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The role of ADP receptors in platelet function

Swaminathan Murugappan [1,3] and Satya P. Kunapuli [1,2,3]

Department of Physiology [1], Pharmacology [2], and Sol Sherry Thrombosis Research Center [3], Temple University School of Medicine, Philadelphia, Pennsylvannia 19140, USA

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1. ABSTRACT

Adenine di-Phosphate (ADP) is an important physiological agonist that plays a vital role in normal hemostasis and thrombosis. The importance of ADP in normal hemostasis is clearly demonstrated in patients suffering from storage pool disease who show excessive bleeding tendencies. It is well established that ADP activates platelets through 3 purinergic receptors, namely P2Y1, P2Y12 and P2X1. The P2Y1 receptor is a Gαq coupled G-protein receptor that is important for platelet shape change, aggregation, thromboxane A_2 generation, procoagulant activity, adhesion to immobilized fibrinogen and thrombus formation under shear conditions. The availability of P2Y1 antagonists and knockout mice have aided in demonstrating the multiple functions of this receptor in platelet function and normal hemostasis. The second ADP receptor, the P2Y12 signals through a Gαi coupled G-protein receptor and has been shown to be important for platelet functions very similar to the P2Y1 receptor. In addition, the P2Y12 receptor is also important for potentiation of platelet activation mediated by other physiological agonists including collagen, von Willebrand and thromboxane A_2. Advances in understanding the importance of the P2Y12 receptor has resulted in the development of drugs like clopidogrel and ticlopidine that is being successfully used clinically in the treatment of thrombotic disorders. The understanding of the function of this receptor has been possible due to the availability of multiple P2Y12 antagonists and the development of the P2Y12 null mice. The third and the final P2X1 receptor is an ion channel that upon activation causes an influx of calcium. Even though the activation of this receptor by itself doesn't lead to aggregation, it causes shape change and aids in the activation process of other agonists. Studies have also shown that it is important for thrombus formation under shear conditions in small arteries. Signaling by this receptor leads to significant ERK activation, which has been shown, to be important for collagen mediated platelet activation. The importance of ADP in hemostasis and thrombosis greatly underscores the significance of understanding the function of these receptors that would enable development of potent and safe anti-thrombotic drugs.

2. INTRODUCTION

Platelets get activated following vascular injury and subsequently lead to the formation of a platelet plug. This process involves shape change, secretion of granule contents, aggregation and generation of lipid mediators like thromboxane A_2 (TXA_2) and platelet activating factor (PAF). Adenosine di-phosphate (ADP) is considered to be an important mediator of platelet function, which is confirmed by studies done in patients exhibiting storage pool deficiencies or defective ADP receptors. Formation of thrombus due to inappropriate activation of platelets results in life-threatening conditions like myocardial infarction and stroke. The importance of ADP receptors in platelet function is further corroborated by the clinical efficacy of ADP receptor antagonists like clopidogrel in preventing and/or treating thrombotic conditions.

Purinergic receptors are categorized as either adenosine receptors (P1 receptors) or nucleotide receptors (P2 receptors). Receptors for nucleotides are subcategorized into two main groups: ligand gated ion channels (P2X) and G protein-coupled receptors (P2Y). This review shall focus on recent advances in the platelet purinergic receptor field, particularly the receptors for ADP in platelets and various functions that have been attributed to these receptors in the overall process of hemostasis. We have proposed the presence of three distinct P2 receptor subtypes on platelets (1): one coupled to inhibition of adenylyl cyclase through activation of the Gαi subunit, the second coupled to mobilization of calcium from intracellular stores through activation of the stimulatory Gαq subunit with resultant phospholipase C (PLC) activation, and the third an ionotropic P2X1 receptor coupled to rapid calcium influx. Several other studies (2-5) independently confirmed the three-receptor model by pharmacological approaches. The ADP receptor coupled to $G\alpha_q$ has been cloned and identified as the P2Y1 receptor (6). The Gαi-coupled ADP receptor has also been cloned and designated the P2Y12 receptor (7-10). Thus, the concept of a single ADP receptor mediating the previously observed effects has evolved into a concept involving three P2 receptor subtypes, viz. P2Y1, P2Y12, and P2X1 receptors, each with distinct functions. The three-receptor model has gained further support from several recent independent studies involving gene disruption approaches (11-13).

