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Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor

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1 The present study was aimed at examining P2 receptor-mediated vasodilatation in human vessels. The isometric tension was recorded in isolated segments of the human left internal mammary artery branches precontracted with 1 μM noradrenaline.

2 Endothelial denudation abolished the dilator responses.

3 The selective $P2Y_1$ agonist, 2-MeSADP, induced a potent vasodilatation ($pEC_{50} = 6.9 \pm 0.1$). The $P2Y_1$ antagonist of 10 μM, MRS 2216, shifted the 2-MeSADP concentration-response curve 1.1 log units to the right. The combined $P2Y_1$ and P2X agonist, 2-MeSATP, stimulated a dilatation with a potency similar to that of 2-MeSADP. Furthermore, MRS 2216 had a similar antagonistic effect on both 2-MeSATP and 2-MeSADP indicating that P2X receptors do not mediate vasodilatation.

4 Both the $P2Y_{2/4}$ agonist, UTPγS and the $P2Y_6$ agonist, UDPβS, stimulated potent dilatations ($pEC_{50} = 7.8 \pm 0.4$ for UTPγS and 8.4 ± 0.2 for UDPβS).

5 The 2-MeSADP-induced nitric oxide (NO)-mediated dilatation was studied in the presence of 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. The involvement of the endothelium-derived hyperpolarising factor (EDHF) was investigated in the presence of 0.1 mM L-NOARG and indomethacin. The involvement of prostaglandins was investigated in the presence of L-NOARG, charybdotoxin and apamin. Both NO, EDHF and prostaglandins mediated 2-MeSADP dilatation with similar efficacy ($E_{max} = 25 \pm 5\%$ for NO, $25 \pm 6\%$ for EDHF and $27 \pm 5\%$ for prostaglandins).

6 In conclusion, extracellular nucleotides induce endothelium-derived vasodilatation in human vessels by stimulating $P2Y_1$, $P2Y_{2/4}$ and $P2Y_6$ receptors, while P2X receptors are not involved. Endothelial P2Y receptors mediate dilatation by release of EDHF, NO and prostaglandins.
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Abbreviations: 2-MeSADP, 2-methylthio adenosine 5′-diphosphate; 2-MeSATP, 2-methylthio adenosine 5′-triphosphate; $\alpha\beta$-MeATP, $\alpha\beta$-methylene adenosine 5′-triphosphate; Ach, acetylcholine; ADP, adenosine diphosphate; ANOVA, the one-way analysis of variance; ATP, adenosine triphosphate; EC, endothelial cells; EC_{50}, effective concentration (e.g. 50% of maximum); EDHF, endothelium-derived hyperpolarising factor; LIMA, left internal mammary artery; L-NOARG, $N\varpi$-nitro-L-arginine; mRNA, messenger ribonucleic acid; MRS 2179, 2′-deoxy-*N*6-methyladenosine-3′,5′-bisphosphate; MRS 2216, 2-chloro-2′-deoxy-6-methyladenosine-3′,5′-bisphosphate; NA, noradrenaline; NO, nitric oxide; t-PA, tissue-plasminogen activator; UDP, uridine diphosphate; UDPβS, uridine 5′-O-2-thiodiphosphate; UTP, uridine triphosphate; UTPγS, uridine 5′-O-3-thiotriphosphate; VSMC, vascular smooth muscle cells

Introduction

Extracellular nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP) regulate vascular tone and blood pressure by stimulating P2 receptors (Gordon, 1986; Kunapuli & Daniel, 1998). Nucleotides induce vasoconstriction stimulating P2 receptors on the vascular smooth muscle cells (VSMCs) (Ralevic & Burnstock, 1998). Conversely, P2 receptors on the endothelium induce vasodilatation by the release of prostaglandins, nitric oxide (NO) and endothelium-derived hyperpolarising factor (EDHF) (Malmsjo *et al*., 1998; Ralevic & Burnstock, 1998; Malmsjo *et al*., 1999a,c). Furthermore, extracellular nucleotides have been shown to mediate both mitogenic effects, angiogenesis, apoptosis and release of tissue-plasminogen activator (t-PA) in endothelial cells (EC) (Satterwhite *et al*., 1999; von Albertini *et al*., 1998; Hrafnkelsdottir *et al*., 2001).

