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The stable pyrimidines UDPβS and UTPγS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery

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1 The contractile and relaxant effects of the different P2 receptors were characterized in the rat isolated mesenteric artery by use of extracellular nucleotides, including the stable pyrimidines uridine 5′-O-thiodiphosphate (UDPβS) and uridine 5′-O-3-thiotriphosphate (UTPγS).

2 The selective P2X receptor agonist, αβ-methylene-adenosine triphosphate (αβ-MeATP) stimulated a potent ($pEC_{50} = 6.0$) but relatively weak contraction ($E_{max} = 57\%$ of 60 mM K^+). The contractile concentration-response curve of adenosine triphosphate (ATP) was biphasic when added in single concentrations. The first part of the response could be desensitized by αβ-MeATP, indicating involvement of P2X receptors, while the second part might be mediated by P2Y receptors.

3 The contractile P2Y receptors were further characterized after P2X receptor desensitization with 10 μM αβ-MeATP. Uridine diphosphate (UDP), uridine triphosphate (UTP) and ATP stimulated contraction only in high concentrations (1–10 mM). The selective $P2Y_6$ agonist, UDPβS, and the $P2Y_2$/$P2Y_4$-receptor agonists UTPγS and adenosine 5′-O-3-thiotriphosphate (ATPγS) were considerably more potent and efficacious ($E_{max} \approx 250\%$ of 60 mM K^+). Adenosine 5′-O-thiodiphosphate (ADPβS) was inactive, excluding contractile $P2Y_1$ receptors.

4 After precontraction with 1 μM noradrenaline, UTP, ADP and ATP induced relaxations with similar potencies ($pEC_{50} \approx 5.0$). UTPγS, ADPβS and ATPγS were approximately one log unit more potent indicating the presence of endothelial $P2Y_1$ and $P2Y_2$/$P2Y_4$ receptors. The $P2Y_6$ receptor agonist, UDPβS, had no effect.

5 UDPβS and UTPγS are useful tools when studying P2 receptors in tissue preparations with ectonucleotidase activity. Contractile responses can be elicited by stimulation of $P2Y_6$ and, slightly less potently, $P2Y_2$/$P2Y_4$ receptors. The P2X response was relatively weak, and there was no $P2Y_1$ response. Stimulation of $P2Y_1$ and $P2Y_2$/$P2Y_4$ receptors elicited relaxation, while $P2Y_6$ did not contribute.

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Abbreviations: ACh, acetylcholine; ADP, adenosine-diphosphate; ADPβS, adenosine 5′-O-thiodiphosphate; A3P5PS, adenosine 3′-phosphate-5′-phosphosulphate; ATP, adenosine triphosphate; ATPγS, adenosine 5′-O-3-thiotriphosphate; αβ-MeATP, αβ-methylene-adenosine triphosphate; NA, noradrenaline; SIN-1, 3-morpholino-synonimine; UDP, uridine diphosphate; UDPβS, uridine 5′-O-thiodiphosphate; UTPγS, uridine 5′-O-3-thiotriphosphate, UTP, uridine triphosphate; VSMC, vascular smooth muscle cell

Introduction

Purines can be released from perivascular nerves, circulating elements such as platelets and erythrocytes, as well as from endothelial and smooth muscle cells during hypoxia and shear stress (Gordon, 1986; Burnstock, 1989). Less is known about the source and vascular effects of pyrimidines, although release has been demonstrated from platelets and other organs during a variety of pathological conditions such as trauma, hypoxia and inflammation (Goez *et al*., 1971; Gordon, 1986).

