ADP Receptor P2Y\textsubscript{12} Is Expressed in Vascular Smooth Muscle Cells and Stimulates Contraction in Human Blood Vessels

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Objective—ADP plays an important role in platelet aggregation by activating P2Y\textsubscript{12} receptors. We assessed the hypothesis that P2Y\textsubscript{12} receptors are expressed in vascular smooth muscle cells (VSMC).

Methods and Results—P2Y\textsubscript{12} receptor mRNA was found to have a high expression among the P2 receptors in human VSMC, significantly higher than the other 2 ADP receptors (P2Y\textsubscript{1} and P2Y\textsubscript{13}, real-time polymerase chain reaction). Western blots gave a band of 50 kD, similar to that in platelets. To unmask a P2Y\textsubscript{12} receptor-mediated vasoconstriction by simulating the in vivo situation, vessels were precontracted to a submaximal level. 2-MeSADP stimulated contractions in vessel segments from internal mammary artery (IM), IM branches and small veins (E\textsubscript{max} = 15 ± 6% of 60mmol/L K\textsuperscript{+} contraction, pEC\textsubscript{50} = 5.6 ± 0.6, E\textsubscript{max} = 21 ± 1%, pEC\textsubscript{50} = 6.8 ± 0.1, and E\textsubscript{max} = 48 ± 9%, pEC\textsubscript{50} = 6.6 ± 0.4). The selective P2Y\textsubscript{12} antagonist AR-C67085 blocked 2-MeSADP contractions. The contraction was not reduced in patients using clopidogrel, a drug inhibiting ADP-induced platelet aggregation by blocking the P2Y\textsubscript{12} receptor. This may be explained by the high instability of the active clopidogrel metabolite that never reaches the systemic circulation.

Conclusion—ADP acting on P2Y\textsubscript{12} receptors not only is important for platelet activation but also stimulates vasospasm. Stable drugs with antagonistic effects on P2Y\textsubscript{12} receptors, affecting both platelets and VSMC, could be of double therapeutic benefit in their prevention of both thrombosis and vasospasm. (Arterioscler Thromb Vasc Biol. 2004;24:1810-1815.)

Key Words: vasoconstriction ■ P2Y receptors ■ platelets ■ ADP

Extracellular nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate, and uridine diphosphate are released from sympathetic nerves, platelets, and endothelial and inflammatory cells.1 Nucleotides induce vasoconstriction-stimulating P2 receptors on vascular smooth muscle cells (VSMC), regulating vascular tone and blood pressure.1,2 Furthermore, extracellular nucleotides have been shown to mediate growth stimulation and migration in VSMC.3,4

P2 receptors can be divided into 2 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 P2Y family is composed of 8 cloned and functionally defined subtypes.5–9

ADP is stored in high concentrations in platelet granula and is released as a positive feedback mechanism in response to most platelet activators. After release, ADP augments platelet aggregation by stimulation of P2Y\textsubscript{12} and P2Y\textsubscript{1} receptors.10 The first clinical application of P2 receptor antagonists has been the use of thienopyridines as platelet aggregation inhibitors. Clopidogrel and ticlopidine are prodrugs that are converted in the liver into irreversible antagonists against P2Y\textsubscript{12} receptors. Clopidogrel is more efficient than aspirin in reducing the composite end point myocardial infarction, stroke, and death in patients with vascular disease.11 Furthermore, the CURE12 and CREDO13 studies have established clopidogrel in combination with aspirin as a valuable treatment for acute coronary syndromes.

Thus, P2Y\textsubscript{12} receptors on platelets are of physiological and clinical importance. Northern blot experiments have suggested that the P2Y\textsubscript{12} receptor has a very restricted expression, being present only in platelets and brain; expression in glioma cells and in rat brain capillary endothelial cells has been verified.6,8,14–16 We have recently established real-time polymerase chain reaction (PCR) quantification methods for most of the P2 receptors and measured their expression in human VSMC and endothelial cells.17 Using these methods we have performed the first quantification of mRNA in platelets and found that the P2Y\textsubscript{12} receptor had the highest expression among the P2 receptors.18

