Research article

Potent P2Y$_6$ receptor mediated contractions in human cerebral arteries

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Abstract

Background: Extracellular nucleotides play an important role in the regulation of vascular tone and may be involved in cerebral vasospasm after subarachnoidal haemorrhage. This study was designed to characterise the contractile P2 receptors in endothelium-denuded human cerebral and omental arteries. The isometric tension of isolated vessel segments was recorded *in vitro*. P2 receptor mRNA expression was examined by RT-PCR.

Results: In human cerebral arteries, the selective P2Y$_6$ receptor agonist, UDPβS was the most potent of all the agonists tested (pEC$_{50}$ = 6.8 ± 0.7). The agonist potency; UDPβS > αβ-MeATP > UTPγS > ATPγS > ADPβS = 0, indicated the presence of contractile P2X$_1$ P2Y$_2$, P2Y$_4$ and P2Y$_6$, but not P2Y$_1$ receptors, in human cerebral arteries. In human omental arteries, UDPβS was inactive. The agonist potency; αβ-MeATP > ATPγS = UTPγS > ADPβS = UDPβS = 0, indicated the presence of contractile P2X$_1$, and P2Y$_2$ receptors, but not P2Y$_1$ or P2Y$_6$ receptors, in human omental arteries. RT-PCR analysis of endothelium-denuded human cerebral and omental arteries demonstrated P2X$_1$, P2Y$_1$, P2Y$_2$ and P2Y$_6$ receptor mRNA expression. There were no bands for the P2Y$_4$ receptor mRNA in the omental arteries, while barely detectable in the cerebral arteries.

Conclusions: P2Y$_6$ receptors play a prominent role in mediating contraction of human cerebral arteries. Conversely, no such effect can be observed in human omental arteries and previous results confirm the absence of P2Y$_6$ receptors in human coronary arteries. The P2Y$_6$ receptor might be a suitable target for the treatment of cerebral vasospasm.

Background

There is evidence of the release of adenosine triphosphate (ATP) from endothelial cells, platelets, and sympathetic nerves as well as from damaged cells in atherosclerosis, hypertension, restenosis and ischemia [1]. Uridine tri-phosphate (UTP) has been shown to be released from endothelial cells in response to flow and shear stress [2], and it has been isolated from platelets [3]. Since UTP is particularly present in the brain and has potent vasoconstrictor actions in human cerebral arteries, it may play a role in

cerebral vasospasm after subarachnoidal haemorrhage [4–6].

Extracellular nucleotides induce vasodilatation by activating P2Y receptors on endothelial cells, while vasoconstriction is mediated by P2Y and P2X receptors on vascular smooth muscle cells. When the endothelial function is damaged in pathological conditions such as subarachnoidal haemorrhage or arteriosclerosis the vasoconstrictor actions dominate, causing cerebral vasospasm [7].

Recent receptor cloning has proven the existence of several different P2X and P2Y receptor subtypes, and there is evidence that five of these elicit vascular response when stimulated by extracellular nucleotides, namely $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ [8,9]. Expression of these receptors in cells has enabled characterisation of their respective pharmacological profile. $P2X_1$ receptors have been shown by immunohistochemistry to be the dominant P2X receptor on smooth muscle cells in the vasculature [10,11]. $P2X_1$ receptors are activated by αβ-methylene-adenosine triphosphate (αβ-MeATP) > ATP with no effect of uridine triphosphate (UDP) or UTP [12]. At the $P2Y_1$ receptor adenosine 5'-O-3-thiodiphosphate (ADPβS) and adenosine diphosphate (ADP) have greater potency than ATP, while UTP and UDP are inactive [13,14]. The $P2Y_2$ receptor is activated with similar potencies by ATP and UTP but not by ADP or UDP; the human $P2Y_4$ receptor is activated most potently by UTP, less potently by ATP, and not at all by nucleotide diphosphates; and the $P2Y_6$ receptor is activated most potently by UDP but weakly by UTP, ADP and ATP [15].