Recent receptor cloning has proven the existence of several different P2X and P2Y receptor subtypes that are activated by purines and pyrimidines, and expression of these receptors in cultured cells has enabled characterization of their respective pharmacological profiles. The $P2X_1$ receptor has been shown to be the most abundant P2X receptor subtype on vascular smooth muscle cells (VSMCs) and is activated by αβ-methylene-adenosine triphosphate (αβ-MeATP) > adenosine triphosphate (ATP) = 2-methylthioadenosine triphosphate (2-MeSATP) (Evans & Kennedy, 1994; Valera *et al*., 1994; Vulchanova *et al*., 1996). P2Y receptors on VSMCs have mainly been shown to be contractile, while P2Y receptors on the endothelium mediate relaxation by release of dilatory mediators such as nitric oxide, prostaglandins and endothelium-derived hyperpolarizing factor (Ralevic & Burnstock, 1998; Malmsjö *et al*., 1998; 1999). At least four P2Y receptor subtypes may mediate the vascular effects of extracellular nucleotides, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ (Harden *et al*., 1998). At the $P2Y_1$ receptor adenosine 5′-O-thiodiphosphate (ADPβS), 2-MeSATP and adenosine-diphosphate (ADP) are more potent than ATP, while uridine diphosphate (UDP) and uridine triphosphate (UTP) are inactive (Léon *et al*., 1997;

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Palmer *et al*., 1998). The $P2Y_2$ and rat $P2Y_4$ receptors are activated with similar potencies by UTP and ATP, but not by UDP or ADP, while the $P2Y_6$ receptor is mainly activated by UDP and weakly by UTP, ADP and ATP (Nicholas *et al*., 1996; Webb *et al*., 1998).

Since there are no selective antagonists the characterization of P2 receptors has been performed by monitoring agonist responses, as described above. This causes difficulties, as the endogenous extracellular nucleotides are neither selective nor stable. When investigations are performed in intact tissues, nucleotide triphosphates are metabolized by ectonucleotidases on the extracellular surface of cells. Commercial nucleotides are contaminated with related compounds, and nucleotide triphosphates are reduced to diphosphates when stored in aqueous solutions. However, stable pyrimidine analogues, uridine 5′-O-thiodiphosphate (UDPβS) and uridine 5′-O-3-thiotriphosphate (UTPγS), that were previously developed by Goody *et al*. (1972) are now being used in the attempts to pharmacologically define the P2 receptor subtypes. These contain a modification of the nucleotide triphosphate group in the form of a thio substitution at the terminal phosphate that provide stability to ectonucleotidase action. UTPγS is a potent and selective agonist at the $P2Y_2/P2Y_4$ receptors (Lazarowski *et al*., 1996). In analogy, UDPβS has recently been proven to be a stable and selective $P2Y_6$ receptor agonist with an EC_{50} value for the $P2Y_6$ receptor of 25 nM and more than 100 μM for the $P2Y_2/P2Y_4$ receptors (Harden *et al*., unpublished). In combination with the previously used stable purines ($\alpha\beta$-MeATP, ADPβS and adenosine 5′-O-3-thiotriphosphate (ATPγS)), these stable pyrimidines may facilitate the characterization of the P2 receptor subtypes in intact tissues.

This study was designed to evaluate the relative contribution of different P2 receptor subtypes involved in the vascular contraction and relaxation of the rat isolated mesenteric artery by use of extracellular nucleotides, including UDPβS and UTPγS.

Methods

Tissue preparation

Female Sprague-Dawley rats weighing 200 g were anaesthetized by inhalation of CO_2, after which they were killed by a cardiac cut. The mesenteric artery was removed gently and immersed in cold oxygenated buffer solution (for composition, see below) and dissected free of adhering tissue under a microscope. In experiments where endothelium denudation was required this was performed by perfusion of the vessel for 5 s with 0.1% Triton X-100 followed by another 5 s of perfusion with a physiologic buffer solution (for composition, see below) using a fine needle. The vessels were cut into cylindrical segments (2–3 mm long) and were immediately used in the experiments. Each cylindrical segment was mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer (FT03C) for continuous recording of the isometric tension, and the other to a displacement device. The position of the holder could be changed by means of a movable unit allowing fine adjustments of the vascular resting tension by varying the distance between the metal prongs. The mounted artery segments were immersed in temperature controlled (37°C) tissue baths containing a bicarbonate based buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. The solution was continuously gassed with 5% CO_2 in O_2 resulting in a pH of 7.4.

