Molecular Basis for ADP-induced Platelet Activation

II. THE P2Y1 RECEPTOR MEDIATES ADP-INDUCED INTRACELLULAR CALCIUM MOBILIZATION AND SHAPE CHANGE IN PLATELETS

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ADP is an important platelet agonist causing shape change from smooth discoid shape to spiculated spheres and platelet aggregation. However, the molecular mechanisms involved in ADP-induced platelet activation have not been elucidated. We demonstrated earlier the existence of two distinct ADP receptors on platelets, one coupled to phospholipase C, P2TPLC, and the other to inhibition of adenyl cyclase, P2T AC (Daniel, J. L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J. B., and Kunapuli, S. P. (1998) J. Biol. Chem. 273, 2024–2029), in addition to the previously described P2X1 receptor. Here we report the cloning of a cDNA clone encoding the P2Y1 receptor from a human platelet cDNA library by homology screening with radiolabeled P2Y1-P2Y6 receptor cDNAs. ADP or 2-methyl(thio)-ADP-induced intracellular calcium increases were inhibited by the P2Y1 receptor-specific antagonists, adenosine 3’-phosphate 5’-phosphosulfate (A3P5PS), adenosine 3’-phosphate 5’-phosphate (A3P5P), and adenosine 2’-phosphate 5’-phosphate (A2P5P), in a concentration-dependent manner, but not by ARL 66096 or α,β-MeATP. A3P5PS, A3P5P, and A2P5P also inhibited the shape change of aspirinated platelets induced by 10 μM ADP or 3 μM 2-methyl(thio)-ADP in a concentration-dependent manner, with complete inhibition occurring at 300 μM. On the other hand ARL 66096 (100 nM), a potent P2T AC antagonist and α,β-methylene-ATP (40 μM), a P2X1 receptor agonist, had no effect on ADP-induced platelet shape change. On the contrary, ADP-induced inhibition of adenylyl cyclase was blocked by ARL 66096, but not by α,β-MeATP or the P2Y1 receptor-specific antagonists, A3P5PS, A3P5P, or A2P5P. These results demonstrate the role of the P2Y1 receptor in ADP-induced platelet shape change and calcium mobilization and support the idea that several P2 receptors are involved in the regulation of different aspects of platelet stimulus-response coupling.

ADP† has been known to cause platelet activation since 1961

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF018284.

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¶¶ The abbreviations used are: ADP, adenosine diphosphate; αβ-MeATP, α,β-methylene adenosine triphosphate; ATP, adenosine triphosphate; HEL, human erythroleukemia; P2T AC, platelet ADP receptor coupled to inhibition of adenylyl cyclase; P2TPLC, platelet ADP receptor coupled to phospholipase C; nt, nucleotide; PPADS, pyridoxal phosphate 6-azophenyl 2’,4’-disulfonic acid; A3P5PS, adenosine 3’-phosphate 5’-phosphosulfate; A3P5P, adenosine 3’-phosphate 5’-phosphate; A2P5P, adenosine 2’-phosphate 5’-phosphate.

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DNA insert from the positive clone was obtained in Bluescript KS vector regions and poly(A) erythroleukemia (HEL) cells with divergent 3-P2Y1 receptor, the short form and the long form, from human flanked by 456 base pairs of 3 contains an open reading frame of 840 base pairs which is termination technique and found to be identical. This clone this approach. Both clones were sequenced by the dideoxy procedures were as described previously (10–13). A3P5PS, A3P5P, and A2P5P were obtained from Sigma. ARL 66096 and SC-52012 were gifts from Astra Research Laboratories, Loughborough, United Kingdom (formerly Fisons) and Searle Research & Development, Skokie, IL, respectively.

Cloning and Sequencing—The human platelet cDNA library in lambda pII vector was screened using XL-1blue as the host strain as described earlier (12, 14), using cDNA inserts from the P2Y1 (14), P2Y2 (11), P2Y3 (12), P2Y4 (15), P2Y5 (16), and P2Y6 (17) (labeled by random priming with [α-32P]dCTP]) as probes. The membranes were finally washed with 2 × SSC containing 0.1% SDS at 50 °C (4 × 30 min). The DNA insert from the positive clone was obtained in Bluescript KS vector by in vitro excision procedures. The complete nucleotide sequence of both strands was determined by dideoxy chain termination using T3 and T7 primers. The nucleotide sequence analysis was performed using the GCG package.

Platelet Shape Change—Agonist-induced platelet shape change was determined using Chronolog Lumi-aggregometer as described earlier (10, 18) except that the effect of platelet aggregation was masked by pretreatment of aspirinated platelets with 1 μM SC-52012, a known inhibitor of platelet aggregation through blocking fibrinogen binding to its receptor (19).