However, the identification of P2 receptors expressed on smooth muscle cells is difficult particularly because of the absence of truly selective agonists and antagonists. Ligand instability complicates the analyses especially when performed in intact tissues as nucleotide triphosphates are rapidly metabolised by ectonucleotidases on the extracellular surface of cells [16]. In addition, commercial nucleotides are impure. Stable nucleotides have recently started to be used in attempts to pharmacologically define the P2Y receptor subtypes. These include uridine 5'-O-thiodiphosphate (UDPβS), uridine 5'-O-3-thiotriphosphate (UTPγS), adenosine 5'-O-thiodiphosphate (ADPβS) and adenosine 5'-O-3-thiotriphosphate (ATPγS) that contain a modification of the nucleotide triphosphate group in the form of a thio substitution at the terminal phosphate, which provides stability to ectonucleotidase action. UTPγS is a potent and enzymatically stable agonist at the human $P2Y_2$ and $P2Y_4$ receptors, while UDPβS has recently shown to selectively activate the $P2Y_6$ receptors [17,18]. It is therefore now possible to discriminate between the vascular effects of different pyrimidine activated P2 receptor subtypes.

In the design of future cerebrovascular therapeutics it is important that the contractile responses of extracellular nucleotides is characterised in human subjects. This study was designed to evaluate the relative contribution of the different P2Y receptor subtypes that mediate the contractile response in human cerebral and omental arteries, which has not been done before by use of the stable pyrimidines UDPβS and UTPγS.

Results
Vasomotor responses
After endothelium removal, the vasodilatory response to acetylcholine in UTP preconstricted arteries was abolished, indicating a properly removed endothelium. Furthermore, vascular smooth muscle cell function was considered intact, since the contractile response to 60 mmol/L K^+ was unaffected. P2X receptors were desensitised by addition of 10 μmol/Lαβ-MeATP. This elicited a transient contraction. After 8 min the tension was back to baseline and if αβ-MeATP was added a second time, no contraction could be observed, indicating desensitised P2X receptors.

The contractile capacity of the arteries was examined by addition of 60 mmol/L K^+. For the cerebral arteries the contractile response to 60 mmol/L K^+ amounted to 2.7 ± 1.3 mN above baseline tension and, for omental arteries, to 8.2 ± 0.8 mN. The nucleotide-induced contractions were thereafter calculated as percent of this K^+-contraction in respective vessel segment.

Human cerebral arteries
αβ-MeATP induced potent contractions of the human cerebral arteries, indicating effects of contractile P2X receptors. After P2X receptor desensitisation (see methods), further experiments were performed to analyse P2Y receptor mediated vasoconstrictions. The endogenous nucleotides, UDP and UTP, elicited contractions of similar potencies (pEC_{50} = 5.5 ± 0.2 and 5.3 ± 0.2, P = n.s.). Further experiments were therefore performed using the stable nucleotide analogues UDPβS, UTPγS, ATPγS and ADPβS. Notably, the selective $P2Y_6$ receptor agonist, UDPβS, was one log unit more potent than UDP in the human cerebral arteries (pEC_{50} = 6.8 ± 0.2 and 5.5 ± 0.2, P < 0.001), making it the most potent of all agonists tested. ATP and ADP induced contractions only at mmol/L concentrations. The stable purine, ATPγS, induced a considerably more potent contraction than ATP (pEC_{50} = 3.0 ± 0.2 for ATP and 4.8 ± 0.2 for ATPγS, P < 0.001). The ATPγS concentration-response curve resembled that of UTPγS, indicating involvement of $P2Y_2$ receptors. On the other hand, ADPβS had no effect, indicating that there are no contractile $P2Y_1$ receptors.

In conclusion, the potency order for the agonists in human cerebral arteries was: UDPβS > αβ-MeATP > UTPγS > ATPγS > ADPβS = 0, indicating involvement of contractile $P2X_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, but not $P2Y_1$ receptors, in human cerebral arteries. All data are presented in Table 1. For data concerning the endogenous nucleotides in the cerebral circulation, see Fig 1a. the stable nucleotides, see Fig 2a.

Human omental arteries

αβ-MeATP induced potent contractions of the human omental arteries, indicating effects of contractile P2X receptors. After P2X receptor desensitisation (see methods), further experiments were performed to analyse P2Y receptor mediated vasoconstrictions. The endogenous nucleotides, UTP and UDP, only induced contraction at a high concentration (mM). Conversely, the stable pyrimidines, UDPβS and UTPγS, had a markedly different pharmacological profile. Unlike the cerebral arteries, UDPβS was inactive in the human omental arteries, indicating that there are no contractile $P2Y_6$ receptors. The potency of UTPγS was one log unit higher than that for UTP (pEC_{50} = 4.3 ± 0.1 and 3.3 ± 0.1, P < 0.001), indicating stability to ectonucleotidase activity. UTPγS and ATPγS induced vasoconstriction might be a result of $P2Y_2$ or $P2Y_4$ receptor activation in omental arteries. Like in the cerebral arteries, ADPβS did not induce vasoconstriction in the omental arteries, indicating that contractile $P2Y_1$ receptors do not play a role.