Twelve ring segments were studied at the same time in separate tissue baths. The artery segments were allowed to stabilize at a resting tension of 2 mN for 1 h before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a K^+-rich (60 mM) buffer solution in which NaCl was exchanged for an equimolar concentration of KCl (for composition, see above). When two reproducible contractions had been achieved the vessels were used for further experiments. Acetylcholine (ACh) induces relaxation by release of endothelial mediators, while 3-morpholino-synonimine (SIN-1) is an NO-donor which stimulates relaxation by a direct effect on the endothelium. Endothelium removal was checked by monitoring responses to ACh in noradrenaline (NA) precontracted vessel segments. Abolished relaxation indicated a properly removed endothelium, while unaffected SIN-1 relaxations suggested intact VSMC-function.

Vascular contraction

Contractile responses were examined in endothelium denuded vessel segments. As the P2X receptors desensitized quickly (basal tension was reached within 8 min), each artery segment was exposed to a single concentration of $\alpha\beta$-MeATP or ATP and the resultant responses of several segments exposed to different concentrations were grouped together to form a concentration-response curve. In this way, each artery segment was exposed to $\alpha\beta$-MeATP or ATP only once and the problem of tachyphylaxia was avoided. These experiments are referred to as 'single-concentration'. To examine the P2Y receptor stimulated contractions without interference of simultaneous P2X responses, UDP, UDPβS, UTP, UTPγS, ADP, ADPβS, ATP or ATPγS were added after desensitization of P2X receptors with 10 μM $\alpha\beta$-MeATP 8 min prior to each experiment (Kasakov & Burnstock, 1983). As P2Y receptors only desensitized very slowly (Ralevic & Burnstock, 1998), these agonists could be added cumulatively to determine concentration-response relationships.

Vascular relaxation

Relaxation to cumulatively added UDP, UDPβS. UTP, UTPγS, ADP, ADPβS, ATP or ATPγS were studied in arteries with intact endothelium, precontracted with 1 μM NA. Recent results show that P2Y receptors induce relaxation that is mediated by release of endothelium derived hyperpolarizing factor that hyperpolarizes VSMCs. This effect is counteracted by simultaneous stimulation by ATP and other nucleotides, of P2X receptors that depolarize VSMCs (Malmsjö *et al*., 1999). Therefore, relaxation was examined after desensitization of P2X receptors with $\alpha\beta$-MeATP. $\alpha\beta$-MeATP was then added 8 min before NA precontraction (as described above).

Drugs

Agonist purity and stability are potential problems when delineating the pharmacological selectivity of P2 receptors especially in intact tissues. Metabolism of nucleotides was therefore prevented by use of more stable compounds like UDPβS, UTPγS, ADPβS and ATPγS. These include a thio substitution at the terminal phosphate which provides stability to ectonucleotidase action (Jacobson *et al*., 1998). UDPβS and UTPγS were gifts from Inspire Pharmaceuticals, Inc. ACh,

ATP, ATPγS, ADP, ADPβS, A3P5PS, αβ-MeATP, NA, UTP, UDP, UTPγS, UDPβS, SIN-1 and Triton X-100 were purchased from Sigma Co., U.S.A. All drugs were dissolved in 0.9% saline.

Calculations and statistics

The negative logarithm of the drug concentration that elicited 50% contraction or relaxation (pEC_{50}) was determined by fitting the data to the Hill equation. R_{max} refers to maximum relaxation calculated as percentage of the corresponding precontraction with 1 μM NA, while E_{max} refers to maximum contraction calculated as percentage of the contractile capacity of 60 mM K^+. For UDP a plateau-phase of the maximum contractile response was not reached within the agonist concentration interval and the real E_{max} value will therefore be similar or higher than the obtained value (marked ⩾ in text and tables). pEC_{50} for UDP was calculated as the negative logarithm of 25% of the maximum response to UDPβS (marked $pEC_{25(UDP\beta S)}$). The Hill coefficients were calculated by use of GraphPad Prism.

