Role of P2Y1 purinoceptor in ADP-induced platelet activation

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Abstract ADP acts as an agonist of platelet aggregation via specific receptors which are still to be characterised. Amplification by PCR of a human platelet cDNA library confirmed the presence of mRNA of the P2Y1 receptor in platelets. In order to determine if these P2Y1 receptors were involved in ADP-induced platelet activation, we determined the effects of A3P5PS, an antagonist of the P2Y1 receptor, on the binding of [^{33}P]2-MeS-ADP, a potent analogue of ADP. We found that A3P5PS displaced about 27% of [^{33}P]2-MeS-ADP binding, a receptor population which has been shown to be resistant to treatment with clopidogrel, a selective anti-ADP agent. A3P5PS specifically inhibited 2-MeS-ADP-induced shape change and calcium increase but did not affect adenylyl cyclase down-regulation. 2-MeS-ADP-induced platelet aggregation was also inhibited by A3P5PS but was restored when platelets were further activated by serotonin, a non-aggregating compound, therefore suggesting that P2Y1-mediated stimulation is an absolute prerequisite for ADP to induce platelet aggregation and a key event for platelet activation and aggregation to occur. These results therefore show that ADP-induced aggregation cannot be attributed to activation of P2Y1 alone, but must be attributed to the simultaneous activation of the high affinity receptor (P2Y1) and a low affinity receptor of ADP (still to be discovered), each of them essential, but neither able to trigger aggregation alone.

Key words: Adenosine diphosphate; P2Y1 purinoceptor; Platelet aggregation

1. Introduction

Adenosine diphosphate (ADP) is the oldest known platelet agonist but the way by which it activates platelets is still not totally understood and, even if it is admitted that it acts on platelets via specific receptors, these receptors have not been characterised and cloned. Progress in the knowledge of ADP-induced events in platelets has been made essentially because of the use of a selective ADP-blocking agent clopidogrel [1]. This new drug has been recently demonstrated to be efficacious in preventing thrombotic events in atherosclerotic patients [2]. Several studies, performed with clopidogrel in different species including rat, rabbit and human, have demonstrated that it antagonised the binding of ADP on its platelet receptors and consequently inhibited specifically ADP-induced aggregation [3].

Although the exact nature of the purinoceptor responsible for ADP-induced platelet aggregation is still unknown, several purinoreceptors have been described in many other cells. This receptor family, named P2, is at present composed of 14 different cloned receptors classified into two subfamilies: P2X (seven members) are pore-forming protein sequences and P2Y (seven members) are G-protein-coupled receptors [4]. Platelet ADP receptors (named P2T) which have not yet been cloned have been classified in the P2Y subfamily. In contrast with the other P2 receptors, diphosphate derivatives of adenosine act as agonists whereas the corresponding triphosphates behave as antagonists [5]. In a recent paper, Léon et al. demonstrated the presence of one of these P2 receptors (P2Y1) in platelets [6] and suggested that it could be the 'unknown' P2T, responsible for the ADP-induced aggregation of platelets. However, up to now, this provocative observation has not been confirmed and most importantly, it is still unknown whether this receptor is the target of clopidogrel: the "low affinity ADP receptor responsible for platelet aggregation" [7] or another purinoceptor responsible for other functions of ADP on platelets such as shape change.

Our work was therefore aimed at determining if the P2Y1 purinoceptor is the receptor responsible for ADP-induced platelet aggregation and the target of clopidogrel.

2. Materials and methods

2.1. Construction of a human platelet cDNA library

Human platelets (obtained from 72 units of outdated platelet concentrates) (Centre de Transfusion Sanguine, Toulouse, France) were purified by repeated centrifugation on Ficoll Paque (Pharmacia, Uppsala, Sweden) and washes in phosphate-buffered saline (PBS). Total RNA was extracted from frozen platelet pellets (10^{12} platelets) with guanidinium thiocyanate lysis followed by ultracentrifugation in a cesium chloride solution as described by Sambrook et al. [8]. About 5 μg of polyA$^+$ mRNA was finally extracted from the total RNA suspension using the Fast Track kit (Invitrogen, Leek, The Netherlands).