In conclusion, the potency order for the agonists in the human omental arteries was; αβ-MeATP > ATPγS = UTPγS > ADPβS = UDPβS = 0, indicating the presence of contractile $P2X_1$ and $P2Y_2$ receptors, but not $P2Y_1$ or $P2Y_6$ receptors, in human omental arteries. All data are presented in Table 1. For data concerning the endogenous nucleotides in the cerebral circulation, see Fig 1b. the stable nucleotides, see Fig 2b.

RT-PCR

Agarose gel electrophoresis of PCR products from endothelium-denuded, human cerebral and omental arteries demonstrated products of the expected size for the corresponding mRNA encoding human $P2X_1$ (383 base pairs), $P2Y_1$ (550 base pairs), $P2Y_2$ (432 base pairs) and $P2Y_6$ receptors (526 base pairs) (Fig. 3). There were no bands for the $P2Y_4$ receptor mRNA (530 base pairs) in the omental arteries, while these were barely detected in the cerebral arteries. No bands were detected in controls without an RT-step.

Discussion

The stable pyrimidines, UTPγS and UDPβS, were here for the first time used to characterise the P2 receptor subtypes that mediate vasoconstriction in human cerebral and omental arteries. The novel findings include that the $P2Y_6$ receptors play a prominent role in mediating contraction of cerebral arteries. Conversely, no such effect can be observed in omental arteries and previous results confirm the absence of $P2Y_6$ receptors in human coronary arteries [26]. The marked effects of the $P2Y_6$ receptor in the cerebral circulation might make it a suitable target for treatment of diseases with cerebral vasospasm.

Study of P2Y receptors has been a challenge due to the paucity of pharmacological tools for identification of receptor subtypes. Since truly selective antagonists have not yet been developed, the characterisation of P2Y receptors has mainly been performed by monitoring agonist responses. This causes difficulties, as the endogenous nucleotides UTP, UDP, ATP and ADP are neither selective nor stable. However, recent studies present the stable pyrimidines UTPγS, UDPβS as unique pharmacological tools that facilitate the characterisation of P2Y receptor subtypes in intact tissues [26,27]. The present results clearly demonstrate the importance of stable nucleotides in the characterisation of receptors in tissue preparations with ectonucleotidase activity: In the cerebral arteries, UTP and UDP were equipotent while the stable pyrimidines demonstrated a 1.5 log unit higher potency for UDPβS as compared to UTPγS. UDP gave contractions in the omental arteries with low potency, but this could not be reproduced by UDPβS, indicating that UDP was enzymatically converted to UTP. Likewise, ADP induced contractions in both cerebral and omental arteries, which could not be reproduced by ADPβS. Thus, the endogenous nucleotides may both underestimate and overestimate the contribution of different P2Y receptor subtypes, and previous studies using non-stable nucleotides should be interpreted with caution.

Extracellular nucleotides are released from blood clots during subarachnoidal haemorrhage [28,29]. Since both UDP and UTP are particularly present in the brain and produce sustained contraction of cerebral blood vessels, these have been postulated to contribute in the genesis of vasospasm after subarachnoidal haemorrhage [4,6]. The present results that UTP and UDP were equipotent in inducing contraction of cerebral arteries has also been shown by others [4,30]. Therefore, stable nucleotides were used to further characterise the pyrimidines sensitive P2Y receptors that mediated this vasoconstrictor response.