P2 receptors can be divided into two classes on the basis of their signal transmission mechanisms and their characteristic molecular structures: ligand-gated intrinsic ion channels, P2X receptors and G-protein-coupled P2Y receptors. The P2X

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receptor family is composed of seven cloned subtypes, all activated by the binding of extracellular ATP ($P2X_1 - P2X_7$) (Ralevic & Burnstock, 1998; Norenberg & Illes, 2000; Khakh *et al*., 2001). The P2Y family is composed of seven cloned and functionally defined subtypes. $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ are ADP receptors specifically activated by the stable analogue 2-methylthio adenosine 5′-diphosphate (2-MeSADP) (Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000; Hollopeter *et al*., 2001). $P2Y_2$ receptors are stimulated by UTP, uridine 5′-O-3-thiotriphosphate (UTPγS) and ATP, while $P2Y_4$ receptors are only stimulated by UTP and UT$P\gamma$S. $P2Y_6$ is activated by UDP and 5′-O-2-thiodiphosphate (UDPβS) (Lazarowski *et al*., 1996; Nicholas *et al*., 1996; Hou *et al*., 2002). The $P2Y_{11}$ receptor is stimulated by ATP, with no selective agonist known so far (Communi *et al*., 1997).

The $P2X_1$ receptor is located on VSMCs and is the main vasoconstrictor among the P2X receptors (Evans & Kennedy, 1994), but in the endothelium pharmacological evidence for P2X-mediated dilatation has been scarce. Recently, high expression levels for the $P2X_4$ receptor were found in the EC by Northern blot analysis and competitive, specific RT – PCR (Yamamoto *et al*., 2000b). Indeed, we have confirmed that $P2X_4$ is by far the P2 receptor having the highest messenger ribonucleic acid (mRNA) level in human EC, even compared to P2Y receptors (Wang *et al*., 2002). Other P2X receptors had considerably lower amounts of mRNA. Furthermore, all P2X subtypes have been detected with immunohistochemistry in human EC, but it is not possible to make any quantitative assessment of their relative expression with this method (Ray *et al*., 2002). The $P2X_4$ receptor is involved in shear stress-mediated EC activation if ATP is present, but any dilator function has not been demonstrated (Yamamoto *et al*., 2000b).

Molecular and functional expressions of $P2Y_1$ and $P2Y_2$ receptors have been shown in EC (Gordon, 1986; Pirotton *et al*., 1996; Kunapuli, 1998; Kunapuli & Daniel, 1998; Rump *et al*., 1998; Viana *et al*., 1998). Recently, we quantified P2Y receptors in human EC and found high mRNA levels of $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ (Wang *et al*., 2002). Thus, mRNA and protein for a large number of P2 receptors in EC have been shown, evidence for a functional role is lacking for several of the subtypes.

The endothelium plays a fundamental role in vascular physiology and pathophysiology, and the P2 receptors could be of major importance in regulating endothelial function. We therefore aimed at characterising the P2 receptors responsible for endothelium-derived vasodilatation in human vessels, using selective agonists and a recently developed antagonist. Furthermore, the involvement of different dilator endothelial mediators was investigated.

Methods

Tissue preparation

Branches of the left internal mammary artery (LIMA) were obtained from 20 patients undergoing coronary artery bypass graft surgery. The patient group consisted of 14 males and six females with an age of 62 ± 9 years (mean $\pm$ s.e.m.). All suffered from ischaemic heart disease and six from diabetes mellitus. All patients were medicated with aspirin, 14 with beta-blockers and two with clopidogrel. Five of the diabetic patients were treated with sulphonylurea and one with both sulphonylurea and insulin. The material was too small for subgroup comparisons. The blood vessels were kept in cold buffer solution (for composition, see below) and transported to the laboratory where they were dissected free of adhering tissue under a microscope. The LIMA branches had a maximal inner diameter of approximately 0.5 – 1 mm. The vessels were cut into 1.5 mm long cylindrical segments. Each 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 isomeric tension (Hogestatt *et al*., 1983). 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 vessel 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. The artery segments were allowed to stabilise at a resting tension of 1.5 mN for 1 h before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a potassium-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 studies. Eight ring segments were studied at the same time in separate tissue baths. Relaxation was examined in vascular segments precontracted by 1 μM noradrenaline (NA). Agonists were added cumulatively to determine concentration – response relations. A functional endothelium was confirmed by monitoring dilator responses to 2-MeSADP after NA preconstriction. If dilator responses to 2-MeSADP were absent, the lack of a functional endothelium could be confirmed by adding acetylcholine. The vessel segments that did not dilate were judged to have a nonfunctioning endothelium and were excluded from the study.

In experiments where endothelial denudation was needed, this was done by rubbing the luminal side of the vessel gently with a needle before mounting in the myograph.