Double-strand platelet cDNAs were obtained from 5 μg of human platelet mRNA using the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Gibco-BRL, Cergy Pontoise, France). After synthesis, ligation of *Bst*XI adapters and digestion with *Not*I as described in the kit, cDNAs were size-fractionated on a 1% agarose gel using the Geneclean II kit (Bio 101, La Jolla, CA, USA). 40 ng of cDNAs migrating above 1000 bp was ligated (16 h at 16°C using 4 units T4 DNA ligase) with 200 ng of a *Bst*XI-*Not*I linearised pZeo-SV vector (Invitrogen) in which the *Hin*dIII-*Xho*I fragment of the polylinker had been replaced by the *Hin*dIII-*Xho*I fragment of the pCDNA3 vector (Invitrogen). The ligation reaction was ethanol-precipitated together with 20 μg of yeast tRNA, resuspended in 20 μl water, and electroporated in 10×40 μl of Electromax DH 10B (*E. coli* strain provided by Gibco-BRL). After each shock, 1 ml of SOC medium was immediately added, and the 10 ml resulting suspension was incubated for 1 h at 37°C with a 225 rpm shaking. Cells were then plated on 20 low salt buffer (LSB) plates containing 25 μg/ml zeocyn and left to grow overnight at 37°C. The 2×10^6 resulting clones were finally pooled in 200 ml LSB containing 20% glycerol, aliquoted and kept frozen at −80°C.

2.2. PCR of the platelet P2Y1 receptor

PCR was performed on a human platelet cDNA library as described by Bouaboula et al. [9]. Sample was amplified by 35 repeated cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 2.2 min. PCR

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reaction mixture (total volume 50 μl) contained 2 U of Taq DNA polymerase (Perkin-Elmer Cetus, Saint Quentin en Yvelines, France) with 10 pmol of the following primers: 5′AAG TCG AGG AGG AGA GAA TG-3′ and 5′-GGA AGA GAG ACT GTG AAA GC-3′. These primers spanned a 1332 bp sequence containing the P2Y1 CDS. The PCR product was analysed by agarose electrophoresis and sequenced.

2.3. ADP receptor-related studies on rabbit platelets

Platelets were obtained from female New Zealand rabbits (2.5–3.0 kg, Lago, France) treated with a single oral administration of clopidogrel (25 mg/kg, p.o.) (Sanofi Recherche, Toulouse, France). Two hours after the administration of clopidogrel or the vehicle, blood was withdrawn into a 3.8% trisodium citrate solution (1:9 volumes of blood) and centrifuged at $180\times g$ for 10 min to prepare platelet-rich plasma (PRP).

Platelet aggregation was monitored by the turbidimetric method of Born [10] on a dual-channel Chrono-Log aggregometer. PRP diluted twofold in saline was equilibrated at 37°C for 1 min under constant stirring (900 rpm) in the presence or absence of adenosine 3′-phosphate-5-phosphosulphate (A3P5PS, Sigma, L'Ile d'Abeau, France), and aggregation was induced by various agonists, including 2-methylthio-ADP (2-MeS-ADP, Bioblok, Illkirch, France), serotonin (5-HT, Sigma) and ADP (Biopool, Umeå, Sweden). Shape change was measured according to Colman et al. [11]. Briefly, PRP (200 μl) diluted in 200 μl Tyrode buffer was incubated for 2 min in an aggregometer under constant stirring (900 rpm) at 37°C. Then, 4 μl EDTA (500 mM) was added and 10 s later, shape change was induced by the tested compounds. When tested, A3P5PS was added 30 s before EDTA. Aggregation and shape change were estimated quantitatively by measuring the maximum deviation above baseline level.