The contractile effect of ATP, added in single-concentrations and cumulative concentrations, without prior P2X receptor desensitization, was analysed according to a two-site model by fitting the following equation to the data by non-linear regression analysis:

$$E = E_{max}\left(\left(\frac{F_H.[A]^{n_{H1}}}{EC_{50\,H}^{n_{H1}} + [A]^{n_{H1}}}\right) + \left(\frac{(1-F_H).[A]^{n_{H2}}}{EC_{50\,L}^{n_{H2}}}\right)\right)$$

In this equation, E_{max} denotes the overall maximum contractile response, F_H denotes the fraction of the response mediated by the high potency component, [*A*] denotes the agonist concentration, EC_{50H} and EC_{50L} denote the high and low potency EC_{50} values, and n_{H1} and n_{H2} denote the Hill coefficient of the high and low potency components, respectively.

Experiments were repeated on six to 10 animals for each substrate and statistical significance was accepted when $P<0.05$, using Student's *t*-test. All differences referred to in the text have been statistically verified. Values are presented as mean ± s.e.mean.

Results

The contractile capacity of the rat mesenteric artery was examined by addition of 60 mM K^+ (5.9 ± 0.1 mN). Vascular relaxations to ACh and each of the P2Y receptor agonists were abolished after endothelium removal, indicating that all relaxation was endothelium-dependent. SIN-1 induced relaxations were unaffected (pEC_{50} = 5.3 ± 0.1 and R_{max} = 99 ± 8% of 60 mM K^+ before, and 5.2 ± 0.1 and 102 ± 9% after endothelium removal), indicating intact VSMC-function after endothelium removal. Vascular contractions were examined in endothelium denuded vessel segments, while it was left intact in experiments where relaxation was studied.

Vascular contraction

Contractile responses to αβ-MeATP Stimulation of P2X receptors with single-concentrations of αβ-MeATP produced equally potent, but relatively weak concentrations (pEC_{50} = 6.1 ± 0.2 and E_{max} = 57 ± 6% of 60 mM K^+), as compared to the efficacious P2Y receptor responses to UDPβS, UTPγS and ATPγS (Figure 1).

Contractile responses to ATP When ATP was added cumulatively over 15 min, without prior P2X receptor desensitization, the concentration-response curve showed a tendency towards being biphasic (pEC_{50} was 4.1 for the first part of the curve and 2.0 for the second part). When added in single-concentrations the ATP concentration-response curve was clearly biphasic (pEC_{50} = 4.1 and 2.4) (Figure 2). The Hill coefficient of the first part of the curve was similar to that of αβ-MeATP (2.4 for ATP and 2.7 for αβ-MeATP), while the midpoint localization of the second part was similar to that of the ATP stimulated P2Y response (see below). The concentration-response curve of ATPγS added in single-concentrations, without prior P2X receptor desensitization, showed no biphasic tendency (data not shown) and the curve was identical to that for ATPγS after P2X receptor desensitization (see below).

P2Y receptor induced responses P2Y receptor mediated contractions were studied after P2X receptor desensitization with αβ-MeATP (see Methods). Contractile responses to UDP, UTP and ATP could be observed only in high concentrations