RESULTS

Cloning of the P2Y1 Receptor cDNA from a Human Platelet Library—We screened a human platelet cDNA library in λZapII by low stringency hybridization with radiolabeled P2Y1-P2Y6 cDNAs. Upon screening nearly 1,000,000 recombinants, two cDNA clones encoding the P2Y1 receptor were isolated by this approach. Both clones were sequenced by the dideoxy termination technique and found to be identical. This clone contains an open reading frame of 840 base pairs which is flanked by 456 base pairs of 3'-untranslated sequence ending in a poly(A)″ tail. We have previously cloned two forms of the P2Y1 receptor, the short form and the long form, from human erythroblasts (HEL) cells with divergent 3'-untranslated regions and poly(A)″ signals (14). The platelet P2Y1 appears to have a unique 3'-untranslated region and a poly(A)″ signal. The sequence of the platelet P2Y1 receptor in relation to the short form and the long form is shown in Fig. 1. Although we also used radiolabeled P2Y2-P2Y6 cDNAs as probes, no signals hybridizing to these probes were seen.

Effect of P2Y1 Antagonists on ADP-induced Intracellular Calcium Mobilization—Since the human P2Y1 receptor is known to be coupled to the phospholipase C pathway, resulting in mobilization of calcium from intracellular stores (9, 20), we investigated the effect of A3P5PS, A3P5P, and A2P5P, known P2Y1 selective antagonists (21), on ADP-induced intracellular calcium increases. As shown in Fig. 2A, ADP increased intracellular calcium levels in platelets, an effect which was inhibited by the P2Y1 specific antagonists, A3P5PS, A3P5P, and A2P5P, in a concentration-dependent manner, with complete inhibition occurring at 30-fold molar excess. Similar inhibition of 3 μM ADP-induced mobilization of calcium from intracellular stores was observed by all the three P2Y1 antagonists (Fig. 2B). ARL 66096 (100 μM) or a,b-32P-ATP (data not shown) (10). These results strongly suggest a correlation between the ADP-induced mobilization of calcium from intracellular stores and platelet shape change.

Effect of P2Y1 Antagonists on Platelet Shape Change—We investigated whether the P2Y1 receptor in platelets is functionally coupled to physiological responses by using the recently described (21) P2Y1 receptor-specific antagonists, A3P5PS, A3P5P, and A2P5P on ADP-induced platelet shape change. To block aggregation and enhance the platelet shape change signal, we used SC52012 at 1 μM, an inhibitor of fibrinogen binding to its receptor (19). A3P5PS inhibited ADP and 2-MeSADP-induced platelet shape change in a concentration-dependent manner (Fig. 3). Similar results were obtained with A3P5PS and A2P5P (data not shown). We have previously demonstrated that the P2TAC receptor-specific antagonist, ARL 66096 (22), neither caused nor inhibited ADP-induced platelet shape change (10), suggesting that the P2TAC receptor does not play any significant role in ADP-induced platelet shape change.

Effect of P2Y1 Antagonists on ADP-induced Adenylyl Cyclase Inhibition—We have previously shown that the ARL 66096, a
Additions were indicated by changes as described under "Experimental Procedures." Various doses of A3P5PS are shown. Agonist-induced platelet shape inhibition of adenylyl cyclase, a P2T PLC receptor, coupled to mobilization of calcium from intracellular stores through activation by reverse transcriptase-polymerase chain reaction analysis of platelet RNA.

We used the low-stringency hybridization approach to isolate the cDNA clone for the P2TPLC receptor from the platelet cDNA library. This strategy resulted in a P2Y1 receptor cDNA clone, representing a P2Y1 mRNA species utilizing a unique polyadenylation signal different from the short and long forms reported previously (14). We failed to isolate any other cDNA clones encoding the P2Y2-P2Y6 receptors in our screening using this strategy. The different forms of P2Y1 purinoreceptor mRNAs result from differential usage of the polyadenylation signals. The 3'-noncoding region of the long form contains 3 consensus polyadenylation sites (AATAAA) at 1477, 1676, and 2149 nt. The short form of cDNA resulted from the use of the first consensus polyadenylation signal (1477 nt) while the platelet form utilized the second polyadenylation signal at 1676 nt. The long form mRNA probably results from the utilization of a different polyadenylation signal, since the 3'-untranslated region extends 275 nucleotides beyond the last consensus polyadenylation site (2149 nt) to end in a poly(A) tail (14). It is interesting to note that the 3'-noncoding region of the long form does not encode a consensus polyadenylation signal prior to the poly(A) tail. Moreover, the long form contains three copies of the AUUUA sequence motif, which is thought to be responsible for destabilization of mRNA (24), while the platelet form contains only one such motif. Thus the long transcript may be more unstable than the platelet form which in turn might be less stable than the short form. Several studies have shown that the 3'-untranslated regions of mRNAs are implicated in many functions, such as translational regulation (25, 26), regulation of cell growth and differentiation (27), mRNA localization during oogenesis (28), and intracellular mRNA localization (29). Although no experimental evidence is currently available, the different lengths of 3'-noncoding regions in these three P2Y1 receptor transcripts could be of functional importance.

