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The stable pyrimidines UDPβS and UTPγS discriminate between contractile cerebrovascular P2 receptors

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Abstract

Extracellular nucleotides were used to characterise the contractile P2 receptors in the rat basilar artery. The isometric tension was recorded in vitro and receptor mRNA expression was examined by reverse transcriptase polymerase chain reaction (RT-PCR) after endothelium-denudation. Transient vasoconstriction was evoked by αβ-methylene-adenosine triphosphate (αβ-MeATP), indicating the presence of $P2X_1$ receptors. The P2Y receptors were analysed after P2X receptor desensitisation with 10 μM αβ-MeATP. Uridine diphosphate (UDP) and uridine triphosphate (UTP) induced sustained contractions of similar magnitude. The stable nucleotide analogue, uridine 5′-*O*-thiodiphosphate (UDPβS), was clearly more potent than uridine 5′-*O*-3-thiotriphosphate (UTPγS), suggesting prominent contractile effects of $P2Y_6$ receptors. $P2Y_2$ and $P2Y_4$ receptors might also be involved in nucleotide responses, since UTPγS and adenosine 5′-*O*-3-thiotriphosphate (ATPγS) were of similar potency. The $P2Y_1$ selective agonists, adenosine 5′-*O*-thiodiphosphate (ADPβS) and 2-methylthioadenosine diphosphate (2-MeSADP) did not induce contractions. RT-PCR analysis demonstrated $P2X_1$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor mRNA expression, while the $P2Y_4$ band was weak. In conclusion, extracellular nucleotides induce contractions of cerebral arteries primarily by activation of $P2Y_6$ receptors on smooth muscle cells, with a lesser contribution of $P2Y_2$ and $P2X_1$ receptors. Although mRNA for the $P2Y_1$ receptor was detected by RT-PCR, it does not mediate contraction.

Keywords: Cerebrovascular circulation; Purine; Pyrimidine; Vasoconstriction; $P2Y_6$ receptor

1. Introduction

The role of nucleotides in the control of cerebrovascular tone is an important consideration in understanding the pathophysiology of migraine, stroke and subarachnoidal haemorrhage (Bryan, 2002). Extracellular nucleotides are released from sympathetic nerves, platelets, endothelial cells and from most cells when damaged. These act through P2 receptors to modulate vascular tone and play an important role in the control of blood pressure. Uridine triphosphate (UTP) is particularly present in the brain and has potent vasoconstrictor actions in the cerebral circulation (Keppler et al., 1970; Ralevic and Burnstock, 1998; Shirasawa et al., 1983; Urquilla, 1978). After subarachnoidal haemorrhage, extracellular nucleotides are released from blood clots and have therefore been suggested to be involved in the pathogenesis of cerebral vasospasm (Sima et al., 1996, 1997; Zhang et al., 1995).

In the brain, dual vasoconstrictor and vasodilator actions of the various nucleotides are integrated to regulate vasomotor tone. P2Y receptors on endothelial cells mediate vasodilatation, while P2Y and P2X receptors on vascular smooth muscle cells mediated vasoconstriction (Miyagi et al., 1996). Recent receptor cloning has proven the existence of several different P2X and P2Y receptor subtypes, and there is evidence that at least five of these elicit vascular responses when stimulated by extracellular nucleotides, namely, $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ (Evans et al., 1998; Harden et al., 1998). Expression of these receptors in cells has enabled the characterisation of their respective pharmacological profile. $P2X_1$ receptors are activated by αβ-methylene-adenosine triphosphate (αβ-MeATP)>adenosine triphosphate (ATP) with no effect of

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uridine diphosphate (UDP) or UTP (Evans and Kennedy, 1994; Valera et al., 1994; Vulchanova et al., 1996). At the $P2Y_1$ receptor adenosine 5′-*O*-thiodiphosphate (ADPβS), 2-methylthioadenosine diphosphate (2-MeSADP) and adenosine-diphosphate (ADP) are more potent than ATP, while UDP and UTP are inactive (Léon et al., 1997; 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).

The identification of P2 receptors that are 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 (Gordon, 1986). 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), 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 $P2Y_6$ receptor (Hou et al., 2002; Lazarowski et al., 1996). It is therefore now possible to discriminate between the vascular effects of the different pyrimidine-activated P2Y receptor subtypes.