Agonists

To stimulate $P2X_1$ receptors αβ-methylene adenosine 5′-triphosphate (α,β-MeATP) was used, 2-MeSADP for $P2Y_1$, 2-methylthio adenosine 5′-triphosphate (2-MeSATP) for P2X and $P2Y_1$ receptor, UTPγS for $P2Y_2$ and $P2Y_4$ ($P2Y_{2/4}$) (Lazarowski *et al*., 1996), UDPβS for $P2Y_6$ (Ralevic & Burnstock, 1998). To study the P2Y receptor-stimulated dilatation without interference of simultaneous $P2X_1$ receptor-induced responses, the extracellular nucleotides were added after desensitisation with 10 μM $\alpha\beta$-MeATP for 10 min (Kasakov & Burnstock, 1982). As the P2Y receptors are only very slowly desensitised, these agonists could be added cumulatively to determine concentration – response relations (Ralevic & Burnstock, 1998). Dilatation induced by acetylcholine (ACh) was studied in the presence and absence of 2-chloro-2′-deoxy-6-methyladenosine-3′,5′-bisphosphate (MRS 2216).

Antagonists

Antagonists were administered 15 min before the application of agonists. To block the $P2Y_1$ receptor, the selective antagonist, the deoxyadenosine bisphosphate derivative MRS 2216 (10 μM), was used (Brown *et al*., 2000).

The vasodilator and hyperpolarising effect of EDHF is antagonised by a combination of the potassium channel inhibitors, charybdotoxin and apamin (Chataigneau *et al*., 1998). Arginine analogues like L-NOARG ($N\varpi$-nitro-L-arginine) inhibit NO synthesis, but do not affect EDHF (Huang *et al*., 1988; Chen *et al*., 1991; Fujii *et al*., 1992). Although potential problems with inhibiting NO-synthetase have been reported, the high concentration of L-NOARG that was used in these experiments has been shown to totally block the release of NO (Zygmunt *et al*., 1994).

The 2-MeSADP-induced NO-mediated dilatation was studied in the presence of 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. EDHF was studied in the presence of 0.1 mM L-NOARG and indomethacin. The involvement of prostaglandins was investigated in the presence of L-NOARG, charybdotoxin and apamin.

Drugs

Agonist selectivity and stability are potential problems when analysing the pharmacological profiles of natural nucleotides in intact tissue. Therefore, more stable compounds were used, UTPγS, UDPβS, 2-MeSADP, 2-MeSATP and $\alpha\beta$-MeATP (for a review see Ralevic & Burnstock, 1998). UTPγS and UDPγS were gifts from Inspire Pharmaceuticals, Inc.; MRS 2216 was a gift from KA Jacobson, Molecular Recognition Section, Bioorganic Chemistry, NIH, Bethesda, MD, U.S.A. $\alpha\beta$-MeATP, 2-MeSADP, 2-MeSATP, NA, ACh, indomethacin, L-NOARG, charybdotoxin and apamin were purchased from SigmaCo, U.S.A. All the drugs were dissolved in 0.9% saline.

Ethics

The Ethics Committee of Lund University approved the project.

Calculation and statistics

Calculations and statistics were performed using the GraphPad Prism 3.02 software. The negative logarithm of the drug concentration that elicited 50% relaxation (pEC_{50}) was determined by fitting the data to the Hill equation. E_{max} refers to maximum relaxation calculated as the percentage of the corresponding precontraction with NA. *n* denotes the number of patients. Statistical significance was accepted when $P<0.05$, using Student's *t*-test analysing the 2-MeSADP and 2-MeSATP data with and without $P2Y_1$ blocker. To analyse the blocking of the dilator mediators (NO, EDHF and PG) the one-way analysis of variance (ANOVA) test followed by the Dunnett multiple comparisons test were used. Values are presented as mean$\pm$s.e.m.

$P2Y_1$ receptor antagonism

Antagonist equilibrium dissociation constant (apparent K_B) for the $P2Y_1$ receptor antagonist, MRS 2216, was determined by Gaddum analysis. This method involves the estimation of equieffective agonist concentrations in the presence and absence of a fixed antagonist concentration (Lazareno & Birdsall, 1993), according to the following equation: $K_B = [\text{antagonist}]_{conc.}/(((EC_{50agonist}$ with antagonist)/$EC_{50\ agonist})-1)$

Results

$P2X_1$ receptor desensitation

A concentration of 10 μM $\alpha\beta$-MeATP induced a transient contraction. When $\alpha\beta$-MeATP was added a second time, no contraction could be observed, indicating desensitised $P2X_1$ receptors.

Endothelial denudation

Relaxation to each agonist was tested after endothelial denudation in vessels from at least three patients, but none of the agonists caused dilatation.