In the present study, we measured the expression of P2Y\textsubscript{12} mRNA in human VSMC. To our surprise, it had a relatively high expression compared with the P2Y receptors, and was in
the same range as the P2Y3 receptor. The P2Y2 receptor mediates uridine triphosphate-stimulated and ATP-stimulated contractile effects in VSMC, which have been confirmed in numerous studies. However, P2Y12-mediated contractile effects have not been reported. We hypothesized that by altering the experimental conditions, we would be able to unmask a P2Y12 receptor-mediated vasoconstriction. The P2Y12 receptor is negatively coupled to adenyl cyclase through G, and inhibits intracellular cAMP levels. These inhibitory effects might go unnoticed if the VSMC in the blood vessel are completely relaxed. Therefore, we precontracted the vessel to a submaximal level using 2 different procedures: using a submaximal concentration of norepinephrine (NE) or with a higher-concentration NE, and afterwards it was relaxed by elevating intracellular cAMP with adenosine or calcitonin gene-related peptide (CGRP). Thereby, the tension in the vessel was balanced. Under both these conditions, P2Y12 receptor stimulation resulted in prominent contractions.

Methods

Tissue

Internal mammary artery (IM) segments, IM branches, and distal internal mammary veins were obtained from 16 patients undergoing coronary bypass surgery (mean age, 71 years; 13 of whom were males and 6 had noninsulin-dependent diabetes mellitus). The blood vessels were kept in cold buffer solution and transported to the laboratory, where they were dissected free of adhering tissue under a microscope. The IM had a relaxed inner diameter of ~1 mm whereas the inner diameters of the IM branches and the veins were ~0.5 mm. The endothelial layer of the IM was removed by gently rubbing the intimal surface with a wooden stick before mounting it in the myograph. The endothelium of the IM branches and veins was removed by perfusion with TritonX (0.1%, 10 seconds). This was confirmed by demonstrating a lack of dilatory effects of acetylcholine. For RNA and protein extraction, the IM were put in a −70°C freezer.

RNA and Protein Extraction

Total cellular RNA and proteins were extracted using TRIzol reagent (Gibco BRL, Life Technology) as described elsewhere.17 RNA samples were stored at −70°C and protein samples were stored at −20°C.

Quantitative Analysis of P2 Receptors by Real-Time Reverse-Transcription PCR

The TaqMan Reverse-Transcription Reagents Kit was used to transcribe mRNA into cDNA. Real-time PCR were performed as previously described.17,18 Primer and probe sequences have previously been reported.17,18 Each primer pair was selected so that the amplicon spanned an exon junction if present to avoid amplification of genomic DNA. Constitutively expressed GAPDH was selected as the endogenous control to correct for potential variation in RNA loading or efficiency of the amplification reaction. To illustrate the expression of the P2 receptors relative to each other, the P2Y2 receptor was arbitrarily chosen as a calibrator for the others, i.e., the other receptors were expressed as a ratio of P2Y2. The target genes were normalized to GAPDH and comparatively analyzed as previously described.17

Western Blot

SDS-PAGE and Western blotting were performed as described elsewhere.17 Protein electrophoresis was performed on 10% Tris-HCl polyacrylamide ready gels (Bio-Rad Laboratories) and electroblotted onto Hybond-C extra nitrocellulose membranes (0.45 μm; Amersham Pharmacia Biotech). Protein loading of 10 μg for each well was diluted with 4× SDS-reducing sample buffer. The membranes were incubated with rabbit antihuman P2Y12 (diluted 1:200) (Alomone Labs, Israel); thereafter, they were incubated with a secondary antibody (antirabbit Ig, horseradish peroxidase-linked, diluted 1:1500, Amersham Life Science). As control, the membranes were incubated with mouse antihuman GAPDH (diluted 1:5000) (Chemicon); thereafter, they were incubated with a secondary antibody (antimouse Ig, peroxidase labeled, diluted 1:10 000, Amersham Life Science). The proteins were visualized by chemiluminescence using the ECLTM Western blotting RPN 2108 system (Amersham Pharmacia Biotech), and the signals were detected by autoradiography with HyperfilmTM ECLTM (Amersham Pharmacia Biotech).