Potent and sustained effects of UTP and UDP on cerebral blood flow (Lewis et al., 2000; Urquilla, 1978) suggest the involvement of pyrimidine sensitive receptors. $P2Y_2$, $P2Y_4$, $P2Y_6$ or a combination of these receptor subtypes may have been responsible for the vasoconstrictor effects. Since these studies were performed in whole tissues, it is likely that some of the UTP and UDP were broken down to UDP and uridine monophosphate (UMP), respectively. In myograph experiments of the rat mesenteric artery in vitro, the stable pyrimidine, UDPβS, was four log units more potent than UDP, while UTPγS was two log units more potent than UTP (Malmsjö et al., 2000a). These results emphasise the importance of using stable nucleotides when studying P2 receptors in intact tissues.

Since only UTP and UDP have previously been used in an attempt to characterise the pyrimidine-sensitive P2Y receptors involved in cerebral vasoconstriction, the receptor subtypes involved remain unclear to a large extent. The present study was designed to evaluate the relative contribution of different P2 receptor subtypes that induce the constriction of rat basilar artery by the use of extracellular nucleotides, including the stable pyrimidines UDPβS and UTPγS.

2. Methods

2.1. Vasomotor responses

Female Sprague–Dawley rats weighing 200 g were anaesthetised by inhalation of CO_2, after which they were killed by a cardiac cut. The basilar artery was removed gently and immersed in cold oxygenated buffer solution (for the composition, see below) and dissected free of adhering tissue under a microscope. The endothelium was removed mechanically using a fine needle. The vessels were then cut into cylindrical segments (1 mm long), and 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 (Högestatt 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 artery segments were immersed in temperature-controlled (37 °C) tissue baths containing 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 segments were allowed to stabilise 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 potassium-rich (60 mM) buffer solution in which NaCl was exchanged for an equimolar concentration of KCl (for the composition, see above). When two reproducible contractions had been achieved the vessels were used for further studies.

Endothelium removal was checked by monitoring the responses to acetylcholine. The dilatory responses were abolished, indicating a properly removed endothelium. Acetylcholine has previously been shown to induce endothelium-dependent dilatation of the rat basilar artery (Schilling et al., 1990). Unaffected K^+-induced contractions after endothelium-denudation indicated intact smooth muscle cell function. As the P2X receptors were quickly desensitised, each artery segment was exposed to a single concentration of αβ-MeATP and the resultant responses of several segments exposed to different concentrations were grouped together. 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, ATPγS and 2-MeSATP were added after P2X receptor desensitisation with 10 μM αβ-MeATP, 8 min prior to each experiment (Kasakov and

Burnstock, 1982). As the P2Y receptors are only very slowly desensitised, these agonists could be added cumulatively to determine the concentration–response relationships.

2.2. RT-PCR

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 the 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 diethylpyrocarbonate (DEPC)-treated water. The RNA concentration was determined spectrophotometrically considering a ratio of $OD_{260:280} \geq 1.6$ as pure.

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 rat $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were constructed based on published nucleotide sequences (Chang et al., 1995; Chen et al., 1996; Tokuyama et al., 1995; Valera et al., 1994; Webb et al., 1998).

	Product length (bp)
$P2X_1$ forward, (5′-AGAGGCACTACTACAAGCAGAA-3′)	
$P2X_1$ reverse, (5′-GGTAAGGCTGTGGGAAAGA-3′)	434
$P2Y_1$ forward, (5′-CATCTCCCCCATTCTCTT-3′)	
$P2Y_1$ reverse, (5′-GTTGCTTCTTCTTGACCTGT-3′)	663
$P2Y_2$ forward, (5′-ACCCGCACCCTCTATTACT-3′)	
$P2Y_2$ reverse, (5′-CTTAGATACGATTCCCCAACT-3′)	538
$P2Y_4$ forward, (5′-TGGGTGTTTGGTTGGTAGTA-3′)	
$P2Y_4$ reverse, (5′-GTCCCCCGTGAAGAGATAG-3′)	464
$P2Y_6$ forward, (5′-GTTATGGAGCGGGACAATGG-3′)	
$P2Y_6$ reverse, (5′-AGGATGCTGCCGTGTAGGTT-3′)	347

First-strand cDNA synthesis was 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 at 95 °C, 1 min at 55–58 °C, 30 s at 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 ethidium-bromide and photographed. DNA Ladder (100 bp; Promega) 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.

2.3. Ethics

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

2.4. Drugs

Acetylcholine, ADPβS, ATP, ATPγS, UDP, UTP, αβ-MeATP and 2-MeSADP were purchased from Sigma (USA). UDPβS and UTPγS were kind gifts from Inspire Pharmaceuticals. All drugs for in vitro pharmacology were dissolved in 0.9% saline. The oligonucleotides were obtained from Gibco BRL. If not stated otherwise, all reagents for the RT-PCR assay were purchased from Sigma.

2.5. 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 mM K^+. The experiments were repeated six times 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 repeated three times.

