The Platelet ATP and ADP Receptors

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Abstract: Adenine nucleotides, ADP and ATP, are coreleased from dense granules during platelet activation, as well as from endothelial cells and damaged red blood cells following vascular injury. Through autocrine and paracrine mechanisms, these extracellular signaling molecules interact with the platelet P2 receptors to amplify ongoing platelet activation. Two receptors for ADP, the G_q-protein-coupled $P2Y_1$ and G_i-protein-coupled $P2Y_{12}$ and one receptor for ATP, the $P2X_1$ ion channel, have been identified on platelets. Due to distinct pharmacological properties and differential regulation, the P2Y and P2X receptors essentially operate on different scales of time and distance and trigger selective intracellular signaling cascades. Recent advances in the understanding of the P2Y receptor physiology have reinforced the concept of these receptors as useful targets for antithrombotic therapy. The function of $P2X_1$ in platelet activation only recently started to be unraveled. This review focuses on recent findings on the physiology of these platelet ADP and ATP receptors, their distinct downstream intracellular signaling pathways as well as on the available agonists, antagonists and inhibitors that allow their pharmacological discrimination.

Key Words: Hemostasis, thrombosis, adenine nucleotides, $P2Y_1$, $P2Y_{12}$, $P2X_1$, antithrombotic therapy.

HISTORY AND CLASSIFICATION OF PURINERGIC RECEPTORS

The first report about the potent actions of an adenine compound, AMP, extracted from heart muscle, was published by Drury and Szent-Györgyi in 1929. Initial investigations focused on effects of adenosine and ATP on the heart and vasculature, or concerned the effects of purines on platelets [1] and on mast cells. Further insights into the physiological role of extracellular purines and pyrimidines were provided *via* the study of their biological sources [2]. In this respect, the detection of nucleotide release from the heart during hypoxia [3] or from skeletal muscle during contraction indicated that secretion of purine compounds might be coupled to metabolic demand to regulate local blood flow. ATP release from sensory nerves suggested that adenine nucleotides play a role as neurotransmitter or neuromodulator in the central and peripheral nervous system [4].

These early publications established that purinergic nucleotides act as extracellular signaling molecules [5]. Nucleotides are now documented to contribute to a diverse range of physiological responses such as smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, immune response, inflammation, platelet aggregation, male reproduction, nociception and modulation of cardiac function [6].

Purines and pyrimidines mediate their effects by interacting with distinct cell-surface receptors. Purinergic receptors were first formally recognized by Burnstock in 1978 [7]. They were divided into two classes: at P1-purinoceptors adenosine is the principal natural ligand, while P2-purinoceptors recognize both purine and pyrimidine nucleotides [8], namely ATP, ADP, UTP and UDP. P1 receptors are further divided into four subtypes (A1, A2A, A2B, A3) and all couple to G proteins [9].

Based on an increasing amount of experimental data concerning signal transduction mechanisms, pharmacological response profiles, desensitization kinetics, tissue distribution and biological effects obtained during the 80-90s, the extensive and heterogeneous group of P2 receptors was subdivided into P2X ligand-gated cation channels and G protein-coupled P2Y receptors [10]. This classification was based almost exclusively on the potency of purine and pyrimidine nucleotides to act as P2 agonists, but it is now known to coincide with the molecular structure and signal transduction mechanism of the underlying receptors, and has been retained.

The possible pitfalls of agonist-based receptor classification are well known and are very relevant to the P2 receptor area. Errors can occur because many nucleotides are metabolically unstable and are degraded by the ecto-nucleotidases present on cells [6]. In addition, ectonucleoside-diphosphokinases on many cells can convert dinucleotides into trinucleotides, making assessment of ADP and UDP activities difficult [11]. Release of endogenous nucleotides may also introduce problems, due to desensitization and down-regulation of the P2 receptor-promoted signaling response [11]. Finally, the presence of contaminating nucleotides in commercial nucleotide sources can produce misleading effects on the observed drug selectivity profiles at P2 receptor subtypes [12, 13].

THE PLATELET $P2Y_1$ AND $P2Y_{12}$ RECEPTORS FOR ADP

Intracellular Signaling Pathways

ADP signaling is often required to complete platelet aggregation. It is released from dense granules in response to

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agonist stimulation and can synergize with other agonists to activate platelets [14, 15]. ADP produces platelet shape change and induces platelet aggregation by converting the platelet fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, to an active conformation. The ability of ADP to trigger secretion from platelets is controversial and may depend on the presence of Ca^{2+} in the medium [16].

