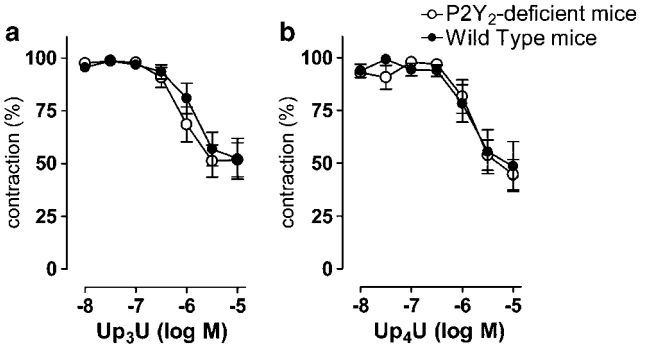

Figure 4 Cumulative concentration–response curves of $P2Y_2$-deficient and WT mice for Up_3U (a) and Up_4U (b). Results are expressed as mean ± s.e.m.; $n = 5$.

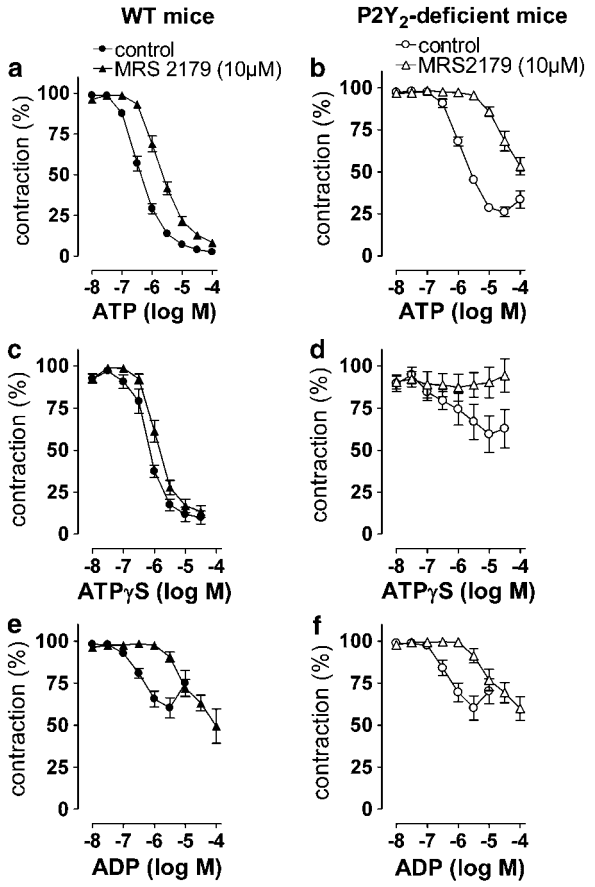

Figure 5 Cumulative concentration–response curves of ATP (a and b), ATPγS (c and d) and ADP (e and f) in WT and $P2Y_2$-deficient mice in the absence or presence of MRS2179 (10 μM).

The responses of ATPγS were even completely inhibited in the knockout mice, whereas MRS2179 hardly influenced the relaxation in the WT mice (Figure 5). The rightward shift of the concentration–response curve for ADP was of the same magnitude in the knockout and WT mice (Figure 5). The responses of UTP were not influenced by MRS2179 in either the knockout mice or in the WT mice (not shown).

Calcium measurements

ATP (10 μM) evoked a transient calcium peak in the knockout as well as in the WT mice. UTP, on the other hand, evoked a biphasic response (a transient spike followed by a sustained plateau) in WT mice, whereas the $P2Y_2$-deficient mice showed only the sustained plateau, without the initial transient spike (Figure 6).

MRS2179 completely inhibited the ATP signal in $P2Y_2$-knockout mice, whereas in WT mice it was only slightly inhibited. In contrast, the calcium peak evoked by UTP was not influenced by MRS2179 in either strain.