3. P2Y1 RECEPTOR

The Gαq-coupled P2Y1 receptor was the first of the ADP receptors to be cloned and was cloned from various cells including human platelets (6), erythroleukemia cells (14) and endothelial cells (15). The corresponding gene was localized to chromosome 3q25.91 (14). P2Y1 receptor is broadly distributed in the central nervous system, peripheral tissues, and blood platelets (16,17). Using polymerase chain reaction techniques, the P2Y1 receptor was found to be present in blood platelets and megakaryoblastic cell lines (18). The P2Y1 receptor consists of 373 amino acids, encoded by a single exon (19). P2Y1 receptor is a G-protein coupled receptor that activates the Gαq/phospholipase C pathway leading to generation of second messengers like inositol tris-phosphate (IP3) and diacylglycerol (DAG) (20). IP3 mobilizes calcium from the intracellular stores while DAG activates the protein kinase C isoforms. This activation leads to platelet shape change (21), and contributes to ADP-mediated platelet aggregation and thromboxane A_2 generation (22).

3.1. Pharmacological profile

P2Y1 is a high affinity receptor for ADP (EC_{50} 0.3 μM). ATP, 2-chloroATP and 2-MeSATP have been shown to be competitive antagonist at the rat and human P2Y1 receptor (18,23), whereas benzoyl-ATP is a non-selective antagonist at both the P2Y1 and P2Y12 receptors (24). Boyer *et al.* (25) have developed specific antagonists for the P2Y1 receptor. These antagonists, adenosine 3'-phosphate-5'-phosphosulfate (A3P5PS), adenosine 3'-phosphate-5'-phosphate (A3P5P), and adenosine 2'-phosphate-5'-phosphate (A2P5P), do not have any effect on the P2Y2, P2Y4, or the P2Y6 receptors, nor do they have any effect on the P2Y12 receptor that mediates ADP-induced inhibition of adenylyl cyclase (26). These bisphosphate nucleotides are competitive, yet not especially potent, antagonists at the P2Y1 receptor.

Another competitive antagonist at the P2Y1 is the riboside bisphosphate MRS2179 (N^6-methyl 2'-deoxyadenosine 3', 5'-bisphosphate) that, like A3P5P above, has no effect on P2Y2, P2Y4, P2Y6, or P2Y12 receptors (27). MRS2179 inhibits ADP-induced platelet shape change and aggregation with no effect on adenylyl cyclase pathways *in vitro*, and also inhibits thrombin-induced platelet aggregation at threshold concentrations in a manner resembling the behavior of platelets from P2Y1-deficient mice (28). MRS2179 has also shown an inhibitory effect at the P2X1 receptor. A recent study reported the development of another high affinity P2Y1 receptor antagonist, MRS2279 (2-chloro-N^6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate) (29), that has similar characteristics as the aforementioned MRS2179.

3.2. Function of the P2Y1 receptor

P2Y1 receptor activation is important for ADP-mediated platelet shape change and aggregation. Antagonizing this receptor using antagonists like A3P5P and A3P5PS inhibited both ADP and 2- MeSADP mediated platelet aggregation in a concentration-dependent manner (4,6,23,30,31). Presence of these antagonists also inhibited shape change and calcium mobilization by ADP suggesting a role for P2Y1 receptors in these responses (6,31). In addition to causing calcium mobilization, P2Y1 receptor activation by ADP or 2-MeSADP results in generation of a factor that mediates activation of p38 MAP kinase with no contribution from either P2Y12 or P2X1 receptors (32). The role of this pathway in platelet activation has not yet been determined. Activation of non-aspirinated platelets by ADP results in thromboxane A_2 generation following phospholipase A_2 activation. 'Outside in' signaling by fibrinogen receptor and 'inside out' signaling from both P2Y1 and P2Y12 receptors is necessary for ADP-mediated activation of phospholipase A_2 and thromboxane A_2