Activation of platelets by ADP results in platelet shape change and aggregation, release of thromboxane A2, and release of granule contents (2, 3). Since P2TAC-specific antagonists, ARL 66096 and ARL 67085, inhibit platelet aggregation and have no effect on the platelet shape change (10, 22), we investigated the role of the P2Y1 receptor in ADP-induced platelet shape change. Until recently, the P2 receptors lacked specific antagonists (and specific agonists). Hence, nonspecific antagonists such as suramin, PPADS, and reactive blue 2 have been used in the past (30). Recently, however, Harden and co-workers (21) identified three adenine nucleotide analogs, A3P5PS, A3P5P, and A2P5P, as specific P2Y1 antagonists, which had no effect at the P2Y2, P2Y4, and the P2Y6 receptors (21). In this study, these P2Y1-specific antagonists were used to demonstrate the functional role of the platelet P2Y1 receptor in ADP-induced mobilization of calcium from intracellular stores and platelet shape change. Furthermore, we have evaluated the contribution of the P2TAC and the P2X1 receptors, depicted in our three receptor model for ADP-induced platelet activation (10), to platelet shape change induced by ADP.

All the three P2Y1 receptor-specific antagonists inhibited ADP-induced mobilization of calcium from intracellular stores (Fig. 2) and platelet shape change (Fig. 3) in a concentration-dependent manner. Furthermore, these antagonists do not block ADP-induced adenylyl cyclase (Fig. 4), indicating that they do not inhibit ADP-induced shape change by acting at the
receptor is the platelet P2TPLC receptor, coupled to inositol P2Y1 receptor selective antagonists demonstrate that the P2Y1 activation of ADP-induced intracellular calcium mobilization by the platelet cDNA library and the concentration-dependent inhibition of phospholipase C and formation of inositol trisphosphate (9, 20). Molecular cloning of the P2Y1 receptor from brain capillary endothelial cells (34) and in B10 rat astrocytoma cells (20). The human P2Y1 receptor has been known to couple to inhibition of adenylyl cyclase. Thus, we conclude that the P2Y1 receptor is not coupled to adenylyl cyclase. Our results demonstrate that the P2Y1 receptor is not coupled to adenylyl cyclase, as the P2Y1 receptor selective antagonists failed to abrogate ADP-induced inhibition of adenyl cyclase, but inhibited ADP-induced calcium mobilization and platelet shape change in a concentration-dependent manner. ARL 66096 failed to inhibit platelet shape change and calcium mobilization induced by ADP (10), while blocking the ADP-induced inhibition of adenyl cyclase. Thus, we conclude that the P2Y1 receptor is not the P2TAC receptor.

A P2Y receptor coupled to inhibition of adenyl cyclase has been characterized in C6-2B rat glioma cells (33) and in B10 rat brain capillary endothelial cells (34). The P2Y1 receptor mRNA sequences were amplified from these cell lines by reverse transcriptase-polymerase chain reaction and no other P2Y receptor mRNA was amplified (34). Based on these findings, Webb et al.

P2TAC Receptor. These results suggest that the P2Y1 receptor plays an important role in ADP-induced platelet shape change and that intracellular calcium increases may be required for platelet shape change. These three P2Y1 receptor-specific antagonists required high concentrations (20-fold molar excess) to completely inhibit functional responses induced by 10 μM ADP. However, other investigators have determined that ATP is a weak antagonist, requiring at least 200-fold molar excess, to block the ADP-induced platelet shape change (31). The pA2 value for ATP at ADP-induced intracellular calcium mobilization in platelets was 5.1 (32). Thus, A3P5PS, A3P5P, and A2P5P are more potent antagonists than ATP at the platelet P2Y1 receptor.