In the human cerebral arteries, UDPβS was 1.5 log units more potent than UTPγS, indicating prominent effects by the $P2Y_6$ receptor. Interestingly, UDPβS did not act as a vasoconstrictor in human omental arteries, which is in accordance with a previous study of human coronary arteries where UDPβS also lacked an effect [26]. It is well known that receptor distribution varies between different
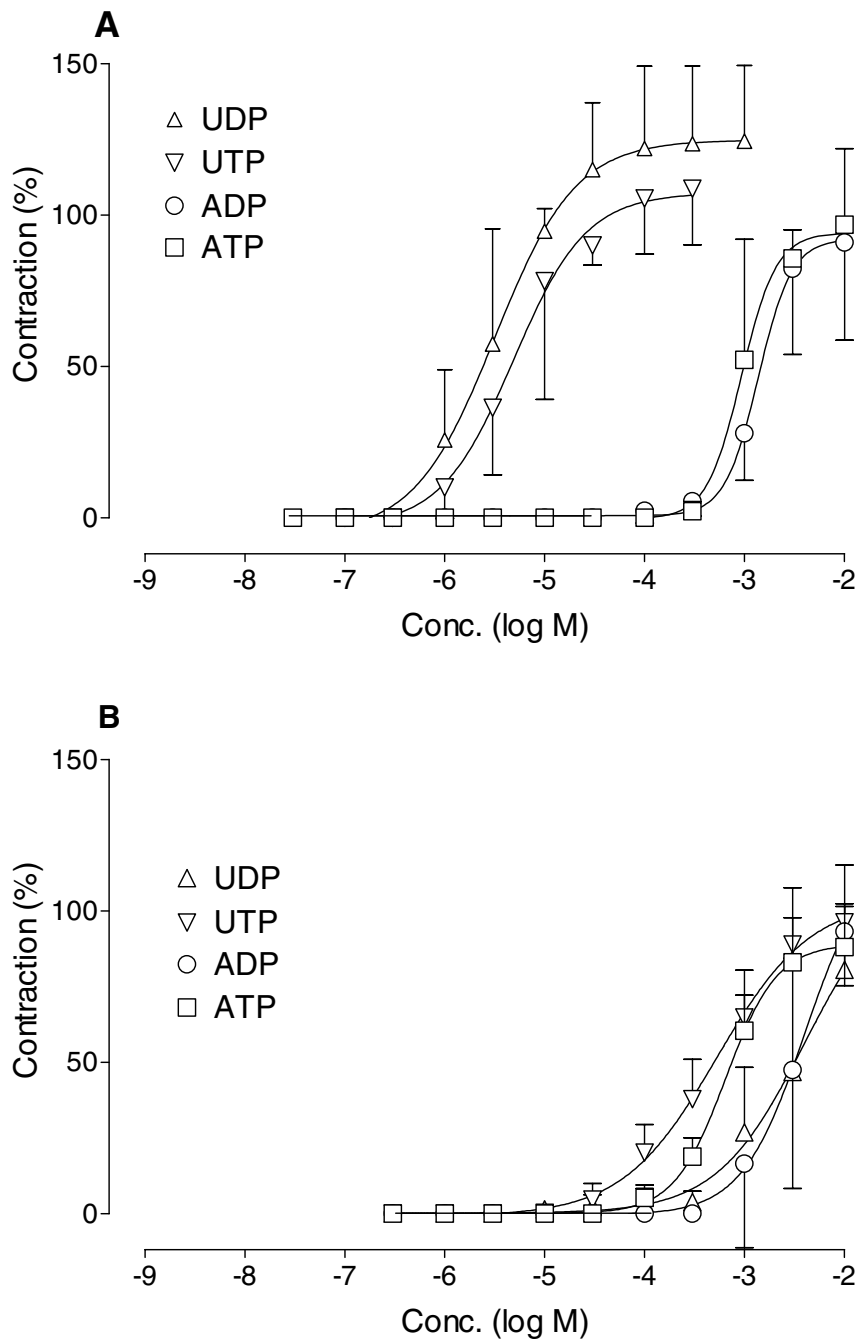
Figure 1
Concentration-dependent contractions to UDP, UTP, ADP and ATP in (**A**) human cerebral arteries and (**B**) human omental arteries. UDP, UTP, ADP and ATP were added after P2X receptor