For measurement of adenylyl cyclase activity, PRP was diluted in the same volume of buffer containing 15 mM Tris, 120 mM NaCl, 4 mM KCl, 1.6 mM $MgSO_4$, 2 mM NaH_2PO_4, 10 mM glucose and 1.5 mM IBMX (Sigma), pH 7.4 at 37°C. Three minutes after adenylyl cyclase activation by 10 μl PGI_2 (Sigma), 500 μl samples were incubated for 3 min with 2.5 μM ADP or 25 nM 2-MeS-ADP, in the presence or absence of 250 μM A3P5PS. The reaction was stopped by adding 50 μl 6 N HCl and 50 μl 0.2 M EDTA and boiling for 5 min. Cyclic AMP was extracted according to Meurs et al. [12] and cAMP in the samples was measured by an assay kit (Amersham, Amersham, UK).

For cytosolic free calcium measurements, platelets were washed and resuspended in buffer PSS (NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, glucose 5.6 mM, HEPES/NaOH 5 mM pH 7.4), containing BSA 0.35%, apyrase 0.1 U/ml, hirudin 1 U/ml (PSSA) and incubated at 37°C for 40 min with fura-2/AM (10 μM). The platelet suspension was washed once with PSSA containing 1 μM PGI_2 to remove extracellular fura-2. Platelets were then resuspended in PSSA at 3×10^8/ml, and kept in the dark at room temperature. Experiments were carried out under constant stirring in a PTI spectrofluorometer using 30 μl of cell suspension diluted in 3 ml PSS containing 1 mM $CaCl_2$ in a fluorescence cuvette at 37°C. Cytosolic free calcium was estimated as the fluorescence ratio $R = F_{340}/F_{380}$ (where F_{340} and F_{380} are the fluorescence intensities of fura-2 measured at 510 nm after excitation at 340 nm and 380 nm respectively) as described by Grynkiewicz et al. [13].

Binding of [^{33}P]2-MeS-ADP (NEN) to rabbit platelets was measured as previously described by us [14] in a total volume of 0.2 ml of fourfold diluted PRP in Tyrode buffer. Triplicate incubations were carried out at 4°C for 15 min in the presence of 0.1 μCi of labelled ligand and different concentrations of the tested compounds. The reaction was terminated by the addition of a 3 ml ice-cold assay buffer followed by rapid vacuum filtration over glass-fibre filters (Filtermats 11734, Skatron Instruments Inc., Sterling, USA). Filters were then washed twice with 5 ml ice-cold incubation buffer, dried and the radioactivity measured by scintillation counting.

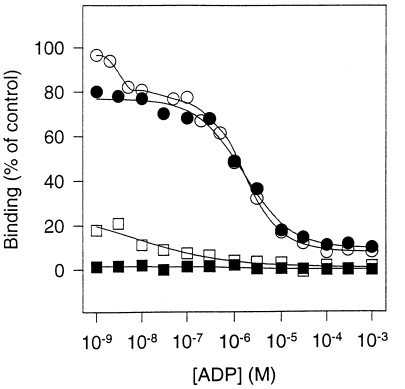

Fig. 2. Effect of A3P5PS on the displacement of [^{33}P]2-MeS-ADP to rabbit platelets by ADP. Platelets from rabbits treated with clopidogrel (25 mg/kg, 2 h) (■, □) or with the vehicle (●, ○) were incubated for 15 min at 4°C with [^{33}P]2-MeS-ADP (0.5 nM) and increasing concentrations of ADP, in the presence (closed symbols) or absence (open symbols) of 250 μM A3P5PS. Each point is the mean of triplicate measurements.