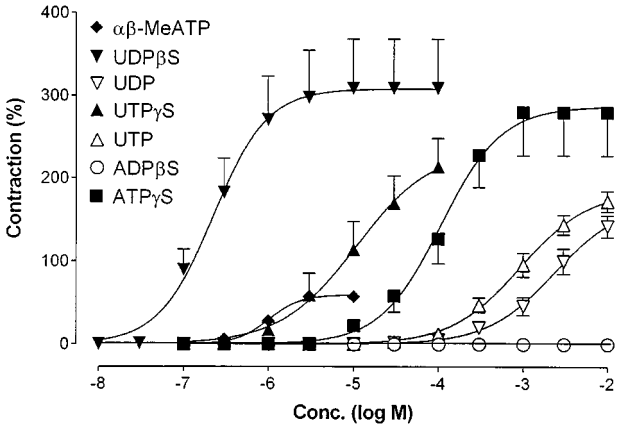

Figure 1 Concentration-dependent contractions to αβ-MeATP, UDPβS, UDP, UTPγS, UTP, ADPβS and ATPγS in the rat mesenteric artery. All nucleotides, except αβ-MeATP, were added after P2X receptor desensitization with 10 μM αβ-MeATP. Contractions are expressed as percentage of an initial contraction induced by 60 mM K^+. Data are shown as means ± s.e.mean of 6–10 experiments.

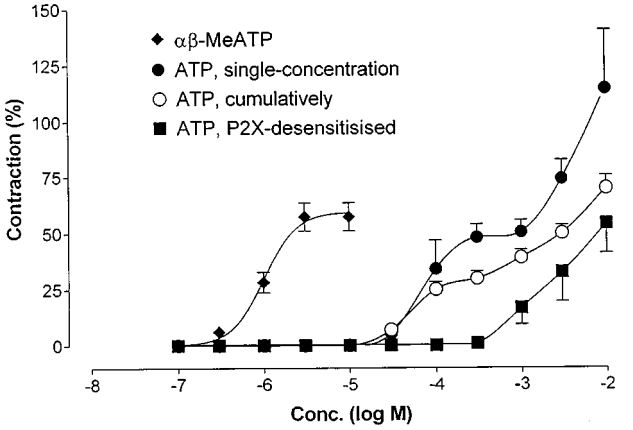

Figure 2 Concentration-dependent contractions to αβ-MeATP and ATP added in single-concentration, ATP added cumulatively and ATP after P2X receptor desensitization with 10 μM αβ-MeATP. Contractions are expressed as percentage of an initial contraction induced by 60 mM K^+. Data are shown as means ± s.e.mean of 6–10 experiments.

(1–10 mM), as compared to the stable nucleotide analogues (Figures 1 and 2). The selective $P2Y_6$ agonist, UDPβS was efficacious and approximately four log units more potent than that for UDP ($pEC_{25} = 7.2 \pm 0.2$, $E_{max} = 309 \pm 60\%$ of 60 mM K^+ for UDPβS and $pEC_{25(UDP\beta S)} = 2.8 \pm 0.2$, $E_{max} \geq 143 \pm 13\%$ for UDP) (Figure 1). UTPγS and ATPγS produced contractions of similar efficacy ($E_{max} = 216 \pm 34\%$ and $280 \pm 52\%$, respectively), but were significantly less potent than UDPβS ($pEC_{50} = 5.3 \pm 0.2$ and 4.1 ± 0.2, UTPγS and ATPγS), $P < 0.01$ (Figure 1). ADP only raised tone slightly at 10 mM ($28 \pm 9\%$ of 60 mM K^+), while the selective $P2Y_1$ agonist (not shown), ADPβS was ineffective. In conclusion, the rank order potency of nucleotides for the P2Y receptor mediated contractile response was; UDPβS > UTPγS > ATPγS > UTP = UDP > ATP = ADP > ADPβS = 0.

Vascular relaxation

Relaxations were studied after precontraction with 1 μM NA. The P2X receptors were first desensitized with $\alpha\beta$-MeATP. The efficacies for the agonists used were similar ($R_{max} = 74 \pm 5\%$ of 60 mM K^+ (UTPγS), $58 \pm 11\%$ (UTP), $66 \pm 3\%$ (ADPβS), $48 \pm 4\%$ (ADP), $52 \pm 8\%$ (ATPγS) and $59 \pm 7\%$ (ATP)). The stable nucleotide analogues were markedly more potent than the endogenous nucleotides ($pEC_{50} = 6.2 \pm 0.1$, 6.0 ± 0.1, 5.8 ± 0.2 for UTPγS, ADPβS and ATPγS, as compared to 4.8 ± 0.2, 5.3 ± 0.2 and 5.4 ± 0.2 for UTP, ADP and ATP) (Figure 3). A high concentration of UDP ($pEC_{50} = 3.8 \pm 0.3$) only decreased tension slightly ($R_{max} = 16 \pm 7\%$), while the selective $P2Y_6$ agonist, UDPβS had no effect. In conclusion, these nucleotides induced relaxations in the following order of potency; UTPγS = ADPβS = ATPγS > ADP = ATP > UTP > UDP. UDPβS had no effect.