P2Y receptor-mediated dilatations

The selective $P2Y_1$ receptor agonist 2-MeSADP caused a maximum vasodilatation of 68$\pm$3% (Figure 1 and Table 1). The $P2Y_1$ antagonist MRS 2216 caused a rightward shift of the concentration – response curve changing the pEC_{50} value from 6.9$\pm$0.1 to 5.8$\pm$0.3 (Figure 1 and Table 1). The pK_B for MRS 2216 was 6.3. A myograph registration of the dilator response to $P2Y_1$ agonist with and without $P2Y_1$ antagonist is shown in Figures 2a and b. There were no significant differences in E_{max} or in pEC_{50} between dilatation for 2-MeSADP and 2-MeSATP either alone or after addition of the $P2Y_1$ antagonist MRS 2216 (Figure 1 and Table 1). Both the $P2Y_{2/4}$ agonist UTPγS and the $P2Y_6$ agonist UDPβS induced potent vasodilatation with low efficacy (see Figure 3 and Table 1). The contractile response to noradrenaline was not affected by the $P2Y_1$ blocker, having an average of 168$\pm$17% and 172$\pm$15% of the initial potassium contraction with and without MRS 2216.

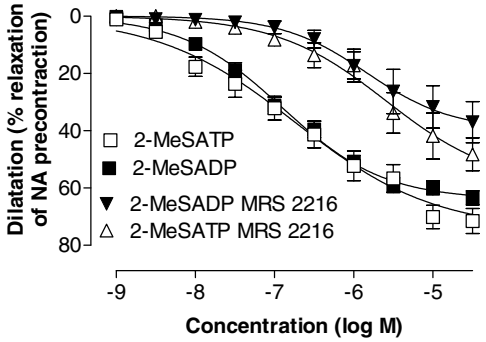

Figure 1 Concentration-dependent dilatation in response to 2-MeSADP and 2-MeSATP with and without pretreatment with the $P2Y_1$ receptor antagonist MRS 2216 (10 μM). The nucleotides were added after desensitisation of $P2X_1$ receptors with $\alpha\beta$-MeATP (10 μM). The dilatations are expressed as percentage relaxation of an initial precontraction induced by NA (1 μM). Data are shown as means$\pm$s.e.m.

There was no significant change in the ACh-induced dilatation by the presence of MRS 2216 ($pEC_{50} = 7.2 \pm 0.2$, $pEC_{50\ (MRS\ 2216)} = 7.3 \pm 0.2$, $E_{max} = 50 \pm 5\%$ and $E_{max\ (MRS\ 2216)} = 57 \pm 4\%$).

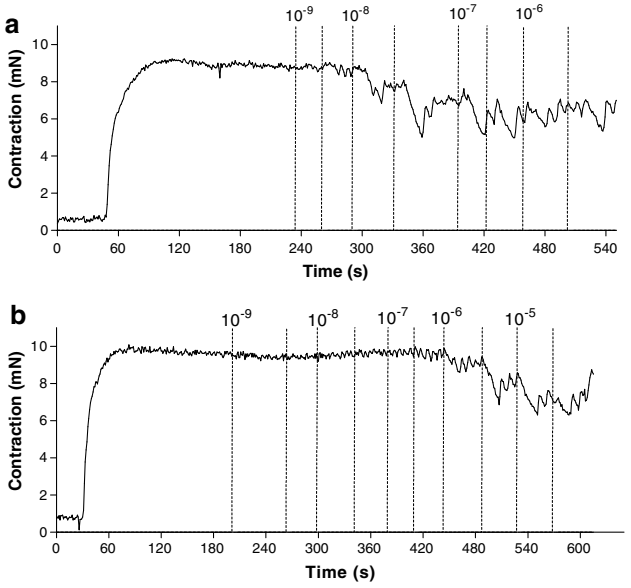

Figure 2 The myograph registration of NA (1 μM) contraction followed by concentration-dependent dilatation to 2-MeSADP with (a) and without (b) pretreatment with the $P2Y_1$ receptor antagonist MRS 2216 (10 μM). The time points for the addition of the respective concentrations of 2-MeSADP are marked in the figure by broken lines. The nucleotides were added after desensitisation of $P2X_1$ receptors with $\alpha\beta$-MeATP (10 μM).