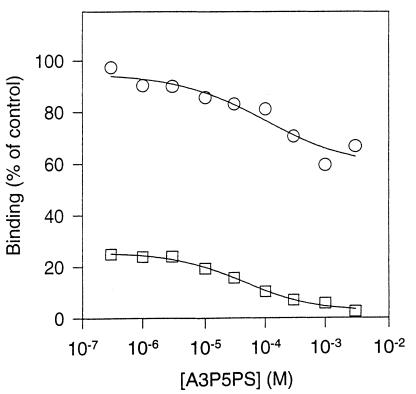

Fig. 1. Effect of A3P5PS on the binding of [^{33}P]2-MeS-ADP to rabbit platelets. Platelets from rabbits treated with clopidogrel (25 mg/kg, 2 h) (□) or with the vehicle (○) were incubated for 15 min at 4°C with [^{33}P]2-MeS-ADP (0.5 nM) in the presence of increasing concentrations of A3P5PS. Bound ligand was then separated from free ligand as described in Section 2. Binding is expressed as a percentage of the specific binding in control platelets, from triplicate measurements.

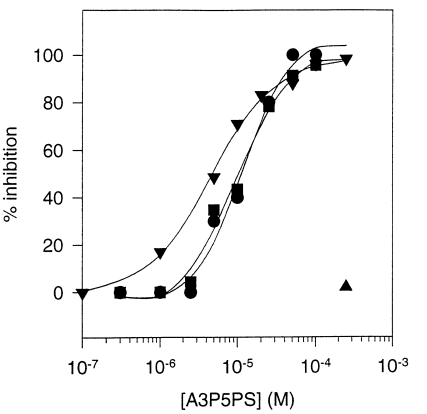

Fig. 3. Effect of A3P5PS on 2-MeS-ADP-induced activation of rabbit platelets. Shape change (■), cytosolic calcium increase (▼), adenylyl cyclase down regulation (▲) and aggregation (●) induced by 25 nM 2-MeS-ADP were measured in the presence of various concentrations of A3P5PS, as described in Section 2. Results (means of triplicate measurements) are expressed as inhibition of the effects obtained in the absence of A3P5PS.