Discussion

Contractile responses

P2 receptors on VSMCs have mainly been shown to be contractile, while stimulation of the same receptors on the endothelium induce release of dilatory mediators such as nitric oxide, prostaglandins and endothelium derived hyperpolarizing factor (Ralevic & Burnstock, 1998; Malmsjö *et al*., 1998; 1999). To eliminate the modulatory effect of the endothelium, it was removed before studying vascular contractions. $\alpha\beta$-MeATP produced a potent contraction indicating the presence of P2X receptors. These are likely to be of the $P2X_1$ subtype, as electrophysiological responses with structure-activity relationships similar to those of the cloned $P2X_1$ receptor have been seen in isolated VSMCs (Evans & Kennedy, 1994). $P2X_1$ receptors have also been visualized by immunocytochemistry on vascular smooth muscle cells of submucosal arteries (Vulchanova *et al*., 1996). $\alpha\beta$-MeATP also activates and rapidly desensitizes $P2X_3$ receptors, although there is yet no evidence of expression in VSMCs (Ralevic & Burnstock, 1998).

When ATP was added cumulatively during 15 min, without prior desensitization of P2X receptors, the concentration-response curve showed a tendency towards being biphasic. Martin *et al*. (1991) suggested that tachyphylaxis of the contractile ATP-response may invalidate the cumulative concentration-response curves and composite curves should therefore be prepared. When ATP was added in single-concentrations, the concentration-response curve became clearly biphasic, suggesting the involvement of two different types of receptors; P2X as the Hill coefficient of the first response was very similar to that of $\alpha\beta$-MeATP, and P2Y receptors as the midpoint localization of the second part of the contraction was similar to that of the ATP stimulated P2Y response. When calculating the sum of the ATP-stimulated P2Y response at 10 mM and that of $\alpha\beta$-MeATP, it equals the contraction elicited by ATP in single-concentration at the same concentration, indicating a combined P2X and P2Y effect of ATP. A closer look at ATP concentration-response curves published revealed biphasic properties that were not discussed (Houston *et al*., 1987; von Kügelgen *et al*., 1987; Lagaud *et al*., 1996; Matsumoto *et al*., 1997). Presumably ATP has activated both P2X and P2Y receptors in these experiments, at low and high concentrations, respectively. It has been demonstrated that after P2X desensitization with $\alpha\beta$-MeATP, the vasoconstrictor responses of ATP were only slightly attenuated, indicating a combined P2X and P2Y effect (von Kügelgen *et al*., 1987; McLaren *et al*., 1998; Hartley & Kozlowski, 1997). When we examined the contractile concentration-response curve of ATPγS added in single-concentrations (without prior P2X receptor desensitization) no biphasic tendency could be observed, and the response was identical to that for ATPγS after P2X receptor desensitization.