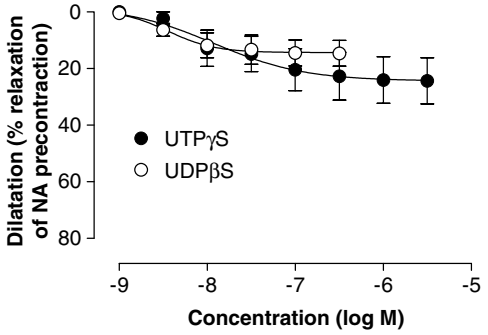

Figure 3 Concentration-dependent dilatation stimulated by UDPβS and UTPγS in human LIMA branches, after desensitisation of $P2X_1$ receptors with $\alpha\beta$-MeATP (10 μM). The dilatations are expressed as percentage of an initial precontraction induced by noradrenaline (1 μM). Data are shown as means$\pm$s.e.m.

Mediators

Pretreatment of the vessel segments with 10 μM indomethacin, 0.1 mM L-NOARG, 50 nM charybdotoxin and 1 μM apamin, abolished all vasodilator responses to the different agonists. In the presence of indomethacin, charybdotoxin and apamin the response stimulated by 2-MeSADP was reduced to $25 \pm 6\%$ relaxation of NA contraction (Figure 3 and Table 2). Inhibition with L-NOARG and indomethacin reduced the dilator response stimulated by 2-MeSADP to $25 \pm 5\%$ of NA contraction (Figure 3 and Table 2). In the presence of L-NOARG, charybdotoxin and apamin the response stimulated by 2-MeSADP was reduced to $27 \pm 5\%$ relaxation of NA contraction (Figure 4 and Table 2). The dilatations were significantly reduced compared to 2-MeSADP alone (Table 2).

In the presence of indomethacin the response to 2-MeSADP was reduced to $36 \pm 10\%$ relaxation of NA contraction, representing the prostaglandins and EDHF-mediated dilatation (Figure 5 and Table 3). In the presence of charybdotoxin and apamin the response to 2-MeSADP was reduced to $30 \pm 7\%$ relaxation of NA contraction, representing the NO and prostaglandins-mediated dilatation (Figure 5 and Table 3). In the presence of L-NOARG the dilator response to 2-MeSADP was reduced to $32 \pm 4\%$ of NA contraction, representing the prostaglandins and EDHF-mediated dilatation (Figure 5 and Table 3). The results differed significantly from the response to 2-MeSADP (Table 3). No significant changes in NA contraction with and without the different blockers were observed in this material.

Discussion

In this study, the dilator P2Y receptors have been characterised in human LIMA branches. In agreement with the initial definition of P2Y receptors by Burnstock & Kennedy (1985), stimulation of endothelial $P2Y_1$ receptors with the selective agonist 2-MeSADP induced the most efficacious dilatation. This indicates an important dilator role for endothelial $P2Y_1$ receptors. Recent real-time PCR measurements have demonstrated high $P2Y_1$ receptor mRNA levels in human EC (Wang *et al*., 2002).

MRS 2216 is a new selective $P2Y_1$ receptor antagonist that has no effect on $P2X_1$ and $P2X_3$ receptors (Brown *et al*., 2000). It has a structure similar to that of 2′-deoxy-*N*6-methyladenosine-3′,5′-bisphosphate (MRS 2179) that has been evaluated in cells selectively transfected with different P2Y receptor subtypes and shown to be a specific antagonist to $P2Y_1$, with

Table 1 Concentration-dependent dilatations stimulated by UTPγS, UDPβS, 2-MeSADP and 2-MeSATP with and without the $P2Y_1$ receptor antagonist MRS 2216 (10 μM)

Agonist/antagonist	E_{max} *(%)*	*pEC$_{50}$ (−log M)*	*n*	
2-MeSADP	68±3	6.9±0.1	12	
2-MeSATP	63±5	6.7±0.3	12	NS
2-MeSADP/MRS 2216	40±7	5.8±0.3	8	
2-MeSATP/MRS 2216	42±8	5.6±0.4	6	NS
UTPγS	24±4	7.8±0.4	6	
UDPβS	14±2	8.4±0.2	9	

The dilatations are expressed as percentage of an initial precontraction induced by NA (1 μM). Data are shown as $E_{max} \pm$s.e.m. and $pEC_{50} \pm$s.e.m. There was no significant difference between 2-MeSADP and 2-MeSATP with or without MRS 2216 with respect to the induced dilatation.