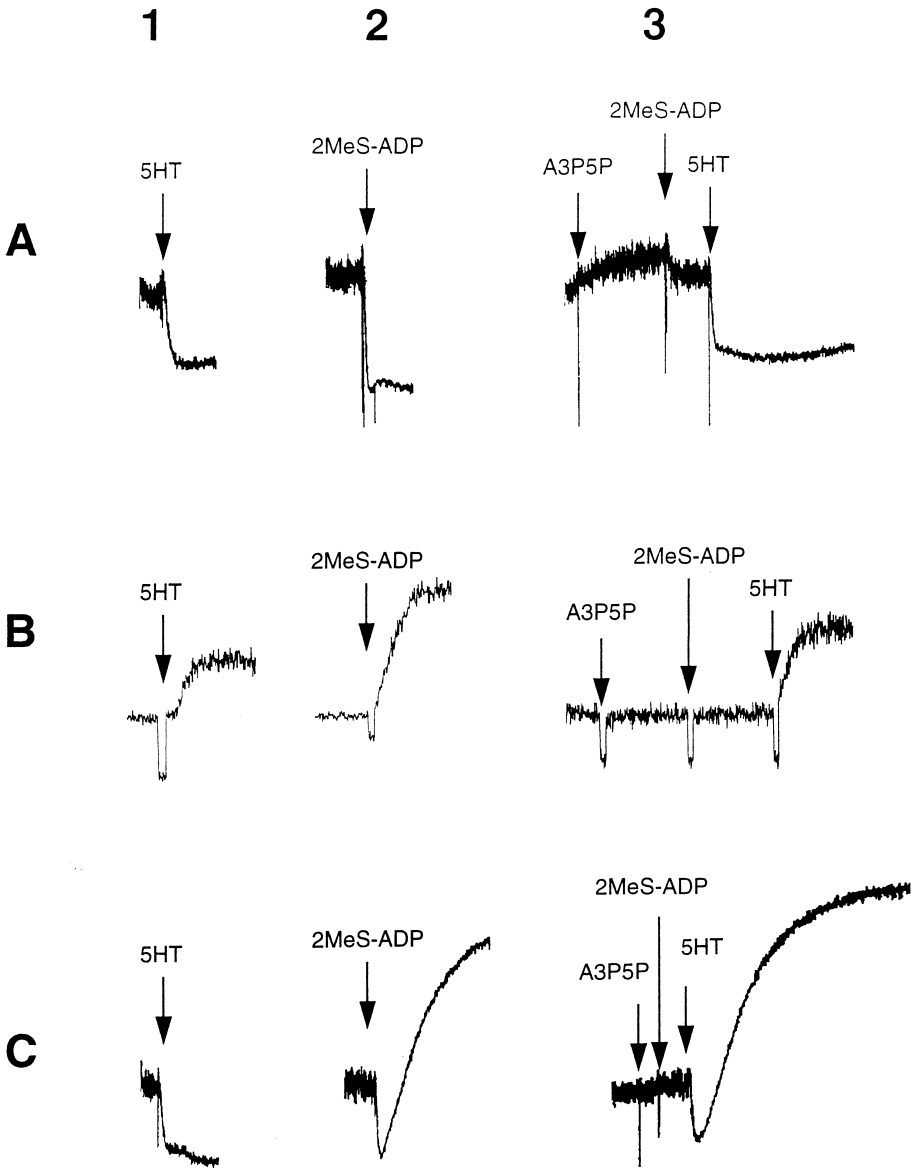

Fig. 4. Effect of 5-HT on the inhibition of 2-MeS-ADP effects by A3P5PS in rabbit platelets. Shape change (A), calcium influx (B) and aggregation (C), recorded as described in Section 2, were induced by 2.5 μM 5-HT (lane 1) or 25 nM 2-MeS-ADP (lane 2). In lane 3, A3P5PS (250 μM) was incubated before activation by 25 nM 2-MeS-ADP, then 2.5 μM 5-HT was added.

3. Results and discussion

Amplification by PCR on our human platelet cDNA library with specific primers for the P2Y1 gene produced only one band at the expected size (1332 bp). Sequencing revealed that this band was homologous to the P2Y1 cDNA, a gene already found in human tissues including prostate, ovary, intestine, placenta and HEL cells [15–17]. This result therefore confirms and extends that indeed, as shown by Léon et al. [6], mRNA encoding the P2Y1 receptor is present in platelets.

In order to determine if the P2Y1 receptor expressed in platelets was involved in ADP-induced platelet activation, we determined the effects of A3P5PS, a specific antagonist of the P2Y1 receptor [18] on the binding of [^{33}P]2-MeS-ADP. This ligand has been shown to bind to two different types of ADP receptors present on the surface of rat platelets [7]: a high affinity ADP binding site (K_i = 15 nM), responsible for shape change which represented about 30% of the total binding of ADP, and a low affinity ADP site (K_i = 486 nM) (about 70% of the total binding sites) responsible for adenylyl cyclase down-regulation and platelet aggregation. A3P5PS displaced about 27% of [^{33}P]2-MeS-ADP binding with an IC_{50} value of 69 μM (Fig. 1), indicating that the P2Y1 receptors only represent one fourth of the total [^{33}P]2-MeS-ADP binding sites on platelets. This finding was confirmed when we compared the displacement of [^{33}P]2-MeS-ADP binding by ADP in the presence or absence of 250 μM A3P5PS (Fig. 2) where two different binding sites for ADP were observed in control rabbit platelets (IC_{50} = 5 and 875 nM). Incubation with A3P5PS strongly decreased the high affinity site (82% inhibition) but did not affect the binding of [^{33}P]2-MeS-ADP to the low affinity ADP sites (Fig. 2).

Work from our group showed that clopidogrel only affects the binding of [^{33}P]2-MeS-ADP to its low affinity sites and does not affect the interaction between [^{33}P]2-MeS-ADP and its high affinity receptors [7]. These 'clopidogrel-resistant' receptors are responsible for ADP-induced shape change, an event which has been shown not to be affected by this compound [7]. This 'clopidogrel-resistant' [^{33}P]2-MeS-ADP binding was totally displaced by A3P5PS (IC_{50} = 41 μM) (Fig. 1), indicating that this class of receptors corresponds to the P2Y1 receptors present on the platelet surface.