desensitisation with 10 μmol/L αβ-MeATP. Contractions are expressed as percentage of the response to 60 mmol/L K$^+$. Data are shown as mean values ± S.E.M of 6 experiments (patients).
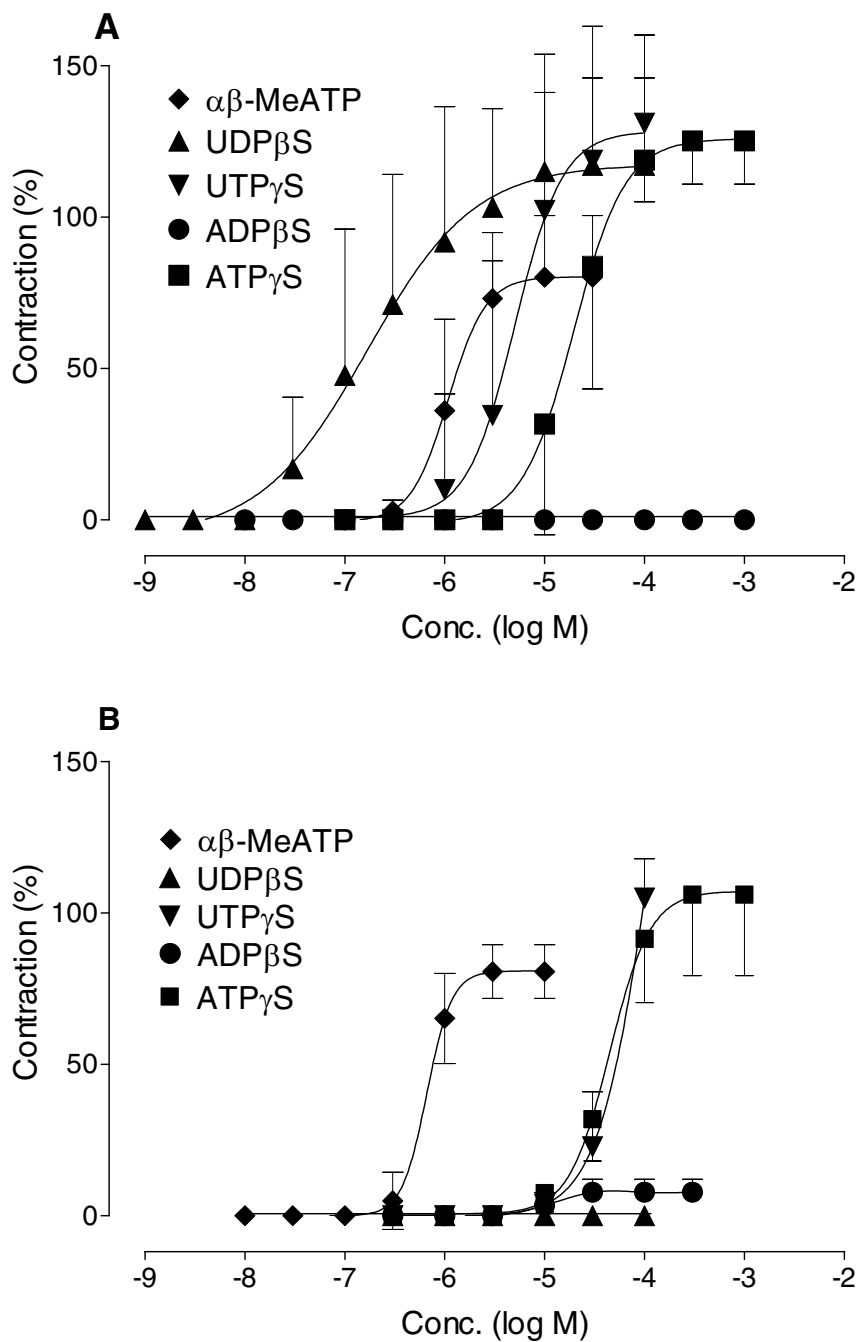
Figure 2
Concentration-dependent contractions to αβ-MeATP, UDPβS, UTPγS, ADPβS and ATPγS in (**A**) human cerebral arteries and (**B**) human omental arteries. UDPβS UTPγS, ADPβS and ATPγS were added after P2X receptor desensitisation with 10 μmol/L αβ-MeATP. Contractions are expressed as percentage of the response to 60 mmol/L K$^+$. Data are shown as mean values ± S.E.M of 6 experiments (patients).