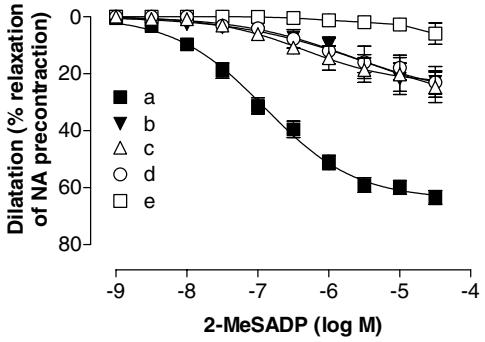

Figure 4 Concentration-dependent dilatation induced by 2-MeSADP after desensitisation of $P2X_1$ receptors with $\alpha\beta$-MeATP (10 μM). (a) Dilatation induced by 2-MeSADP. (b) Dilatation induced by 2-MeSADP in the presence of 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. (c) The dilatation induced by 2-MeSADP in the presence of 10 μM indomethacin and 0.1 mM L-NOARG. (d) Dilatation induced by 2-MeSADP in the presence of 0.1 mM L-NOARG, 50 nM charybdotoxin and 1 μM apamin. (e) Dilatation induced by 2-MeSADP in the presence of 10 μM indomethacin, 0.1 mM L-NOARG, 50 nM charybdotoxin and 1 μM apamin. The dilatations are expressed as percentage of an initial precontraction induced by noradrenaline (1 μM). Data are shown as means $\pm$ s.e.m.

no effect on the other P2Y receptor subtypes (Jacobson *et al*., 2002). MRS 2179 is 11-fold selective for $P2Y_1$ *versus* $P2X_1$ receptors (Brown *et al*., 2000). The dilator effects of 2-MeSADP were inhibited by 10 μM MRS 2216 in the present study, indicating that it acts as a potent $P2Y_1$ antagonist in intact blood vessels. Furthermore, the specificity was supported by a lack of effect on ACh-mediated vasodilatation. This is one step towards the development of a $P2Y_1$ receptor antagonist for human use, for which an important role as an inhibitor of platelet aggregation can be anticipated.

Ligand instability is a problem especially when investigations are performed in intact tissues as nucleotide triphosphates are metabolised by ectonucleotidases on the cell membranes (Malmsjo *et al*., 2000a,b). The vascular actions of the pyrimidine-sensitive receptors were not possible to evaluate until the stable pyrimidine analogues UTPγS and UDPβS were characterised on selectively transfected P2Y receptor subtypes (Lazarowski *et al*., 1996; Hou *et al*., 2002). UTPγS is selective for $P2Y_2$ and $P2Y_4$ receptors but cannot discriminate between them (Lazarowski *et al*., 1996). UDPβS is selective for $P2Y_6$ receptors with no effects on the other P2 receptors (Hou *et al*., 2002). It has been demonstrated that UTP mediates endothelium-derived dilatation in human coronary arteries, but it was not possible to discriminate between the pyrimidino-receptor subtypes (Hansmann *et al*., 1998).

Previous studies have not been able to demonstrate any $P2Y_6$ receptor-mediated dilatations, but $P2Y_6$ receptors on VSMCs have been repeatedly shown to mediate vasoconstriction (Malmsjo *et al*., 2000a). Surprisingly, $P2Y_6$ receptors were expressed to a similar extent in EC as in VSMC at the protein level (Wang *et al*., 2002). In the present study, the selective $P2Y_{2/4}$ agonist, UTPγS and the selective $P2Y_6$ receptor agonist UDPβS induced potent vasodilatations with low efficacy. The low maximum dilatation is probably because of counteracting contractile $P2Y_{2/4}$ and $P2Y_6$ receptors on VSMCs. Both

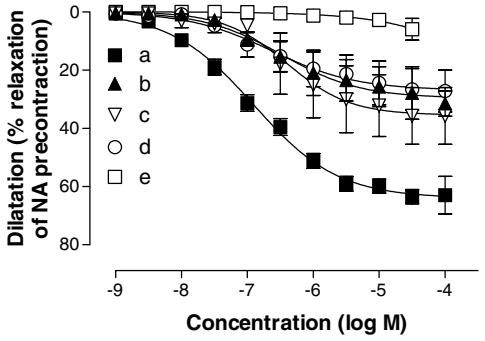

Figure 5 Concentration-dependent dilatation in response to 2-MeSADP after desensitisation of $P2X_1$ receptors with $\alpha\beta$-MeATP (10 μM). (a) Dilatation induced by 2-MeSADP. (b) Dilatation induced by 2-MeSADP in the presence of 0.1 mM L-NOARG. (c) Dilatation induced by 2-MeSADP in the presence of 10 μM indomethacin. (d) Dilatation induced by 2-MeSADP in the presence of 50 nM charybdotoxin and 1 μM apamin. (e) Dilatation induced by 2-MeSADP in the presence of 0.1 mM L-NOARG, 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. The dilatations are expressed as percentage of an initial precontraction induced by noradrenaline (1 μM). Data are shown as means $\pm$ s.e.m.