3. Results

3.1. Vasomotor responses

The contractile response to 60 mM K^+ amounted to 1.1±0.2 mN.

3.1.1. Endothelium removal

After endothelium-denudation, the vasodilatory response to acetylcholine was abolished, indicating a properly removed endothelium. Vascular smooth muscle cell function was considered intact, since the contractile response to 60 mM K^+ was unaffected.

3.1.2. P2X receptors

Transient vasoconstrictions were evoked by the P2X receptor agonist, αβ-MeATP. When added in single-concentrations a concentration–response curve could be constructed (see Methods). Eight minutes after a single-concentration of αβ-MeATP, the vessel tension was back to baseline and if αβ-MeATP was added a second time, no contraction could be observed, indicating desensitised P2X receptors.

3.1.3. P2Y receptors

The following P2Y receptor-mediated vasoconstrictions were examined after P2X receptor desensitisation with 10 μM αβ-MeATP (see Methods). The endogenous pyrimidines, UDP and UTP, elicited sustained contractions of similar magnitude (Fig. 1A). Conversely, the stable $P2Y_6$

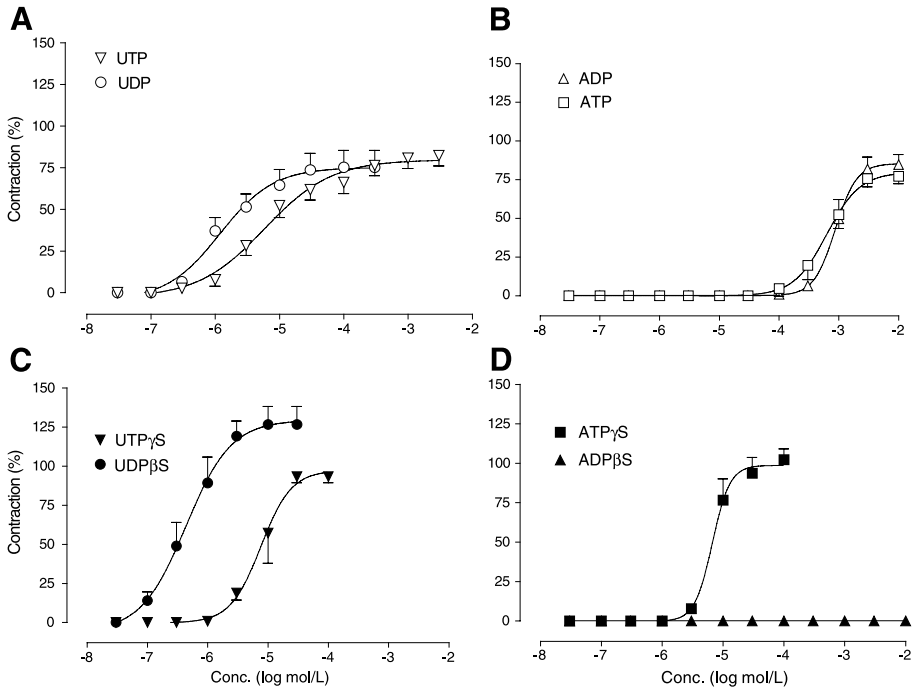

Fig. 1. Concentration-dependent contractions to the endogenous nucleotides UTP and UDP (A), ADP and ATP (B), and the stable nucleotides UTPγS and UDPβS (C), ATPγS and ADPβS (D) in the rat basilar artery. All nucleotides were added after P2X receptor desensitisation with 10 μM αβ-MeATP. Contractions are expressed as percentage of the response to 60 mM K^+. Data are shown as mean values±S.E.M. of six experiments.

receptor agonist, UDPβS, was significantly more potent than both UDP and UTPγS (Fig. 1C). A similar discrepancy in response between the endogenous and the stable nucleotides could also be observed for the purines. ATP and ADP induced comparable contractions only at micromolar concentrations (Fig. 1B). ATPγS, on the other hand, was 1.8 log units more potent than ATP, and induced a contraction that resembled those of UTPγS (Fig. 1D). The $P2Y_1$ receptor agonists ADPβS and 2-MeSADP had no effect. Thus, the potency order was UDPβS>αβ-MeATP>UTPγS=ATPγS>ADPβS=2-MeSADP=0, indicating the presence of contractile $P2Y_6$, $P2X_1$ and $P2Y_2$/$P2Y_4$ but not $P2Y_1$ receptors in the rat basilar artery (Table 1, Fig. 2).