**Table 1: Contractile responses to extracellular nucleotides in human cerebral and omental arteries.**

	Cerebral arteries		Omental arteries	
	pEC_{50} (-log mol/L)	E_{max} (%)	pEC_{50} (-log mol/L)	E_{max} (%)
$\alpha\beta$-MeATP	6.0 ± 0.1	80 ± 8	6.2 ± 0.1	81 ± 2
UDP	5.5 ± 0.2	128 ± 16	2.7 ± 0.2	81 ± 9
UDPβS	6.8 ± 0.2	117 ± 8	-	0 ± 0
UTP	5.3 ± 0.2	110 ± 10	3.3 ± 0.1	96 ± 8
UTPγS	5.3 ± 0.1	131 ± 10	4.3 ± 0.1	105 ± 7
ADP	2.9 ± 0.1	91 ± 23	2.6 ± 0.2	96 ± 7
ADPβS	0.0 ± 0.0	-	-	8 ± 4
ATP	3.0 ± 0.2	97 ± 25	3.2 ± 0.1	88 ± 6
ATPγS	4.8 ± 0.2	125 ± 8	4.4 ± 0.1	106 ± 11

All nucleotides, except $\alpha\beta$-MeATP, were added after desensitisation of P2X receptors with 10 μmol/L $\alpha\beta$-MeATP. Contractions are expressed as percentage of an initial contraction induced by 60 mmol/L K^+. Data are shown as E_{max} ± S.E.M. and pEC_{50} ± S.E.M of 6 experiments (patients).
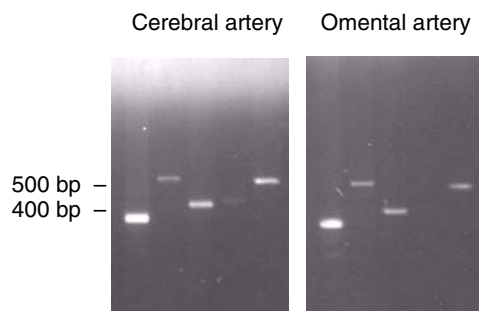
Figure 3
Electrophoresis of RT-PCR products corresponding to mRNA encoding human $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (shown from left to right) in human cerebral and omental arteries. The amplified products were of the predicted size for the human $P2X_1$ (383 base pairs), $P2Y_1$ (550 base pairs), $P2Y_2$ (432 base pairs), $P2Y_4$ (530 base pairs) and $P2Y_6$ receptors (526 base pairs). Samples were analysed on a 2% agarose / ethidium bromide gel at 5 V/cm, and photographed.

vascular beds in order to obtain a specific blood flow regulation. UTP has been shown to produce prominent contractions in the cerebral circulation, as compared to in the periphery [29]. Furthermore, cerebral arteries were more sensitive to ATP than systemic arteries from the dog heart and mesentery [31].

The similarity in potency between ATPγS (after P2X receptor desensitisation) and UTPγS suggest the presence of contractile $P2Y_2$ receptors in both cerebral and omental arteries. An effect by the $P2Y_4$ receptor cannot be excluded since it is also activated by UTPγS. However, RT-PCR analysis could not detect $P2Y_4$ mRNA expression in the omental arteries. These results indicate that the $P2Y_4$ receptor is of none or minor importance in mediating vasoconstriction. Similar results have been reported from rat pial arteries where mRNA transcripts for the $P2Y_2$, but not the $P2Y_4$ receptor could be amplified [32].

The $P2Y_2$ effect was less prominent than that for the $P2Y_6$ receptors in human cerebral arteries. UTP has previously been proposed to elicit sustained vasoconstriction and vasospasm after subarachnoidal haemorrhage in cerebral arteries [4,6]. In the present experiments, part of the UTP may have been degraded to UDP by ectonucleotidases on the extracellular surface of cells before eliciting vasoconstriction. It is therefore probable that the UTP effects seen in previous reports were really mediated by UDP sensitive $P2Y_6$ receptors, with a lesser contribution of UTP sensitive $P2Y_2$ receptors.

$\alpha\beta$-MeATP induced a transient contraction both in the human cerebral and omental arteries indicating the presence of P2X receptors. Since the $P2X_1$ receptor is rapidly desensitised and has been shown to be the dominant subtype in cerebral vascular smooth muscle cells, these are likely to be $P2X_1$ receptors [32,33]. In the omental arteries, $\alpha\beta$-MeATP induced a transient contraction that was 2 log units more potent than that of UTPγS and ATPγS. In the cerebral circulation, the case was different. The $\alpha\beta$-MeATP induced contraction was 1 log unit less potent than that of UDPβS as well as 30 % less efficacious than the vasoconstriction mediated by $P2Y_6$ and $P2Y_2$ receptors. Thus, con-

Table 2: Specific primers for the human P2X$_1$, P2Y$_1$, P2Y$_2$, P2Y$_4$ and P2Y$_6$ receptors were constructed based on published nucleotide sequences [21–25], as follows.

Primer	Nucleotide sequence	Product length × (base pairs)
P2X$_1$ forward	5'-GTTTGGGATTCGCTTTGA-3'	
P2X$_1$ reverse	5'-GGCTGAGAGGGTAGGAGAC-3'	383
P2Y$_1$ forward	5'-ACGGGCTTCCAGTTTTAC-3'	
P2Y$_1$ reverse	5'-CCAAGGGGACACAGAACA-3'	550
P2Y$_2$ forward	5'-CTCTGCTTCCTGCCATTC-3'	
P2Y$_2$ reverse	5'-GCACAAGTCCTGGTCCTCT-3'	432
P2Y$_4$ forward	5'-CAGCACCAAAGGGACCAC-3'	
P2Y$_4$ reverse	5'-GCTTGCCACCACCACAGA-3'	530
P2Y$_6$ forward	5'-ACAGGCATCCAGCGTAAC-3'	
P2Y$_6$ reverse	5'-CGGACACAATGGCAAATA-3'	526

tractile P2Y receptors play a dominating role in the cerebral circulation, as compared to the P2X receptor effects.