Table 2 Concentration-dependent dilatation induced by 2-MeSADP with and without blocking of the mediators

	Blockers	E_{max} *(%)*	pEC_{50} *(−log M)*	*n*	*Versus control*
a	Control (no blockers)	68 $\pm$ 3	6.9 $\pm$ 0.1	12	
b	10 μM indomethacin 50 nM charybdotoxin 1 μM apamin	25 $\pm$ 6	5.4 $\pm$ 1.0	6	**
c	10 μM indomethacin 0.1 mM L-NOARG	25 $\pm$ 5	6.3 $\pm$ 0.3	7	**
d	0.1 mM L-NOARG 50 nM charybdotoxin 1 μM apamin	27 $\pm$ 5	5.7 $\pm$ 0.7	6	**

(a) Dilatation induced by 2-MeSADP. (b) Dilatation induced by 2-MeSADP in the presence of 10 μm indomethacin, 50 nm charybdotoxin and 1 μm apamin. (c) The dilatation induced by 2-MeSADP in the presence of 10 μm indomethacin and 0.1 mm l-NOARG. (d) Dilatation induced by the 2-MeSADP in the presence of 0.1 mm l-NOARG, 50 nm charybdotoxin and 1 μm apamin. The dilatations are expressed as percentage of an initial precontraction induced by noradrenaline (1 μm). Data are shown as $E_{max} \pm$ s.e.m. and $pEC_{50} \pm$ s.e.m.

Table 3 Concentration-dependent dilatation in response to 2-MeSADP with and without blocking of the mediators

	Blockers	E_{max} *(%)*	pEC_{50} *(−log M)*	*n*	*Versus control*
a	Control (no blockers)	64±3	6.9±0.1	12	
b	0.1 mM L-NOARG	32±4	6.5±0.3	3	**
c	10 μM indomethacin	36±10	6.4±0.3	5	**
d	50 nM charybdotoxin 1 μM apamin	30±7	6.6±0.3	11	**

NO, prostaglandins and EDHF with 0.1 mM L-NOARG, 10 μM indomethacin, 50 nM charybdotoxin and 1 μM apamin. The table shows the remaining dilator response to 2-MeSADP after (b) blockade of EDHF with 50 nM charybdotoxin and 1 μM apamin, (c) blockade of NO with 0.1 mM L-NOARG and (d) blockade of prostaglandins with 10 μM indomethacin. The dilatations are expressed as percentage of an initial precontraction induced by NA (1 μM). Data are shown as E_{max} ± s.e.m. and pEC_{50} ± s.e.m.

UTPγS and UDPβS induced vasoconstriction (data not shown), indicating the presence of $P2Y_{2/4}$ and $P2Y_6$ receptors also on VSMCs in these LIMA branches. The observation of a vasodilator effect induced by UDPβS is important, because it implies that a selective $P2Y_6$ receptor agonist could have hypotensive effects, particularly if administered only at the endothelial side of the vessel. On the other hand, it could be valuable for vasodilation or t-PA release.

UTPγS induced potent dilatations with low efficacy, indicating the presence of dilator $P2Y_2$ or $P2Y_4$ receptors on the endothelium. $P2Y_2$ receptor mRNA has been detected to a level similar to that of $P2Y_1$ receptor mRNA in human EC, the level of $P2Y_4$ receptor mRNA was detected to a lower extent (Wang *et al*., 2002). These results suggest that the UTPγS dilatation was mediated via $P2Y_2$ and not $P2Y_4$ receptors. Real-time PCR experiments have also demonstrated the presence of $P2Y_{11}$ receptor mRNA in EC (Wang *et al*., 2002). $P2Y_{11}$ is an ATP receptor for which selective agonists have not yet been developed. Therefore, the importance of this receptor in inducing vasodilatation cannot be elucidated yet.

Endothelial P2X receptors

Although considerable levels of mRNA of P2X receptors in EC has been shown, no dilator effects of P2X receptors have yet been demonstrated (Yamamoto *et al*., 2000a). The highest level of P2 receptor mRNA corresponded to the $P2X_4$ receptor. One difficulty in studying P2X receptors is that no selective P2X receptor antagonists have yet been developed. From the present study it can be concluded that P2X receptors do not induce vasodilatation in human LIMA branches. Firstly, αβ-MeATP did not elicit vasodilatation, indicating no effect of $P2X_1$ and $P2X_3$ receptors. Secondly, the selective $P2Y_1$ receptor agonist, 2-MeSADP, induced dilatations that were similar to those for the combined $P2Y_1$ and P2X receptor agonist, 2-MeSATP. Thirdly, the selective $P2Y_1$ receptor antagonist, MRS 2216, had the same inhibitory effect of the dilatation induced by 2-MeSADP, as that induced by 2-MeSATP. Thus, even if the $P2X_4$ receptor has the highest mRNA level in EC we could not find evidence of any dilator action. However, it may have other roles such as angiogenesis, t-PA release or even apoptosis (von Albertini *et al*., 1998; Satterwhite *et al*., 1999; Hrafnkelsdottir *et al*., 2001).