Platelets possess at least two P2Y receptors whose combined action is required for full activation and aggregation in response to ADP [17-23] (Fig. **1**). One of these, $P2Y_1$, is coupled to the heterotrimeric GTP-binding protein G_q and to phospholipase C-β activation; it induces mobilization of cytoplasmic Ca^{2+} and mediates shape change followed by an initial wave of rapidly reversible aggregation. Whether it also leads to stimulation of Rho/Rho kinase *via* $G\alpha_{12/13}$ is unclear [24]. Nevertheless, the study by Paul *et al*. [25] indicates that both Ca^{2+}/calmodulin-stimulated myosin light chain kinase and p160 Rho-associated coiled-coil-containing protein kinase independently contribute to ADP-induced myosin light chain phosphorylation and to platelet shape change, through Ca^{2+}-sensitive and Ca^{2+}-insensitive pathways, respectively. It was also described that $P2Y_1$ mediates p38 mitogen-activated protein kinase (p38MAPK) activation, an event requiring an aspirin-sensitive cofactor [26]; however, the functional significance of this signaling cascade in platelets and the downstream target of this kinase are not known.

The other receptor, $P2Y_{12}$ [27], is negatively coupled to adenylyl cyclase through G_i; it mediates progressive and sustained platelet aggregation in the absence of shape change and plays an important role in the potentiation of secretion induced by several agonists *via* its interaction with released ADP. This process is independent of the formation of large aggregates and of thromboxane A_2 synthesis [28, 29]. $P2Y_{12}$-mediated activation of the phosphoinositide 3-kinase (PI 3-K) pathway contributes to stabilize thrombin-induced platelet aggregates [30], although thrombin and thrombin-related peptides can cause platelet aggregation independently of G_i signaling [31]. The concurrent activation of the G_q and G_i pathways during full platelet aggregation is highlighted by the observation that normal aggregation responses to ADP can be restored in $P2Y_{12}$-deficient platelets by adrenaline, which is negatively coupled to adenylyl cyclase through G_z [32]; likewise, serotonin, which is coupled to G_q, can substitute for ADP in $P2Y_1$-null mouse platelets [33, 34]. However, from studies performed on $G\alpha_q$-deficient murine platelets, it appeared that the activation of G_{12}/G_{13}- and G_i-mediated pathways by the thromboxane A_2 mimetic and ADP, respectively, is sufficient to induce $\alpha_{IIb}\beta_3$ activation [35]. This indicates that the efficient induction of platelet aggregation through G-protein-coupled receptors is an integrated response triggered by various converging G-protein-mediated signaling pathways involving G_q, G_i as well as G_{12}/G_{13}. Of interest is the observation that high ADP concentrations are able to induce partial $\alpha_{IIb}\beta_3$-dependent aggregation without shape change for $P2Y_1$-null mouse platelets, through $P2Y_{12}$-mediated PI 3-K activation [36]. Such aggregation was strongly potentiated by adrenaline.

The small GTPase Rap1B is activated by ADP through both G_i- and PI 3-K-dependent and G_q- and Ca^{2+}-dependent mechanisms, which eventually may promote $\alpha_{IIb}\beta_3$ activation [37]. In this respect, Larson *et al*. [38] have recently shown that the collagen receptor GPVI-mediated Rap1 activation largely depended on ADP signaling through $P2Y_{12}$, but not through $P2Y_1$. In the same context, another study showed that, during thromboxane A_2 mimetic-induced platelet aggregation, signaling through the $P2Y_{12}$ receptor by secreted ADP causes positive feedback on platelet secretion through a PI-3 kinase pathway [39]. Thus, $P2Y_{12}$-mediated PI 3-K activation seems to be instrumental during platelet aggregation triggered by most platelet agonists (Fig. **1**). Finally, a recent study shows an essential role for $P2Y_{12}$/Gi signaling pathways, *via* secreted ADP, in activating the PI 3-K-dependent serine-threonine kinase Akt during platelet responses to thrombin, PAR1- or PAR4-activating peptides [40].