Tissue Bath

The pharmacological responses were analyzed using tissue baths. The experimental setting has previously been described.19,20 Contraction was examined in vascular segments precontracted by 1 μmol/L NE and relaxed with adenosine (1 mmol/L) or CGRP (0.1 μmol/L). Agonists were added in single doses to determine concentration-response relationships. Contraction was also examined in vascular segments precontracted by a submaximal concentration of NE (0.05 to 0.1 μmol/L).

To stimulate P2X receptors, α,β-methylene adenosine 5′-triphosphate (α,β-MeATP) was used; 2-methylthio adenosine 5′-diphosphate (2-MeSADP) was used for P2Y1, P2Y2 and P2Y13. The natural ligand ADP was also tested. To study the response to 2-MeSADP without interference of simultaneous P2X receptor-induced responses, the extracellular nucleotides were added after desensitization of the P2X receptors with 10 μmol/L αβ-MeATP for 10 minutes.21 Contraction was studied in the presence and in the absence of the P2Y1 antagonist 2′-deoxy-β-N6-methyladenosine-3′,5′-bisphosphate (MRS 2179) and the P2Y12 antagonist AR-C67085. The contractile response to 2-MeSADP was examined in patients medicated with clopidogrel and compared with patients who had not received this drug. Antagonists were administered 15 minutes before the application of agonists.

cAMP Assay

IM were dissected and demuded according to methods described in the Tissue section of this article. After 1 to 2 hours of resting at 37°C, vessel segments of IM were stimulated with 2-MeSADP (10−6 M) for 10 minutes. The concentration of cAMP was determined in duplicate using the cAMP Enzyme Biotrak (EIA) System (Amersham Biosciences).

Drugs

αβ-MeATP, 2-MeSADP, NE, and adenosine were purchased from Sigma. MRS 2179 and CGRP were purchased from Tocris. AR-C67085 was a gift from Astra-Zeneca. All the drugs were dissolved in 0.9% saline. PCR consumables were purchased from Life Technologies or Perkin-Elmer Applied Biosystems.

Ethics

The Ethics Committee of Lund University approved the project. All the patients had submitted written consent to participation in the study.

Calculation and Statistics

Calculations and statistics were performed using the GraphPad Prism 3.02 software. EMax refers to maximum contraction calculated as the percentage of the contraction with potassium (60 mmol/L); n denotes the number of patients. Statistical significance was accepted when P<0.05, using Student t test analyzing the 2-MeSADP data. Analysis of the blocking of the contraction was performed by means of the 1-way ANOVA test followed by the Dunnett multiple comparisons test. Values are presented as mean±SEM. Statistical analysis of gene expression was performed with a 1-way ANOVA, followed by
Results

Real-time PCR of IM demonstrated relatively high levels of P2Y<sub>12</sub>, the second highest mRNA expression among the P2Y receptors, slightly lower than P2Y<sub>1</sub> (Figure 1). Among the 3 ADP receptors, P2Y<sub>12</sub> was significantly higher than P2Y<sub>1</sub> and P2Y<sub>13</sub> (P<0.05; n=5 to 6).

To examine the protein expression of the P2Y<sub>12</sub> receptor, Western blotting was performed with specific antibodies for the P2Y<sub>12</sub> receptors. A 50 kDa band was detected, similar to that found in platelets. (Figure 2)

The contractile response to 2-MeSADP was investigated in IM (diameter, 2 to 3.5 mm), IM branches (1 mm), and veins (0.5 to 1 mm). The vessels were pretreated with NE followed by adenosine. 2-MeSADP induced contractile responses. 2-MeSADP was administered in single doses. The small veins were the most reactive vessels. The contractile responses for IM were E<sub>max</sub>=15±6% of 60 mmol/L K<sup>+</sup> contraction, pEC<sub>50</sub>=5.6±0.6; for IM branches were: E<sub>max</sub>=21±1%, pEC<sub>50</sub>=6.8±0.1; and for small veins were: E<sub>max</sub>=48±9%, pEC<sub>50</sub>=6.6±0.4. Furthermore, contractions induced by the endogenous ligand ADP (1 mmol/L) were observed (36±19%, n=3). The contractions obtained are shown in Figure 3. Contraction to 2-MeSADP was also obtained using a submaximal concentration of NE as pretreatment. There was no significant difference in contraction to 2-MeSADP between these 2 pretreatment methods, NE contraction followed by adenosine-induced dilatation or just a submaximal NE contraction (vein 32±10% versus 22±11%, NS; and IM 12±4 versus 10±6, NS; n=5 to 6) (Figure 4a). An example of the 2 different preconditioning steps and the contractions are shown in Figure 4b. The contractile response to 2-MeSADP using NE to contract the vessel followed by dilatation was independent of the cAMP elevating substance, CGRP, or adenosine (18±4% versus 20±8%, n=6).