generation (22). Blocking either the P2Y1 or the P2Y12 receptor completely blocked the thromboxane A_2 generation mediated by ADP. More recently, it was shown that the Src family of tyrosine kinases are activated downstream of P2Y1 but not P2Y12 stimulation (33). This study showed the calcium mobilization mediated by the P2Y1 receptor is potentiated by the P2Y12 receptor through a PI3-kinase dependent but cAMP-independent pathway and further the signaling via the P2Y1 receptor negatively regulates the P2Y12 mediated potentiation of calcium. The dependence of ADP-mediated Src activation on the P2Y1 receptor independent of the P2Y12 receptor was demonstrated by measuring the phosphorylation of the Y416 residue on the Src kinases. But unpublished data from our lab demonstrated that Src tyrosine activation occurs downstream of P2Y12 receptor as well which was shown through the use of P2Y1 knockout mice platelets. In this study, activation of the P2Y12 receptor by ADP in the P2Y1-deficient platelets resulted in significant activation of the Src family tyrosine kinases. In addition to this study, a previous study from our lab has shown that ADP mediated Src activation can occur downstream of the P2Y12 or the Gz-coupled α2A- adrenergic receptor stimulation (34). Both of the above studies showed that Src activation can occur downstream of P2Y12 receptor which contradicts the results published by the previous study.

Platelets from mice-deficient in P2Y1 receptor showed no shape change or aggregation in response to normal concentrations of ADP confirming the role of this receptor in shape change and aggregation as demonstrated using pharmacological approaches (11,12). However, partial aggregation was seen when high concentrations of ADP were used. In addition to ADP, the platelet response to other agonists was also impaired. The inhibition of adenyly cyclase was still preserved in mice platelets in response to ADP indicating and confirming the lack of P2Y1 receptor activation in this signaling pathway (12). Over expression of P2Y1 receptor in transgenic mice also resulted in increased platelet aggregation response to ADP and low concentrations of collagen. In addition, in contrast to wild type platelets, these transgenic mice platelet released dense granules in response to ADP (35).

P2Y1 receptor has also been implicated in procoagulant function of platelets following vascular injury. P2Y1-deficient mice platelets were resistant to thrombosis induced by injection of agonists like ADP, epinephrine or collagen or tissue factor (TF) (11,12,28). Also mice platelets treated with P2Y1 antagonist MRS2179 showed decreased localized thrombus formation following ferric chloride-induced vascular injury (36). Finally over expression of P2Y1 receptor in transgenic mice induced hyperactivity of platelets (35). These studies suggest an important role of P2Y1 receptor in platelet procoagulant function as well. In addition to mediating ADP-induced platelet activation, P2Y1 receptor activation is also important for platelet activation in whole blood in response to other agonists like collagen, serotonin, epinephrine and thrombin receptor activating peptide (TRAP) (37). More recently, studies have focused on the role of P2Y1 receptor in flow conditions that exist in the body. Results from such studies show that antagonism of P2Y1 receptor can significantly inhibit platelet aggregation under shear conditions (38). Furthermore, activation of P2Y1 receptor by ADP is also essential for effective platelet adhesion on immobilized fibrinogen in physiological flow conditions (39).

4. THE P2Y12 RECEPTOR

The P2Y12 receptor has been cloned by different groups (8-10) and is shown to couple to the $G\alpha i_2$ member of the Gi family of G proteins. This leads to inhibition of adenylyl cyclase (40) and decreasing levels of cAMP. However, Yang *et al.* (41) and our laboratory (42) have shown that inhibition of adenylyl cyclase is not the signaling event that contributes to P2Y12-mediated platelet responses. More recently it was shown that the P2Y12 receptor doesn't result in lowering of the basal levels of cAMP, but infact inhibits the elevation of cAMP mediated by PGE2 or forskolin (41). P2Y12 stimulation results in potentiation of platelet aggregation, procoagulant activity, and dense granule secretion caused by other platelet agonists. Specifically, P2Y12 has been implicated in mediating irreversible aggregation by protease-activated receptor-1 and playing an integral role in thromboxane- and collagen-mediated aggregation. We have shown that PI 3-kinase is an important signaling molecule in the P2Y12 receptor-mediated potentiation of dense granule release (43). Although mice lacking PI 3-kinase γ represent a model that confers protection from thromboembolism, their platelets did not show significantly reduced aggregation in response to high concentrations of ADP (44). Other signaling molecules contributing to the P2Y12-mediated platelet function remain to be elucidated but recent studies have implicated a role for G-protein coupled inwardly rectifying potassium channels (GIRKs) (45) and Src family tyrosine kinases (34) in mediating the responses induced by the P2Y12 receptor activation in human platelets. It is known that, in contrast to the wide distribution of the P2X1 and P2Y1 receptors, P2Y12 receptor is found only on the platelet surface and in brain. Given the potentiating role of P2Y12 in various platelet functions and its limited localization, the P2Y12 receptor is a viable target for therapeutic intervention.