Dilatation mediators

Activation of P2Y receptors on EC induces vasodilatation by release of prostaglandins and NO (Ralevic & Burnstock, 1998). In animal studies, P2Y receptors have also been shown to stimulate EDHF-mediated dilatation (Malmsjo *et al*., 1998, 1999c). Infusion of UTP and ATP in humans reduces forearm vascular resistance independent of prostaglandins and NO (Hrafnkelsdottir *et al*., 2001). Nonetheless, the factor responsible for the remaining dilatation has not been characterised in humans in accordance with all the requirements needed for identifying EDHF.

Although the chemical identity of EDHF remains unknown, certain criteria have been postulated to identify it. EDHF is released by the endothelium, not inhibited by antagonists of NO synthetase or cyclooxygenase pathways, that hyperpolarises and relaxes VSMCs. The dilator effect of EDHF can be antagonised by the potassium channel inhibitors charybdotoxin and apamin (Corriu *et al*., 1996; Zygmunt & Hogestatt, 1996; Chataigneau *et al*., 1998; Zygmunt *et al*., 1998). In the mesenteric artery, activation of P2Y receptors induces endothelium-derived vasodilatation mediated by a factor distinct from NO and prostaglandins that is blocked by charybdotoxin and apamin, indicating that it is EDHF (Malmsjo *et al*., 1998). This was confirmed by electrophysiological experiments in which both ACh and P2Y receptor agonists induce endothelium-derived hyperpolarisation of VSMCs (Malmsjo *et al*., 1999c). The hyperpolarisation was antagonised by charybdotoxin and apamin, confirming the use of this combination to inhibit EDHF.

In the present study, 2-MeSADP induced endothelium-derived dilatations of the LIMA branches. Pretreatment of the vessel segments with L-NOARG, indomethacin, charybdotoxin and apamin abolished all vasodilator responses to the agonist, indicating that no dilator mediator other than NO, prostaglandins or EDHF was involved. The dilatations turned out to be mediated by a similar contribution of EDHF, nitric oxide and prostaglandins. This indicates that EDHF is at least as important as NO in mediating vasodilatation to extracellular nucleotides in man. The evidence is in line with our findings in the human forearm where we were unable to block UTP-mediated reduction in vascular resistance by L-NAME and indomethacin (Satterwhite *et al*., 1999; Hrafnkelsdottir *et al*., 2001). Furthermore, it is supported by previous evidence of EDHF-mediated dilatation in LIMA for other agonists (Liu *et al*., 2000; He & Liu, 2001). Prostaglandins were at least as important as NO in mediating vasodilatation to extracellular nucleotides in man. This stands in contrast to recent animal studies where prostaglandins played only a minor role in mediating P2Y receptor induced dilatation (Malmsjo *et al*., 1998). Furthermore, the dilatation mediated by prostaglandins may have been underestimated in these patients, because all were medicated with aspirin. It should be noted that this study was performed on arteries from patients with atherosclerotic

disease where the endothelium function could be altered, although the vessels used did not show any signs of atherosclerosis. For obvious reasons, the availability of arteries from healthy individuals is limited. The importance of EDHF in inducing dilatation has been shown to be greater in rats with congestive heart failure (Vargas *et al*., 1996; Malmsjo *et al*., 1999b). On the other hand, in hypertensive patients, the ATP induced vasodilatation did not differ from healthy controls *in vivo* (Nilsson *et al*., 2000).

Conclusion

The $P2Y_1$ receptor selective agonist, 2-MeSADP, induced the most potent and efficacious dilatation in human arteries. The $P2Y_{2/4}$ agonist UTPγS also induced vasodilatation. Experiments with UDPβS demonstrated for the first time a role for $P2Y_6$ receptors in stimulating endothelium-derived relaxation in humans. The UTPγS and the UDPβS dilatations were less efficacious than that induced by 2-MeSADP, probably because of the counteracting contractile effects of the two former agonists. The highest expressed P2 receptor in EC, the $P2X_4$ receptor, did not mediate endothelium-derived dilatation. This study is the first to provide thorough evidence that nucleotides induce dilatation of human arteries via EDHF, in addition to the previously studied NO and prostaglandins.

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