Table 1
Contractile responses in the rat basilar artery

	pEC_{50} (−log M)	E_{max} (%)
αβ-MeATP	6.2±0.1	70±3
UDP	5.9±0.1	76±10
UDPβS	6.4±0.2	127±12
UTP	5.2±0.1	82±6
UTPγS	5.2±0.2	105±35
ATP	3.3±0.1	77±5
ATPγS	5.1±0.1	102±7
ADP	3.1±0.1	85±6
ADPβS	0±0	–
2-MeSADP	0±0	–

All nucleotides, except αβ-MeATP, were added after the desensitisation of P2X receptors with 10 μM αβ-MeATP. Contractions are expressed as percentage of an initial contraction induced by 60 mM K^+. Data are shown as E_{max}±S.E.M and pEC_{50}±S.E.M. of six experiments.

3.2. RT-PCR

Agarose gel electrophoresis of PCR products from rat basilar arteries demonstrated products of the expected size for the corresponding mRNA encoding rat $P2X_1$ (434 bp), $P2Y_1$ (663 bp), $P2Y_2$ (538 bp), $P2Y_4$ (464 bp) and $P2Y_6$ receptors (347 bp) (Fig. 3). The band for the $P2Y_4$ receptor

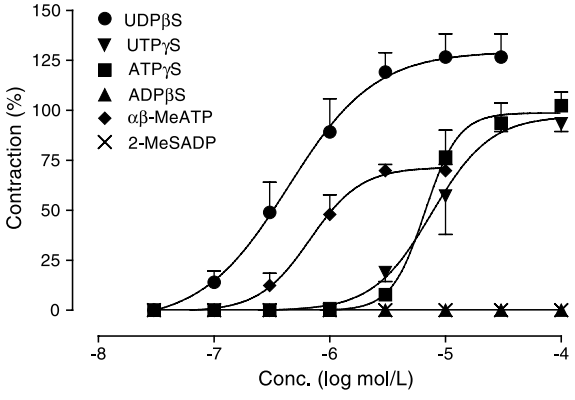

Fig. 2. Concentration-dependent contractions to αβ-MeATP, UDPβS UTPγS, ADPβS and ATPγS in the rat basilar artery. All nucleotides (except αβ-MeATP) were added after P2X receptor desensitisation with 10 μM αβ-MeATP. Contractions are expressed as percentage of the response to 60 mM K^+. Data are shown as mean values±S.E.M. of six experiments.

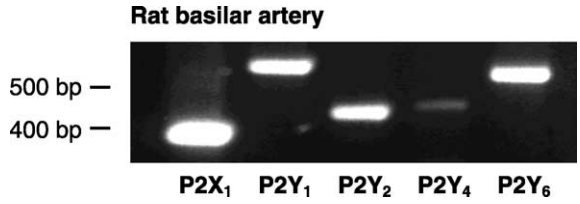

Fig. 3. Electrophoresis of RT-PCR products corresponding to mRNA encoding rat $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in the rat basilar artery. The amplified products were of the predicted size for the rat $P2X_1$ (434 bp), $P2Y_1$ (663 bp), $P2Y_2$ (538 bp), $P2Y_4$ (464 bp) and $P2Y_6$ receptors (347 bp). Samples were analysed on a 2% agarose/ethidium bromide gel at 5 V/cm and photographed.

mRNA was weak. No bands were detected in controls without a RT-step.

4. Discussion

Pyrimidines induce sustained vasoconstriction of cerebral arteries (Shirasawa et al., 1983; Urquilla, 1978). Due to ligand instability and lack of selective antagonists, the P2Y receptor subtypes that mediate these effects have not been thoroughly characterised before. The stable pyrimidines UTPγS and UDPβS have been used here to identify the P2 receptor subtypes that mediate vasoconstriction in the rat basilar artery. Novel findings show that vasoconstriction is mediated primarily by $P2Y_6$ receptors in the rat basilar artery, with a lesser contribution of $P2Y_2$ and $P2X_1$ receptors, while a role for $P2Y_4$ can be questioned. $P2Y_1$ does not mediate vasoconstriction.

The 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 characterization of P2Y receptors has mainly been performed by monitoring agonist responses (Ralevic and Burnstock, 1998). 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 and UDPβS, as unique pharmacological tools that facilitate the characterisation of P2Y receptor subtypes in intact tissues (Malmsjö et al., 2000a,b). The present results clearly demonstrate the importance of stable nucleotides in the characterisation of receptors in tissue preparations with ectonucleotidase activity: UDPβS was 0.5 log units more potent than UDP. Likewise, ATPγS was 1.8 log units more potent than ATP. These endogenous nucleotides were most certainly degraded by ectonucleotidases to UMP, ADP and AMP that apparently lacked a contractile effect. Conversely, the contractile effect of UTP and UTPγS was of similar potency and efficacy. Although it is probable that UTP was degraded, its effects were not attenuated, which might be explained by prominent contractile effects by its degradation product, UDP, mediated by $P2Y_6$ receptors. ADP induced contractions with low potency, but this could not be reproduced by ADPβS, indicating that ADP was enzymatically converted to ATP. Thus, the endogenous nucleotides may both underestimate and overestimate the contribution of different P2Y receptor subtypes, and previous studies using nonstable nucleotides should be interpreted with caution.