Discussion

Agonist

Previously, we documented the presence of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors in the murine aorta. The receptors involved in endothelium-dependent relaxation were tentatively identified based on pharmacological criteria: $P2Y_1$ for ADP, $P2Y_2$ for ATP and $P2Y_6$ for UDP. For UTP it was unclear whether $P2Y_2$, $P2Y_6$ or yet another subtype was involved, but $P2Y_4$ was excluded by experiments on $P2Y_4$-null mice (Guns *et al*., 2005). In view of the lack of specific nucleotide agonists and antagonists, we used $P2Y_2$ receptor-deficient mice to clarify the involvement of the $P2Y_2$ receptor subtype in nucleotide-induced vasodilatation. The $P2Y_2$-deficient mice model has been described and used previously (Homolya *et al*., 1999; Matos *et al*., 2005). First of all, it should be mentioned that relaxations evoked by non-$P2Y_2$ agonists, such as ADP, UDP and ACh, were not different between $P2Y_2$-deficient and WT mice. These findings indicate that the receptors for those agonists are functioning normally in the knockout mice. In fact, this makes the $P2Y_2$-deficient mice an ideal and suitable model for investigating the role of $P2Y_2$ receptors in nucleotide-induced vasodilatation. However, the pD_2 values of ATPγS and particular ATP were lower in the present study (Table 1) in comparison with the previous one (Guns *et al*., 2005). This could be due to the different genetic background of the mice (B6D2/SV129 *versus* C57Bl6), which could lead to subtle differences in the activity of nucleotidases or other properties of the vessel wall. The cumulative concentration–response curves for ATP, ATPγS and UTP are most interesting, as they are all potential ligands for the $P2Y_2$ subtype. In $P2Y_2$-deficient mice, ATP was less potent and less

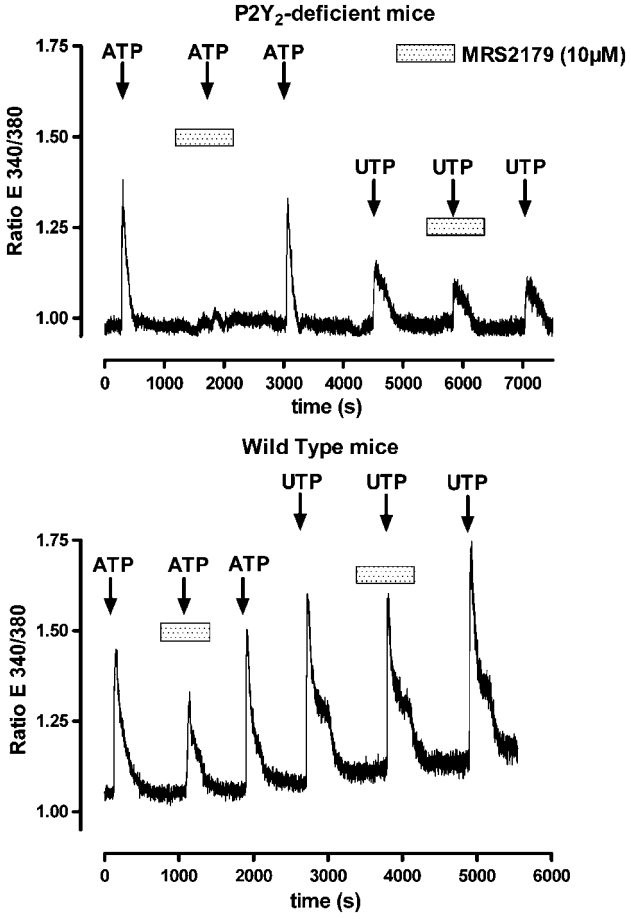

Figure 6 Intracellular calcium signals (E 340/380 ratio) during stimulation (5 min) with ATP and UTP (both 10 μM) in $P2Y_2$-deficient and WT mice. For each agonist, the calcium signal was measured twice in the absence of MRS2179 and once in the presence of MRS2179. Tracing is representative of three different experiments.

efficient to evoke relaxation in comparison to the WT strain. These results clearly prove a disturbed ATP response in the absence of the $P2Y_2$ receptor subtype. Moreover, the WT mice displayed a sustained vasodilator response to ATP and ATPγS, whereas in $P2Y_2$-deficient mice these relaxations were only transient. As the $P2Y_1$ agonist ADP evoked similar, transient relaxations in both strains, it seems likely that the ATP-evoked relaxation in the $P2Y_2$-deficient mice is mediated directly or indirectly (upon conversion to ADP) by the $P2Y_1$ receptor subtype. Furthermore, the concentration–response curve of ATPγS was severely impaired in $P2Y_2$-deficient mice, much more than that of ATP. This can be explained by the fact that ATPγS is quite resistant to ATP-degrading enzymes. Therefore, we suppose that the (small) relaxing effect of ATPγS is due to (slow) conversion of ATPγS into ADP, whereas the relaxing effect of ATP is due to direct and/or indirect (upon conversion to ADP) interaction with the $P2Y_1$ receptor. Indeed, the $P2Y_1$-selective antagonist MRS2179 clearly antagonized the ATP- and ATPγS-evoked relaxation response in the $P2Y_2$-deficient mice, whereas in the WT mice MRS2179 induced only a small rightward shift. This indicates that in $P2Y_2$-deficient mice, the action of ATP and ATPγS is mediated by $P2Y_1$ receptors. Furthermore, the involvement of the $P2X_4$ receptor that is highly expressed in human endothelial cells (Yamamoto *et al*., 2000; Wang *et al*., 2002) seems unlikely, as MRS2179 clearly antagonized the ATP response in the knockout mice but is inactive at rat $P2X_4$ (and $P2X_2$) receptors (Brown *et al*., 2000). As expected, the effect of MRS2179 on the $P2Y_1$-selective agonist ADP was similar for both strains.