The contractile P2Y responses were in the present work characterized after desensitization of P2X receptors with $\alpha\beta$-MeATP (Kasakov & Burnstock, 1983). UDP stimulated contraction only in high concentrations (1–10 mM), while the stable analogue UDPβS elicited an efficacious response reaching 309% of the response to 60 mM of K^+, and that was approximately four log units more potent than that for UDP. The potency of UDPβS in these experiments ($pEC_{50} = 6.1$) is lower than the potency of UDPβS-stimulated inositol phosphate formation in cells stably expressing $P2Y_6$ receptors ($EC_{50} = 25$ nM, Harden *et al*., unpublished). However, considering the abundant presence of ectonucleotidases in intact blood vessels the high potency for UDPβS induced vasoconstriction indicates that this contractile response is indeed mediated by $P2Y_6$ receptors. Effects of UDP in arteries have previously been shown by Hartley *et al*. (1998). These responses were insensitive to antagonism by suramin thereby indicating the presence of $P2Y_6$ receptors in vascular myocytes. Our results suggest that extracellular nucleotides mediate contraction most potently by stimulation of $P2Y_6$ receptors. In analogy, high concentrations (1–10 mM) of ATP and UTP

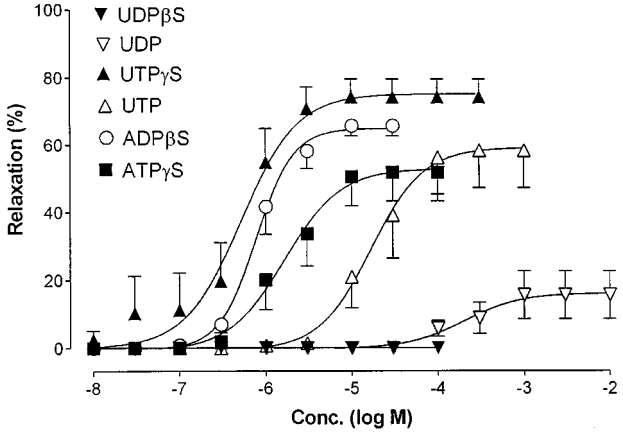

Figure 3 Concentration-dependent relaxations to UDPβS, UDP, UTPγS, UTP, ADPβS, ATPγS and in the rat mesenteric artery. All nucleotides were added after P2X receptor desensitization with 10 μM $\alpha\beta$-MeATP. Relaxations are expressed as percentage of an initial contraction induced by 1 μM NA. Data are shown as means ±s.e.mean of 6–10 experiments.

were needed to stimulate contraction, while UTPγS and ATPγS were markedly more potent and produced almost as efficacious contractions as UDPβS, although with a slightly lower potency. These results suggest a role also for contractile $P2Y_2/P2Y_4$ receptors. Although ADP produced an apparent, but weak contraction, the selective $P2Y_1$ agonist, ADPβS was inactive, excluding a contractile effect by $P2Y_1$ receptors. Contaminating ATP in the ADP solution may in these experiments be responsible for the contraction observed. Desensitization with αβ-MeATP has previously shown to abolish the contractile responses to 2-MeSATP in isolated rabbit coronary arteries (Corr & Burnstock, 1994), proving that $P2Y_1$ receptors are not contractile.

It has so far been shown that ATP is more potent at inducing vascular contraction than UTP in the rat mesenteric artery, and that the ATP response can be abolished after desensitization with αβ-MeATP (Ralevic & Burnstock, 1991), suggesting that the contractile effects of extracellular nucleotides in blood vessels are stimulated mainly by P2X receptors with a contribution of P2Y receptors. By using stable nucleotide analogues we could here demonstrate that αβ-MeATP produced a potent but relatively weak contraction of 57% of 60 mM K^+, while the P2Y receptor responses to UDPβS, UTPγS and ATPγS reached a maximum contraction of approximately 300%. These results suggest that in the contractile response to extracellular nucleotides P2Y receptors might be of primary importance, as compared to the less efficacious P2X receptors.