The P2Y$_1$ receptor agonist, ADPβS, had no contractile effect neither in the cerebral nor in the omental arteries. Studies in animals demonstrate that P2Y$_1$ receptors only mediate endothelium-dependent dilatation in cerebral arteries [32]. Furthermore, 2-MeSADP was recently shown to induce potent dilatations in preconstricted human left internal mammary artery branches, indicating an important role for endothelial P2Y$_1$ receptors [34]. This dilatory response was abolished after the endothelium was removed, providing evidence that there were no dilatory P2Y$_1$ receptors in these human vascular smooth muscle cells [34]. Likewise, when the endothelium was removed from the rat mesenteric arteries, the ADPβS induced dilatation was abolished [27]. PCR analysis demonstrated presence of P2Y$_1$ receptor mRNA. Although, the endothelium was functionally removed this does not necessarily mean that there are no endothelial cells and mRNA from the same, which might explain the absence of effect by ADPβS and presence of P2Y$_1$ receptor mRNA. Also, presence of receptor mRNA does not necessarily mean there is functional receptor-protein on the cell surface.

Our results show that the selective P2Y$_6$ receptor agonist UDPβS induce an efficacious and potent constriction of human cerebral arteries, while no such effect can be observed in human omental or coronary arteries [26]. Conversely, mRNA for P2Y$_6$ receptors was detected in both cerebral and omental arteries. Apparently the P2Y$_6$ mRNA does not encode functional receptors in the omental arteries. Similar results were previously obtained in coronary arteries [26]. Both UTP and UDP have previously proven to induce dilatation of cerebral arteries [35], although when vasodilatation has been studied by use of the stable pyrimidines, UTPγS and UDPβS, no dilatory ef-

fect of the P2Y$_6$ receptor have be observed [26]. Taken together, it is plausible that only vasoconstriction is mediated by P2Y$_6$ receptors and that this effect is especially prominent in the cerebral circulation.

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 [36]. In cell systems, where the influence of ectonucleotidases has been minimised, UTP and UTPγS are equally potent [18], confirming that the increased efficacy and potency by the stable pyrimidines in the present experiments is not due to a structural change that alters the effect at the receptor.

Conclusions

The stable pyrimidines UTPγS and UDPβS are useful tools in the pharmacological P2 receptor characterisation in intact tissues with ectonucleotidase activity. Extracellular nucleotides stimulated contractions of human cerebral arteries primarily by activation of P2Y$_2$, P2Y$_6$ and P2X$_1$ receptors, while a role for P2Y$_1$ can be excluded. A similar pattern was seen in the omental arteries, except that no P2Y$_6$ receptor mediated contractions was seen. These results indicate that antagonists of the P2Y$_6$ receptor, but also the P2Y$_2$ and P2X$_1$ receptors may be useful in the treatment of vasospasm.

Methods

Patients

Cerebral and omental arteries were explanted during tumour surgery from 12 patients that were between 17 and 64 years of age. The cerebral arteries looked macroscopically healthy and were removed from the temporal-parietal cortex.

Vasomotor studies
Tissue preparation
The cerebral and omental arteries were immediately immersed in cold oxygenated buffer solution (for composition, see below), transported to the laboratory and dissected free from adhering tissue under a microscope. The endothelium was removed from both the cerebral and omental arteries by perfusion 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 [19]. The vessels were then cut into cylindrical segments (1 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 [20]. 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 bicarbonate based buffer solution of the following composition (mmol/L): NaCl 119, NaHCO$_3$ 15, KCl 4.6, MgCl$_2$ 1.2, NaH$_2$PO$_4$ 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 segments were allowed to stabilise at a resting tension of 4 mN (omental arteries) and 2 mN (cerebral arteries) for 1 h before the experiments were started. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mmol/L) 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.