The human P2Y1 receptor has been known to couple to mobilization of calcium from intracellular stores through activation of phospholipase C and formation of inositol triphosphate (9, 20). Molecular cloning of the P2Y1 receptor from platelet cDNA library and the concentration-dependent inhibition of ADP-induced intracellular calcium mobilization by the P2Y1 receptor selective antagonists demonstrate that the P2Y1 receptor is the platelet P2TAC receptor, coupled to inositol triphosphate formation and mobilization of calcium from intracellular stores, proposed earlier (10). Since myosin phosphorlylation has been shown to play a role in ADP-induced platelet shape change (18), we predict that the P2Y1 receptor mediated signaling events will lead to the phosphorylation of the myosin light chain.

α,β-MeATP has earlier been shown to have no effect at the P2Y1 receptor stably expressed in Jurkat cells (9) or in 1321N1 astrocytoma cells (20). α,β-MeATP, causing an increase in intracellular calcium through P2X1 receptor-mediated rapid calcium influx in the presence of 1 mM extracellular calcium, failed to elicit platelet shape change (Fig. 5) suggesting that these increases are insufficient to evoke shape change. Apparently, intracellular calcium increases and/or other signal transduction events, coupled to the P2Y1 receptor, are required for this physiological response. Thus, contrary to the proposal of Gachet et al. (23), the P2X1 receptor does not play a significant role in the ADP-induced shape change. Pretreatment with α,β-MeATP did not inhibit ADP-mediated shape change suggesting that the P2X1 receptor does not in any way modulate the effects mediated through the P2Y1 receptor.

ARL 66096, a P2TAC specific antagonist, failed to inhibit ADP-induced platelet shape change and mobilization of calcium from intracellular stores, while blocking the ADP-induced adenyl cyclase. Furthermore, even at high concentrations, A3P5PS, A3P5P, and A2P5P did not block ADP-induced inhibition of adenyl cyclase (Fig. 4). These results conclusively demonstrate that the P2TAC receptor does not play a role in the ADP-induced platelet shape change. Leon et al. (9) proposed that the P2Y1 receptor is indeed the elusive platelet ADP receptor coupled to inhibition of adenyl cyclase. Our results demonstrate that the P2Y1 receptor is not coupled to adenyl cyclase, as the P2Y1 receptor selective antagonists failed to abrogate ADP-induced inhibition of adenyl cyclase, but inhibited ADP-induced calcium mobilization and platelet shape change in a concentration-dependent manner. ARL 66096 failed to inhibit platelet shape change and calcium mobilization induced by ADP (10), while blocking the ADP-induced inhibition of adenyl cyclase. Thus, we conclude that the P2Y1 receptor is not the P2TAC receptor.

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(34) suggested that the P2Y1 receptor couples to inhibition of adenyl cyclase in different cells. However, since the pharmacological agents used in their studies are not specific for the P2Y1 receptor, we believe that C6-2B cells and B10 cells express multiple P2 receptor subtypes: the P2Y1 receptor, that was detected by reverse transcriptase-polymerase chain reaction and at least one other P2 receptor coupled to inhibition of adenyl cyclase. Boyer et al. (35) demonstrated differential effects of P2 receptor antagonists, suramin, reactive blue 2, and PPADS, on the turkey erythrocyte P2Y receptor, coupled to phospholipase C, and the C6-2B P2Y receptor, coupled to adenyl cyclase. PPADS inhibited 2-MeSATP-induced phospholipase C activation in turkey erythrocytes, while even at 100 μM it had no effect on 2-MeSATP-induced adenyl cyclase inhibition in C6-2B cells (35). Furthermore, it has been shown earlier that the P2 receptors in HEL cells, from which the human P2Y1 receptor has been cloned (14), do not couple to inhibition of adenyl cyclase (36), suggesting that the P2Y1 receptor coupled to adenyl cyclase is not expressed in these cells, while ADP-induced calcium mobilization from intracellular stores, a P2 receptor response, was observed. We ruled out the possibility of a splice variant of the P2Y1 receptor which may be coupled to the inhibition of adenyl cyclase, since previous studies from our laboratory have demonstrated that the human P2Y1 receptor is encoded by an intronless gene at chromosome 3q25 (37). We speculate that the P2 receptor in B10 and C6-2B cells, coupled to adenyl cyclase, may be the same receptor as the P2TAC receptor in platelets.

In conclusion, we have cloned a P2Y1 receptor cDNA clone from a human platelet cDNA library and demonstrated that this receptor mediates ADP-induced mobilization of calcium from intracellular stores and shape change in platelets. The other two ADP receptors on platelets, the P2TAC receptor, coupled to the inhibition of adenyl cyclase, and the P2X1 receptor, coupled to rapid calcium influx, do not play a role in platelet shape change induced by ADP.

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