Pyrimidines have been postulated to contribute to the genesis of vasospasm after subarachnoid haemorrhage since these are released from blood clots and may produce sustained constriction of cerebral blood vessels (Shirahase et al., 1988; Shirasawa et al., 1983; Sima et al., 1996; Urquilla, 1978). In the present experiments, UTP and UDP were equipotent at inducing vasoconstriction, which has previously been shown by other works (Shirasawa et al., 1983; Sima et al., 1997). Although the stable pyrimidines show a markedly different pharmacological profile, which enabled the characterisation of the P2Y receptor subtypes involved. UDPβS was 1.5 log units more potent than UTPγS, indicating prominent effects by the $P2Y_6$ receptor. Potent and efficacious vasocontractile effects of $P2Y_6$ receptors have previously been demonstrated in the rat mesenteric artery by the use of UDPβS (Malmsjö et al., 2000a). Furthermore, $P2Y_6$ receptors do not seem to mediate vasodilatation (Malmsjö et al., 2000a). Even when UDP was applied luminally to evaluate the endothelial receptor effects without interactions from the contractile receptors located on the smooth muscle cells, no dilatation could be observed (Malmsjö et al., 2002). $P2Y_6$ thus seems to be an effective vasocontractile receptor.

The similarity in potency between the ATPγS- (after P2X receptor desensitisation) and UTPγS-induced vasoconstrictions suggests the effects mediated by $P2Y_2$ receptors, although less prominent than those for the $P2Y_6$ receptor. An effect by the $P2Y_4$ receptor cannot be excluded since it is also activated by UTPγS. However, RT-PCR analysis only demonstrated weak bands corresponding to $P2Y_4$ receptor mRNA expression. These results indicate that the $P2Y_4$ receptor is of minor importance in mediating vasoconstriction in cerebral arteries. 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 (Lewis et al., 2000).

Transient vasoconstrictions were evoked by αβ-MeATP, 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 (Bo et al., 1998; Lewis et al., 2000). $P2X_1$ receptors have previously been believed to account for the main part of the nucleotide-induced vasoconstriction in cerebral arteries (Lewis et al., 2000). Experiments with stable pyrimidines showed otherwise. The αβ-MeATP-induced contraction was 0.2 log units less potent than that of UDPβS as well as 50% less efficacious than the vasoconstriction mediated by $P2Y_6$ and $P2Y_2$ receptors. Thus, contractile P2Y receptors play a dominant role in cerebral circulation, as compared to the P2X receptor effects.

The specific $P2Y_1$ receptor agonist ADPβS did not induce vasoconstriction. This is in agreement with the previous suggestions that $P2Y_1$ receptors only mediate endothelium-dependent dilatation of cerebral blood vessels (Lewis et al., 2000; You et al., 1999).

There are two conditions were a prominent $P2Y_6$-receptor-mediated contractions in cerebral arteries could be especially important. The vasospasm seen after subarachnoid haemorrhage can be mediated at least in part by the release of nucleotides from aggregating platelets and erythrocytes. Specific antagonists of the $P2Y_6$ receptor, but also antagonists of the $P2Y_2$ and the $P2X_1$ receptors, could be useful to prevent or diminish vasospasm. The other possible therapeutic implication of the findings is the use of a selective and stable $P2Y_6$ receptor agonist in the treatment of migraine.

In conclusion, pyrimidines have been postulated to contribute in the genesis of vasospasm after subarachnoidal haemorrhage (Shirasawa et al., 1983; Urquilla, 1978), although the receptors involved have not been identified due to ligand instability and lack of specific antagonists. UDP and UTP induced vasoconstriction of similar magnitude. The P2Y receptor subtypes could first be characterised after having used UDPβS and UTPγS, which provide stability to ectonucleotidase activity and therefore possess a markedly different pharmacological profile. It was concluded that the contractile effects of extracellular nucleotides are primarily mediated by $P2Y_6$ receptors, with a lesser contribution of $P2Y_2$ and $P2X_1$ receptors, while a role for $P2Y_4$ can be questioned. $P2Y_1$ does not mediate contraction.

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