4.1. Pharmacological profile of the P2Y12 receptor

Due to its importance in platelet function and the limited distribution in the body, the P2Y12 receptor presents an attractive target for developing anti-thrombotic drug. ADP and 2-MeSADP are both agonists at P2Y12 receptors. While P2Y1 has a higher affinity towards ADP, the P2Y12 receptor has a 100-fold more affinity for 2-MeSADP compared to ADP (7,10). BeZATP is a non-specific antagonist at both the P2Y1 and P2Y12 receptors (24), while the methylated nucleotide 2-MeSAMP is a selective antagonist at the P2Y12 receptor (5,10). ATP was found to be a weak competitive antagonist of ADP-induced platelet aggregation. This lead to the development of a series of substituted purine derivatives like AR-C66096, AR-C67085 and AR-C69931MX, which acted like a reversible antagonist at the P2Y12 receptor in platelets (1,6,30,46,47). The potential for AR-C69931MX as an anti-

thrombotic drug is under investigation and is currently in phase II clinical trials (48,49).

The search for other compounds that targets the P2Y12 receptor lead to the development of thienopyridine derivatives that includes clopidogrel, ticlopidine (50), and CS747 (51). In contrast to the AR-C group of compounds, the thienopyridine compounds act as irreversible antagonists at the P2Y12 receptor. These drugs have to be metabolized in the body to an active metabolite that has the observed effects on platelets, thus limiting the use of these compounds to *in vivo* studies. The IC_{50} of these compounds for irreversible inhibition of 2 Me-SADP- and ADP-induced platelet aggregation were 0.53 μM and 1.8 μM, respectively. The specificity of the drugs towards the P2Y12 was demonstrated by the inhibition of ADP-mediated decrease in cAMP levels (52). Recently studies done using site-directed mutagenesis and by using the thiol group reagent p- chloromercuribenzene sulfonate (PCMBS) have shown that the residues C17 and C270 on the platelet P2Y12 receptor might be important targets for the active metabolites of clopidogrel and CS-747.

4.2. Role of the P2Y12 receptor in platelet responses

P2Y12 receptor is one of the purinergic (P2) receptors that is considered important for platelet responses. The role of this receptor in platelet function was demonstrated using both pharmacological and gene disruption studies. Using P2Y12 receptor antagonists like AR-C66096, AR-C67085 and AR-C69931MX, studies have shown that the P2Y12 receptor is essential for ADP and thromboxane A_2 mediated platelet aggregation (1,6,30,42,43,53). These studies also showed that activation of this receptor is not required for ADP-mediated shape change but causes potentiation of TXA_2 mediated dense granule secretion in a PI3-kinase dependent manner (43). Studies done in platelets from P2Y12 receptor- deficient patient showed absence of ADP-induced inhibition of adenylyl cyclase and defective aggregation (54). In addition to ADP, the P2Y12 receptor plays an important role in platelet responses, including aggregation and procoagulant function, in response to agonists like the thromboxane A2 analogue U46619, PAR1 activating peptide SFLLRN and collagen (37,53,55-62). Quinton *et al* have shown that PKC and calcium-pathways can independently synergize with P2Y12-mediated Gi signaling to cause platelet aggregation (58). Recently studies have also shown that P2Y12 receptor and the G12/13 pathways can synergize and cause fibrinogen receptor activation and platelet aggregation (63,64). In addition to playing a significant role in platelet aggregation and thromboxane generation, the Gαi-coupled P2Y12 receptor is also important for mediating the alpha granule release resulting from activation of platelets using ADP or U46619 (65). The P2Y12 receptor activation has also been demonstrated to be important for the activation of protein kinase B (PKB) or Akt. ADP-mediated activation of Akt was completely dependent on the P2Y12 receptor independent of the P2Y1 receptor. Also the Akt activation mediated by other agonists like thrombin was dependent on the signaling through the P2Y12 receptor that occurred following its stimulation through the secreted ADP (66). Finally studies done in our lab have shown that the requirements for the Gαq and Gαi - mediated aggregation and thromboxane generation responses are different compared to that mediated by the combination of Gαi and Gαz pathways (34). Whereas the Gαq plus Gαi mediated platelet responses occurred in a calcium-dependent but Src-independent manner, the Gαi plus Gαz mediated platelet responses occurred in a Src-dependent but calcium-independent manner. These above mentioned studies suggest the important role played by the P2Y12 receptor in the process of platelet activation.