The contractile response to 1 μmol/L 2-MeSADP was not affected by the P2Y<sub>1</sub> blocker, MRS 2179 (25±9 versus 21±7%, NS), but it was totally blocked by the P2Y<sub>12</sub> antagonist AR-C67085 (10 μmol/L). At high concentrations, >1 μmol/L, AR-C67085 have been reported to have antagonistic effects at the P2Y<sub>1</sub> receptor, as well. Therefore, lower concentrations were also tested, and even at 0.1 μmol/L, AR-C67085 caused an almost total inhibition of 2-MeSADP–mediated contraction (3±1%, n=5), indicating a P2Y<sub>12</sub> receptor-mediated effect (Figure 5a). An example of blocking the 2-MeSADP contraction using AR-C67085 is shown in Figure 5b. The NE contraction was not affected by AR-C67085 (71±12% versus 70±14% with AR-C67085, n=6; NS). The contraction to 1 μmol/L 2-MeSADP was not attenuated in patients on clopidogrel medication (25±9% versus 18±2%, n=4 to 6) (Figure 6).

Experiments were performed, measuring cAMP level in vessel segments of IM in which 2-MeSADP decreased cAMP levels. After resting for 1 hour, the cAMP level was lower than in the reference vessel (72±16% of control, n=4; NS).

Discussion

Previous Northern blotting has indicated that the only tissues expressing the ADP stimulated P2Y<sub>12</sub> receptor are platelet and brain. In this study, we show that P2Y<sub>12</sub> is also expressed in the human VSMC and reveal contractile effects mediated by the receptor.
Even if P2Y₁₂ expression has not been previously found in VSMC, agonists of P2Y₁₂ have been used extensively in vascular studies. 2-MeSADP has been used as a vasodilator by stimulating P2Y₁ receptors on endothelial cells to release nitric oxide, the endothelium-dependent hyperpolarizing factor, and prostaglandins.⁵,¹⁹ However, there are no reports of 2-MeSADP having contractile effects. Recently, contractile responses in human vessels were examined, and only in one patient did 2-MeSADP stimulate vasoconstriction.²⁰ In the previous studies, the endothelium was intact, enabling counteracting dilator effects, thus masking the contractile response in the VSMC. Furthermore, agonists acting by Gᵢ and cAMP inhibition are dependent on the basal intracellular level of cAMP. The P2Y₁₂ and receptor couple to Gᵢ and inhibit adenylyl cyclase. In vivo, the vasculature is under vasodilator and vasodilator influences. When a vessel segment is taken out of its natural milieu, it is deprived of vasoconstrictor and vasodilator influences. When a vessel is equilibrated. The vessel segments were pretreated using 2 different methods to achieve a submaximal tension in the vessel segment. In the first method, the vessel was precontracted with NE to increase inositol triphosphate and intracellular Ca²⁺, causing vasoconstriction. This was followed by adenosine, naturally occurring in vessels, that increases the intracellular cAMP level, causing vasorelaxation. After this pretreatment, the vessel was washed and the P2Y₁₂ blocker AR-C69085 was added to the vessel shown. No 2-MeSADP contraction was then obtained. The lower figure shows a control vessel of the upper vessel, showing that the contraction is reproducible.
Besides the P2Y12 receptor, there are 2 more subtypes of the P2 receptors on the VSMC that are stimulated by ADP, P2Y1, and P2Y13. These are selectively activated by the stable analogue 2-MeSADP. P2Y12 has not been shown in VSMC before. We found that the P2Y12 receptor had a high expression among the P2Y receptor subtypes in human IM VSMC. The mRNA expression of P2Y1 and P2Y13 was significantly lower than for P2Y12. Contamination can be neglected because of carefully washed tissue and the very low content of mRNA in platelets.