Vasomotor responses
Endothelium removal was checked by monitoring responses to acetylcholine at the end of the experiment, in arteries preconstricted by 1 mM UTP. Abolished dilatation indicated a properly removed endothelium [19]. The range of acetylcholine-dilatation that can be observed when the endothelium is intact amounts to 30–70%, for both the human cerebral and omental arteries, depending on the quality of the endothelium after surgery (not published data). Unaffected K$^+$ induced contractions indicated intact smooth muscle cell function. As the P2X receptors were quickly desensitised, each artery segment from both the cerebral and omental arteries was exposed to a single concentration of αβ-MeATP and the resultant responses of several segments exposed to different concentrations were added up. In this way, each artery segment was exposed to αβ-MeATP only once and the problem of tachyphylaxia was avoided. These experiments are referred to as 'single-concentration'. To study the P2Y receptor stimulated contractions without interference of simultaneous activation of P2X receptors, uridine diphosphate UDP, UDPβS, UTP, UTPγS, ADP, ADPβS, ATP and ATPγS were added after P2X receptor desensitisation with 10 μmol/L αβ-MeATP, 8 min prior to each experiment. As the P2Y receptors are only very slowly desensitised, these agonists could be added cumulatively to determine concentration-response relationships.

The experimental protocol
First 60 mM K$^+$ was added twice to each vessel segment. Washout was performed for one hour both in between the K$^+$ induced responses and before the experiments were started. Thereafter, one concentration of αβ-MeATP was added to each vessel segment. When the effect of this had been monitored for 3 min, 10 μmol/L of αβ-MeATP was added to all vessel segments. This was left in the tissue bath for 8 min before a P2Y receptor agonist was added in increasing concentrations. Only one P2Y receptor agonist was tested per arterial segment. Washout was performed for one hour. Thereafter, 1 mM UTP was used to preconstrict the vessel before acetylcholine was added to examine endothelium function.

RT-PCR
RNA extraction
The arteries were carefully dissected and the endothelium was removed (see above). The arteries were snap-frozen in liquid nitrogen immediately after acquisition and total cellular RNA was extracted using TRIzol reagent (Gibco BRL) following the supplier's instructions. The resulting RNA pellet was finally washed with 70% ice-cold ethanol, air-dried and redissolved in 10 μl diethyl-pyrocarbonate (DEPC) treated water. The RNA concentration was determined spectrophotometrically considering a ratio of OD$_{260:280}$ ≥ 1.6 as pure.

RT-PCR
RT-PCR was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA, USA) on a GeneAmp PCR system 2400 (Perkin-Elmer). Specific primers for the human P2X$_1$, P2Y$_1$, P2Y$_2$, P2Y$_4$ and P2Y$_6$ receptors were constructed based on published nucleotide sequences [21–25] (see Table 2).

First-strand cDNA synthesis carried out with the Amplitaq RNA-PCR kit (Perkin Elmer) in a 20 μl volume using random hexamers. Amplification was performed using a modified profile (2 min at 95°C followed by 30 cycles of 1 min 95°C, 1 min 55–58°C, 30 sec. 72°C and a final extension step of 7 min at 72°C). The products were separated on a 2% agarose gel containing 1.0 μg/mL ethidiumbromide and photographed. The DNA Ladder 100 bp (Promega Co.) was used as molecular weight marker. As

these P2 receptors are intronless within their coding regions, PCR without the RT-step was always used to exclude genomic DNA contamination.

Ethics

The project was approved by the Ethics Committee of Lund University in Sweden.

Drugs

Vasomotor studies

Acetylcholine, ADPβS, ATP, ATPγS, UDP, UTP and αβ-MeATP were purchased from Sigma Co. (USA). UDPβS and UTPγS were kind gifts from Inspire Pharmaceuticals, Inc. All drugs were dissolved in 0.9% saline.

RT-PCR

Oligonucleotides were obtained from Gibco, BRL. If not stated otherwise, all reagents for the RT-PCR assay were purchased from Sigma Co. (USA).

Calculations and statistics

The negative logarithm of the drug concentration that elicited 50% contraction (pEC_{50}) was determined by linear regression analysis using the values immediately above and below half-maximum response. E_{max} refers to maximum contraction calculated as percent of the contractile capacity of 60 mmol/L K^+. The experiments were performed on arteries from six patients 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 means ± S.E.M. RT-PCR experiments were performed on three patients.

Authors' contributions

Malin Malmsjö: Designed the study and performed the pharmacological experiments and wrote the manuscript.

Mingyan Hou: Performed the RT-PCR experiments.

William Pendergast: Supplied the stable pyrimidines and proof-read the manuscript.

David Erlinge: Conceived the study and participated in writing the manuscript.

Lars Edvinsson: Guided throughout the study and participated in writing the manuscript.

Acknowledgements

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