Studies done under physiological flow conditions have also demonstrated that the P2Y12 receptor plays an important role in platelet activation response under such conditions. P2Y12 receptor antagonism by AR-C69931MX significantly decreased the extent of platelet aggregation under different shear rates (38,67). This function of P2Y12 receptor was further confirmed by studies done using platelets from patients deficient in P2Y12 receptor. The thrombus formed in such patients was small and loosely packed compared to platelets from normal subjects (39). P2Y12 receptor also plays a differential role in thrombus and embolus formation *in vivo*. The size of the initial thrombus was significantly reduced, although its stability was unaffected (68). In addition to platelet activation functions, the P2Y12 receptor has been demonstrated to have important role in procoagulant function as well. Studies with P2Y12 receptor antagonist AR-C69931MX has shown that this receptor is involved in thrombin-induced exposure of phosphatidyl serine (PS) on isolated platelets and consequently in Tissue factor (TF) -induced thrombin formation in platelet-rich plasma (69). *Ex vivo* inhibition of the P2Y12 ADP receptor by clopidogrel administration diminished the rapid exposure of tissue factor (TF), suggesting a role for P2Y12 receptor in procoagulant activity of platelets (70). The importance of P2Y12 receptor in thrombin generation was further shown, wherein antagonizing the P2Y12 receptor using the receptor specific antagonist significantly reduced both thrombin and collagen-mediated thrombin generation (71). All the above studies confirmed the vital role of P2Y12 receptor in thrombus formation following activation by flow and thus maintaining normal hemostasis.

Even though extensive studies have demonstrated the role of P2Y12 receptor in platelet function, the functional effector/s system downstream of this receptor has not yet been delineated completely. The P2Y12 receptor couples to the inhibitory Gαi pathway (40). This was confirmed by a recent study done in platelets lacking $G\alpha i_2$, which showed impaired ADP-induced platelet aggregation and loss of inhibition of adenylyl cyclase (72). Studies have shown that inhibition of cAMP levels following P2Y12 receptor activation is not directly responsible for the effects mediated by this receptor. Following P2Y12 receptor activation, the regulatory βγ dimmer activates the gamma isoform of phosphatidylinositol 3-kinase (PI3K γ). Platelets from P2Y1 deficient mice showed partial aggregation in response to ADP in a PI3-kinase-dependent manner (73). From the above studies it was hypothesized that PI3-kinase could be the downstream effector of P2Y12 receptors.

However, studies done using PI3-kinase γ knockout mice platelets showed that there is only a slight reduction in platelet aggregation mediated by high concentrations of ADP, even though there is some degree of protection from thromboembolism (44). More recently, Shankar *et al* (45) has shown the presence of G-protein coupled inwardly rectifying potassium (GIRK) channels in human platelets and that these channels are important for mediating the P2Y12-mediated platelet responses. This study used two structurally different blockers of the GIRK channels and showed the role played by these channels in platelet function. But what was intriguing was that the inhibition seen with these compounds was not complete, though significant. This showed the existence of other signaling effectors that might be important for mediating the P2Y12 responses downstream of ADP in platelets.

P2Y12 deficient mice platelets aggregated poorly in response to ADP and did not respond to thienopyridine compounds like clopidogrel. These platelet changed shape normally indicating that the P2Y12 receptor had no role in platelet shape change (13). Using an *in vivo* mesenteric artery injury model and real-time continuous analysis of the thrombotic process in P2Y12 deficient mice platelets, Andre *et al* showed that the P2Y12 receptor plays an important role in platelet adhesion/activation, thrombus growth, and stability (74).