Relaxant responses

In healthy vasculature, nucleotides act on endothelial P2Y receptors and stimulate the release of nitric oxide and prostaglandins resulting in vascular relaxation. Removal of the endothelium abolished relaxations in our preparation indicating that the P2Y receptors studied were located on the endothelium. We recently showed that relaxation by P2Y receptors may be mediated by release of EDHF, and that this effect is counteracted by simultaneous stimulation of P2X receptors on VSMCs by agents like ATP and other nucleotides (Malmsjö *et al*., 1998; 1999). Therefore, relaxation was studied after P2X receptor desensitization. Previous reports have demonstrated coexisting $P2Y_1$ and $P2Y_2$ receptors on the endothelium in among others the mesenteric arterial bed, which has been confirmed by molecular biology studies (Wilkinson *et al*., 1994; Motte *et al*., 1993; Henderson *et al*., 1995; Gödecke *et al*., 1996; Ralevic & Burnstock, 1996). In our preparation with raised tension the selective $P2Y_1$ receptor agonist, ADPβS was a potent stimulator of vascular relaxation, suggesting a primary role for $P2Y_1$ receptors. Although UTP and ATP elicited relaxation, the stable nucleotide analogues UTPγS and ATPγS were significantly more potent, indicating involvement of $P2Y_2$ or $P2Y_4$ receptors. UDP elicited weak relaxation only at a high concentration and the stable $P2Y_6$ agonist UDPβS was ineffective. These results suggest that $P2Y_6$ receptors do not mediate vascular relaxation of the rat mesenteric artery.

Another possible way in which to discriminate between P2Y receptor subtypes that mediate relaxation is by use of adenosine-3′-phosphate-5′-phosphosulphate (A3P5PS), which has been shown to selectively antagonize $P2Y_1$ receptors in turkey erythrocyte membranes (Boyer *et al*., 1996). In our preparation A3P5PS not only inhibited the ADPβS-relaxation, but also attenuated the response to ACh and UTP (unpublished observations). We therefore considered the compound non-selective, possibly due to its degradation by ectonucleotidases, and it was not further used in our attempts to characterize the vascular response. These findings have recently been confirmed in precontracted rat aorta (Bultmann *et al*., 1998).

The influence of ectonucleotidases

An interesting observation is the difference in potency in these experiments between the endogenous (UDP and UTP) and the stable nucleotides (UDPβS and UTPγS) and the importance of ectonucleotidases for pyrimidine degradation, as suggested by Lazarowski *et al*. (1997). In cell systems where the influence of ectonucleotidases has been minimized, UTP and UTPγS are equally potent (Lazarowski *et al*., 1996). Since more potent responses were induced by stable nucleotides in both intact (relaxation experiments) and endothelium denuded (contraction experiments) vessel segments in our preparation it seems likely that ectonucleotidases on both the endothelium and on the VSMCs are responsible for rapid degradation of endogenous nucleotides, which results in low potency. Furthermore, the difference in potency between stable and endogenous nucleotides was more pronounced for the contractile effects as compared to the endothelium-mediated relaxant effect. A possible explanation might be that the extracellular nucleotides have to penetrate several cell layers to reach the VSMCs and is thereby more exposed to ectonucleotidases, as compared to when they stimulate receptors on the monolayer of endothelial cells. By analogy, UTPγS stimulated relaxation in concentrations that were similar to those of UTPγS-stimulated inositol phosphate formation in astrocytoma cells stably expressing $P2Y_2$ receptors ($pEC_{50} = 6.4$ and 6.6) (Lazarowski *et al*., 1996), while higher concentrations were required to stimulate contractile responses ($pEC_{50} = 5.3$).

Conclusion

Finally, we would like to emphasize the importance of stable nucleotide analogues in the characterization of P2 receptors in intact tissues with ectonucleotidase activity, as there is yet no selective P2 receptor antagonists. UDPβS and UTPγS are useful tools when trying to discriminate between the pyrimidine sensitive P2Y receptors. The most potent and efficacious contractile response was obtained by stimulation of $P2Y_6$ receptors, although $P2Y_2/P2Y_4$ receptor agonists mediated similar contractions but with a lower potency. In comparison, the P2X receptor responses were relatively weak, while $P2Y_1$ receptors were not contractile. Stimulation of $P2Y_1$ and $P2Y_2/P2Y_4$ receptors elicited relaxations, but $P2Y_6$ receptor agonists had no relaxant effect.

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