In contrast to ATP, the relaxation evoked by UTP did not differ between $P2Y_2$-deficient and WT mice, thereby proving that the UTP-evoked relaxation is not exclusively mediated by the $P2Y_2$ subtype. Further, we evaluated the relaxation potency of the dinucleotides Up_3U and Up_4U. At recombinant human receptors, these compounds are $P2Y_2$ (Up_4U) and $P2Y_6$ (Up_3U) selective (Pendergast *et al*., 2001). Although they are less potent than UTP and UDP on those receptors, they have the advantage of being less rapidly degraded by ectonucleotidases than UTP or UDP. In accordance with the latter assumption, the Up_3U action was not different in WT and $P2Y_2^{-/-}$ mice. However, the current experiments also did not show differences between $P2Y_2$-deficient mice and WT mice for Up_4U. There are two possible explanations. Although Up_4U is more resistant to ectonucleotidases than UTP, it could nevertheless be degraded into UDP to a significant extent (Yerxa *et al*., 2002). On the other hand, Up_4U itself might be an agonist of the murine $P2Y_6$ receptor.

Taken together, the action of UTP and Up_4U, both described to be ligands of the $P2Y_2$ receptor subtype, is not (exclusively) mediated by $P2Y_2$ receptors. The involvement of $P2Y_4$ receptors in the action of UTP has been excluded previously in experiments on $P2Y_4$-null mice (Guns *et al*., 2005). Further, $P2Y_1$ receptors can be excluded by the lack of effect of MRS2179 on UTP responses. Therefore, the action of UTP is possibly mediated by a $P2Y_6$(like) receptor, as proposed previously (Vial & Evans, 2002; Malmsjo *et al*., 2003; Guns *et al*., 2005; Vonend *et al*., 2005). Fully compatible with this 'P2Y6(like) receptor hypothesis' is the observation that the cumulative concentration–response curves of UTP and UDP are identical in $P2Y_2$-deficient and WT mice.

In addition, intracellular calcium signalling was measured directly in endothelial cells *in situ*. In the WT mice, UTP evoked a biphasic response, a fast and transient initial spike, followed by a sustained plateau. In contrast, in the $P2Y_2$-deficient strain, UTP evoked (only) a sustained plateau without the preceding fast initial peak. Hence, we suggest that the initial transient spike seen in the WT mice might be due to the $P2Y_2$ receptor subtype, whereas the sustained plateau can probably be attributed to the $P2Y_6$(like) receptor. This could be related to the particular time course of the $P2Y_6$ receptor, characterized by a slow onset and little desensitization (Robaye *et al*., 1997). Moreover, these data suggest that although UTP might activate $P2Y_2$ receptors and give rise to a calcium peak, this UTP–$P2Y_2$ interaction does not seem to be related to the relaxation response, as the sustained plateau on its own (as demonstrated in the knockout mice) evoked complete relaxation.

Further, MRS2179 clearly inhibited the ATP-elicited calcium spike in $P2Y_2$-deficient mice, confirming that ATP activates $P2Y_1$ receptor subtypes in the knockout mice, whereas in WT mice, the ATP-evoked calcium spike was only slightly inhibited. In contrast, the UTP-evoked calcium signal was not influenced by MRS2179 in both strains. These results are fully compatible with the result of the vasomotor study.

In conclusion, ATP- and ATPγS-evoked relaxation of the murine aorta is mediated by $P2Y_2$ receptors, as the ATP and ATPγS responses were severely impaired in the knockout mice. Moreover, experiments with MRS2179 clearly showed that the residual ATP/ATPγS-induced relaxation in the $P2Y_2$-deficient mice was mediated by the $P2Y_1$ receptor subtype directly or indirectly, after conversion to ADP. In contrast, the UTP-evoked relaxation remained normal in the $P2Y_2$-knockout mice and was not influenced by MRS2179. Therefore, it appears that the endothelium-dependent relaxation by UTP is mainly mediated by a receptor other than $P2Y_2$, most probably the $P2Y_6$ receptor.

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