5. P2X1 RECEPTOR

P2X receptors belong to the purinergic (P2) receptor family that mediates ligand -gated ion influx. These receptors have been shown to exist in the plasma membrane of various tissues. Seven genes for the P2X receptors have been identified on different chromosomes. Among these, only P2X1 is found in human platelets and megakaryocytes. The presence of P2X1 in human platelets was initially proposed by pharmacological methods and subsequently confirmed by demonstrating the presence of P2X1 mRNA (75-78). The gene for this receptor was later identified and localized to chromosome 17p13.2 in megakaryocytes.

5.1. Pharmacological profile of the P2X1 receptor

Activation of P2X1 receptor by agonists resulted in an inward current due to calcium influx in platelets. Both ADP and ATP were believed to be agonists that could activate P2X1 in platelets. Mahaut-Smith and coworkers subsequently showed ATP, but not ADP, is the true agonist at the P2X1 receptor (79). This was confirmed by demonstrating that a pure preparation of ADP had no agonist's activity at the P2X1 receptor in platelets. In addition to ATP, α,β-methylene ATP is a very selective agonist of the P2X1 receptor compared to other P2X receptors. Both ATP and α,β-methylene ATP had similar EC50 for the P2X1 receptor. 2', 3'-O-(benzoyl-4-benzoyl)-ATP (BzATP) is also an effective agonist. It is particularly potent when calcium flux is measured, with an EC_{50} in the low nanomolar range. Surprisingly, ADP acted as an antagonist at the P2X1 receptor in the presence of α,β-methylene ATP (80). Selective antagonists like MRS 2220 (cyclic pyridoxine-α 4,5-mono-phosphate-6-azo-phenyl-2', 5'-disulfonate) were subsequently developed that blocked the P2X1 receptor-mediated inward currents at around 10 μM but had no effect on currents evoked at P2X2 or P2X4 receptors (81). Selective agonists like pyridoxine-α5-monophosphate (MRS 2219) were also developed exhibiting selective P2X1 receptor activity in rats (81).

5.2. Role of P2X1 receptor in platelet responses

The role of P2X1 receptors in platelet function has been difficult to study because of the rapid desensitization of the receptor. Experiments done in the presence of apyrase, which prevents this desensitization, has revealed important functions of these receptors in the platelet activation process (82,83). Activation of P2X1 receptor results in influx of extracellular calcium into platelets contributing to the calcium increase following platelet activation. This increase in intracellular calcium due to P2X1 receptor activation is very short-lived and lower in magnitude compared to calcium increase resulting from activation of other receptors like P2Y1 (84). This P2X1-mediated calcium is not enough to cause aggregation but is sufficient to cause shape change (85) independently of fibrinogen receptor activation (86). The calcium increase due to P2X1 is potentiated in the presence of P2Y1 signaling suggesting a synergy between the two pathways (84). However, similar synergy was not seen between the P2X1 and P2Y12 pathways (87).

The P2X1 receptor has been shown to play an important role in regulating platelet responses to other platelet receptors. Studies have shown that platelet aggregation and shape change induced by low dose collagen requires activation of P2X1, since desensitizing or antagonizing this receptor results in inhibition of shape change and aggregation (88). In addition to calcium increase, P2X1 activation can also result in the activation of extracellular signal-regulated kinase 2 (ERK2) (89) Activation of the extracellular signal-regulated kinase 2 (ERK2) downstream of P2X1 receptor activation plays an important role in mediating the effects of P2X1 on collagen induced platelet responses including aggregation and secretion (89). The effect on secretion seems to be mediated via activation of the extracellular signal-regulated kinase 2 (ERK2) and subsequent myosin light chain kinase activation (90).