To investigate a possible involvement of P2Y1 receptors in the contractile effects, we used the selective P2Y1 antagonists MRs 2179 (K_i = 100 nmol/L). However, the contractile effect of 2-MeSADP was not affected, even when a high dose of the antagonist was used (10 μmol/L).

AR-C67085 was developed as an antagonist against P2Y12 receptors in nanomolar concentrations. Recent data suggest that it may also act as an antagonist at P2Y1 receptors in micromolar concentrations. AR-C67085 totally blocked the contractions to 2-MeSADP, and the inhibition was almost total, even at 100 nmol of the antagonist, indicating a P2Y12 (and not a P2Y13) receptor-mediated effect. The main part of the study was performed on IM branches because of the clinical relevance for this vessel.

An increasingly large number of patients with cardiovascular disease are medicated with clopidogrel (Plavix). This is a prodrg that is inactive in vitro. A biotransformation by the liver is necessary to change the precursor into an active metabolite, which, in turn, binds irreversibly to P2Y12 receptors on platelets, antagonizing the aggregating effects of ADP. However, in the present study, the contraction to 2-MeSADP was not affected by clopidogrel medication. The reason for this lack of effect is probably the short half-life of the active metabolite of clopidogrel. Previous studies have shown that platelet-poor plasma from clopidogrel-treated animals and humans lacks an anti-aggregating activity, indicating a considerable instability of the metabolite. The highly labile active metabolite binds irreversibly with P2Y12 receptors when platelets pass through the liver. Because the platelets are unable to produce new receptors, the antagonism remains for the rest of their life-span (~10 days). The VSMC, however, are not reached by the active metabolite; furthermore, they have the ability to produce new P2Y12 receptors. It could be argued that the antagonistic metabolite of clopidogrel is washed away in vitro. However, it is known to bind irreversibly to the receptor, making a wash-out effect less likely.

A stable P2Y12 blocker with more traditional pharmacokinetic properties with activity in the systemic circulation will block P2Y12 receptors on the smooth muscle cells in the vascular wall. This may result in blood pressure reduction, which may be of benefit for patients with ischemic heart disease. Furthermore, such a compound may give a double benefit in the ruptured plaque by prevention of platelet aggregation and vasospasm. In fact, P2Y12 receptor blockade by AR-C69931 sustains coronary artery recanalization and improves myocardial tissue perfusion. It is possible that inhibition of ADP-mediated vasoconstriction contributed to that effect. To our knowledge, at least 4 major pharmaceutical companies have stable P2Y12 blockers in their “pipeline.” It will be important to study their vascular effects when it comes to clinical trials.

The finding of contractile effects by a platelet aggregating receptor is logical, because vasoconstriction is an important part of platelet-mediated primary hemostasis. This is in accordance with the effects of thromboxane and 5-hydroxytryptamine (5-HT), which stimulate platelet aggregation and vasoconstriction. The contractile effects could have effect in situations of thrombogenic vasospasm, such as unstable angina or subarachnoidal bleeding. The IM vessel used in the present study is used in coronary artery bypass surgery in which vasospasm and possible growth stimulatory effects are of importance for their patency.

**Conclusion**

In the present study, we show that VSMC express the P2Y12 receptor, which, in turn, mediates contractile function after stimulation with ADP. At mRNA level, the P2Y12 receptor is the highest expressed ADP receptor and the second highest expressed P2 receptor in human IM VSMC. The contractions were not inhibited in patients medicated with clopidogrel. Drugs with antagonistic effects on P2Y12 receptors, affecting both platelets and VSMC, could be of double therapeutic benefit in their prevention of thrombosis and vasospasm.

**Acknowledgments**

The study has been supported by the Swedish Heart and Lung Foundation, Franke and Margareta Bergqvist Foundation, the Wiberg Foundation, the Bergwall Foundation, the Zoegas Foundation, the Tore Nilsson Foundation, and Swedish Medical Research Council grant 13130.
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Arterioscler Thromb Vasc Biol. 2004;24:1810-1815; originally published online August 12, 2004; doi: 10.1161/01.ATV.0000142376.30582.ed

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