Activation of the ADP receptors by ADP or 2-MeS-ADP is followed by several changes in platelets including shape change [19], cytosolic calcium increase [20], adenylyl cyclase down-regulation [21], protein phosphorylation [22,23], Gp IIb-IIIa activation [24] and aggregation [10]. Some of them (adenylyl cyclase down-regulation, Gp IIb-IIIa activation, aggregation and some phosphorylations) are inhibited by clopidogrel treatment indicating that they are the consequence of activation of the low affinity binding site [7,23]. ADP-induced shape change, calcium increase, MLCK and PKC activation, attributed to activation of the high affinity ADP binding sites, have been shown not to be affected by clopidogrel treatment [7,23].

The observation that P2Y1 was the clopidogrel-resistant, high affinity binding site of ADP was confirmed by the effect of A3P5PS which specifically inhibited 2-MeS-ADP-induced shape change and calcium increase (Fig. 3). On the other hand, adenylyl cyclase down-regulation, which was previously attributed by us to activation of the low affinity ADP receptors [7], was not affected by A3P5PS (Fig. 3). Surprisingly, 2-MeS-ADP-induced platelet aggregation was also inhibited by A3P5PS but the full aggregating activity of 2-MeS-ADP was restored in A3P5PS-treated platelets when platelets were further activated with 5-HT, a non-aggregant agonist (Fig. 4). Similar results were obtained with ADP instead of 2-MeS-ADP (not shown), suggesting that P2Y1-mediated stimulation is an absolute prerequisite for ADP to induce platelet aggregation.

This work therefore demonstrates for the first time that the P2Y1 purinoceptor is involved in ADP-induced shape change and calcium increase. However, the inhibition of ADP-induced aggregation observed with A3P5PS indicates that P2Y1 stimulation is a key event for platelet activation and aggregation to occur. The anti-aggregating activity of clopidogrel and its selective antagonism of the low affinity ADP receptors further demonstrate the involvement of another pathway of platelet activation, triggered by the low affinity ADP receptors. A double pathway of platelet activation was first suggested by some authors who studied the potentiation of epinephrine by 5-HT, low concentrations of ADP or protein kinase C activation [25,26]. In these experiments, the authors showed that platelet aggregation was the result of different activation pathways acting in synergy [25,26]. Our results indicate that this scenario can also be applied to the ADP activation pathway.

Among the P2Y1-related events, cytosolic calcium increase seems to be a major event for aggregation, whereas shape change has been demonstrated not to be required [27]. Several enzymatic systems, mainly protein phosphokinases, are known to be calcium-regulated and cytosolic calcium increase has been considered as a necessary but not sufficient event in the platelet aggregation process [28].

Therefore, from the results shown in this paper, it is clear that ADP-induced aggregation cannot be attributed to activation of the P2Y1 alone, but must be attributed to the simultaneous activation of the high affinity receptor (P2Y1) and the low affinity receptor of ADP (not known), both of them essential but neither able to trigger aggregation alone. This other ADP receptor, which is clopidogrel-sensitive, has still to be identified. In a recent paper, we demonstrated that P2X1, which is present on rat platelets, was not involved in the aggregation process, nor antagonised by clopidogrel treatment [29], and therefore was not the low affinity ADP receptor. Like the high affinity receptor (P2Y1), the low affinity receptor could be a seven transmembrane domain receptor, coupled to G-proteins as suggested by its ability to regulate adenylyl cyclase through a Gi protein [30].

In conclusion, one platelet ADP receptor involved in the ADP-induced aggregation (shape change) has been found, but the other one, which is the target of clopidogrel, co-responsible for the aggregating activity of ADP, has still to be discovered.

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