In vivo studies in P2X1 knockout mice show that P2X1 activity is necessary for thrombus formation under high shear rates as seen in small arteries (91). Such mice had decreased mortality rates as compared to normal control mice following laser induced vessel injury. The same study showed that thrombus removal was also rapid in these knockout mice (91). These knockout mice appear healthy and have no hemostatic defects. *In vitro* studies using platelets from P2X1 knockout mice show a 50% reduction in ATP-induced calcium currents suggesting a role for the secreted ATP in platelet activation (84). Studies done with mouse platelets overexpressing P2X1 receptors show an increase in collagen induced platelet aggregation, secretion and extra cellular signal-regulated kinase 2 (ERK2) phosphorylation (92). These transgenic mouse platelets also showed increased aggregation under shear

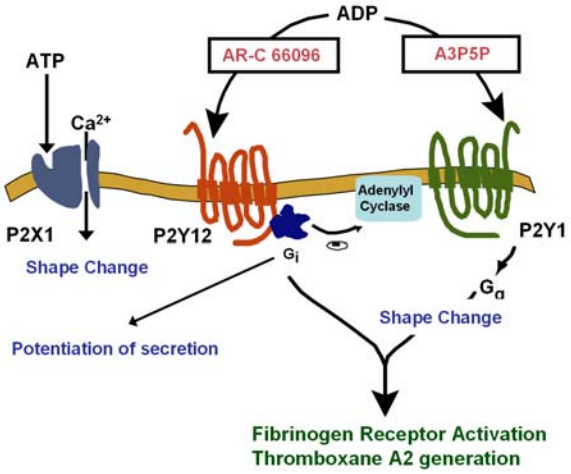

Figure 1. Three-Receptor model for ADP-mediated Platelet Activation. The P2Y1 receptor couples to Gq pathways and is important for platelet shape change and initiation of aggregation through integrin activation. The P2Y12 receptor couples to G1 pathways and is essential for gull aggregation and integrin activation. The P2Y12 receptor is also important for potentiation of dense granule secretion resulting from other agonists. Both the P2Y1 and P2Y12 receptor activation is essential for complete integrin activation by ADP. The P2X1 is an ion channel that causes calcium influx and results in platelet shape change. ARC 66096 and A3P5P are P2Y12 and P2Y1 antagonists, respectively.

conditions that failed to induce any response in platelets from normal mice. Finally the mortality in transgenic mice was higher compared to normal mice in *in vivo* conditions of platelet activation. These results suggested that overexpression of P2X1 receptor induces a prothrombotic phenotype in platelets (92).

6. CONCLUSIONS AND FUTURE DIRECTIONS

The concept of a single P2T receptor being resolved into three P2 receptor subtypes, i.e. the P2Y1, P2Y12 and P2X1 receptors, helped to explain the intracellular and physiological effects of ADP on platelets (Figure 1). The coordinated interaction of signaling pathways mediated by the P2Y1 and P2Y12 receptors is a novel mechanism of physiological response and could be a general mechanism of GPIIbIIIa integrin activation by most physiological agonists. We have shown that this is also the mechanism of activation for another platelet integrin, alphaVbeta3. The P2Y12 receptor has already been proven to be a useful target for anti-thrombotic agents. The extensive applications of the P2Y12 drugs, namely clopidogrel and ticlopidine in the treatment of thrombotic disorders underscores the potential of understanding the mechanism of ADP receptors. The P2Y1 receptor could also potentially be target for anti-platelet drugs. However, the expression of the P2Y1 receptor in several tissues other than platelets could pose problems in the drug evaluation. Despite the ubiquitous expression of the P2Y1 receptor, mice deficient in the P2Y1 receptor have been shown to breed and survive normally and hence the P2Y1 receptor is a viable alternative target for anti-platelet drugs. A combination of P2Y1 and P2Y12 receptor antagonists may prove effective in preventing thrombotic events compared with either antagonist alone. Given the negligible contribution of the P2X1 receptor in platelet plug formation, this receptor might not be a good anti-thrombotic drug target. The exact signaling mechanisms and pathways mediated by these three P2 receptor subtypes will provide a better understanding of ADP–mediated physiological responses in platelets and, provide new anti-thrombotic drug targets.

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Send correspondence to: Dr Satya P. Kunapuli, Temple University School of Medicine, 224 OMS, 3420 N. Broad Street, Philadelphia, PA 19140, Tel: 215-707-4615, Fax: 215-707-4003, E-mail: spk@temple.edu

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