Pharmacology

The order of potency for agonist stimulation of $P2Y_1$ is 2-methyl-thio-ADP (2MeS-ADP) >> ADP = ADPαS > ADPβS and of $P2Y_{12}$ is 2MeS-ADP >> ADP = ADPβS; ADPαS is an antagonist at $P2Y_{12}$. Structure-activity studies have previously been reviewed extensively [14, 41]. ADP receptor numbers have been quantified with radiolabeled 2MeS-ADP, which binds to between 500 and 1000 sites per platelet [29, 42-44]. Evaluation of $P2Y_1$ binding sites is enabled by studies performed under conditions in which one receptor is blocked or absent. In the two well-characterized patients lacking $P2Y_{12}$, the number of 2MeS-ADP binding sites fell to about 30 for one patient [42] and to about 170 for the other [43]. These low values fit with the difficulties to detect $P2Y_1$ in flow cytometry by using available antibodies; in the $P2Y_{12}$-deficient SP1999 mouse model, no specific binding for 2MeS-ADP could be measured [45]. In fact, a recent study [46] described the existence of internal membrane pools of $P2Y_1$ receptors, on membranes of α-granules and of the open canalicular system (OCS), and on a network of thin channels that ramify from the surface into the interior of the platelet. Although the functional significance of the internal pools is unknown, pharmacological antagonism of ADP during antithrombotic therapy may need to take into account blockade of such internal receptor pools.

Platelets treated with ADPβS become refractory to ADP stimulation [47]; this platelet refractory state was entirely due to desensitization of the $P2Y_1$ receptor, the $P2Y_{12}$ receptor remaining functional. The decrease in [^{33}P]2-MeSADP binding sites measured on refractory platelets corresponded to the disappearance of the $P2Y_1$ sites with no change in the number of $P2Y_{12}$ sites, suggesting internalization of $P2Y_1$. This was confirmed by flow cytometric analysis of Jurkat cells expressing an epitope-tagged $P2Y_1$ receptor, where ADPβS treatment resulted in complete loss of the receptor from the cell surface. It was concluded that the $P2Y_1$ and $P2Y_{12}$ receptors are differently regulated during platelet activation.

The $P2Y_1$ Receptor

The $P2Y_1$ receptor was the first P2Y to be cloned and identified as a metabotropic receptor for adenine nucleotides [47]. Since its discovery, many more P2Y receptor subtypes have been identified [48] and the current series now extends to $P2Y_{13}$ [49]. $P2Y_1$ couples to heterotrimeric G proteins ($G_q/G\alpha_{11}$) and, through this link, stimulates phospho-

inositide turnover and release of intracellular calcium. Human $P2Y_1$ is particularly sensitive to ADP and 2MeS-ADP; whether ATP is an agonist of $P2Y_1$ is highly controversial and may depend on receptor expression levels. The tissue distribution for $P2Y_1$ protein, and messenger RNA, is ubiquitous –found in peripheral sensory nerves and central nervous system, most physiological systems and many cell types including blood platelets. $P2Y_1$-deficient mice [33, 34] displayed impaired platelet aggregation and increased resistance to pulmonary thromboembolism induced by infusion of a mixture of collagen and adrenaline. Also, using tissue factor (TF) to promote thrombin dependent thromboembolism, these mice exhibited higher thromboresistance and a significantly reduced *in vivo* thrombin generation [51]. These observations have led the authors to present the platelet $P2Y_1$ receptor as a potential target for antithrombotic therapy.

The 2-Chloro-N^6-methyl-(*N*)-methanocarba-2' deoxyadenosine-3', 5'-biphosphate (MRS 2279) developed in the Molecular Recognition Section (NIH, Bethesda, MD) is a potent highly selective and competitive antagonist for the $P2Y_1$ receptor with a K_d value of 9 nM at the platelet receptor [52, 53]. This antagonist provides the means to study the physiological role of native $P2Y_1$ receptors. MRS 2279 potently inhibits ADP-induced aggregation of human platelets *in vitro*, although the current utility of the compound is limited to being a research tool, not an antithrombotic agent. The origins of this compound can be traced back to work on the inhibitory actions of simple bisphosphate nucleotides (e.g. adenosine 3'-phosphate 5'-phosphate) at the $P2Y_1$ receptor - where these agents acted as competitive, yet not especially potent, antagonists [54]. Others drew attention to the "limited subtype specificity and non-P2 receptor effects" of such bisphosphate nucleotides [55, 56]. The next landmark compound was MRS 2179 (N^6-methyl 2'-deoxyadenosine 3', 5'-biphosphate), another competitive antagonist at the $P2Y_1$ receptor [57-60]. But hopes for MRS2179 were dashed following observations of inhibitory activity for this compound at recombinant $P2X_1$ and $P2X_3$ receptors [61]. Thereafter, the focus switched to non-ribose compounds

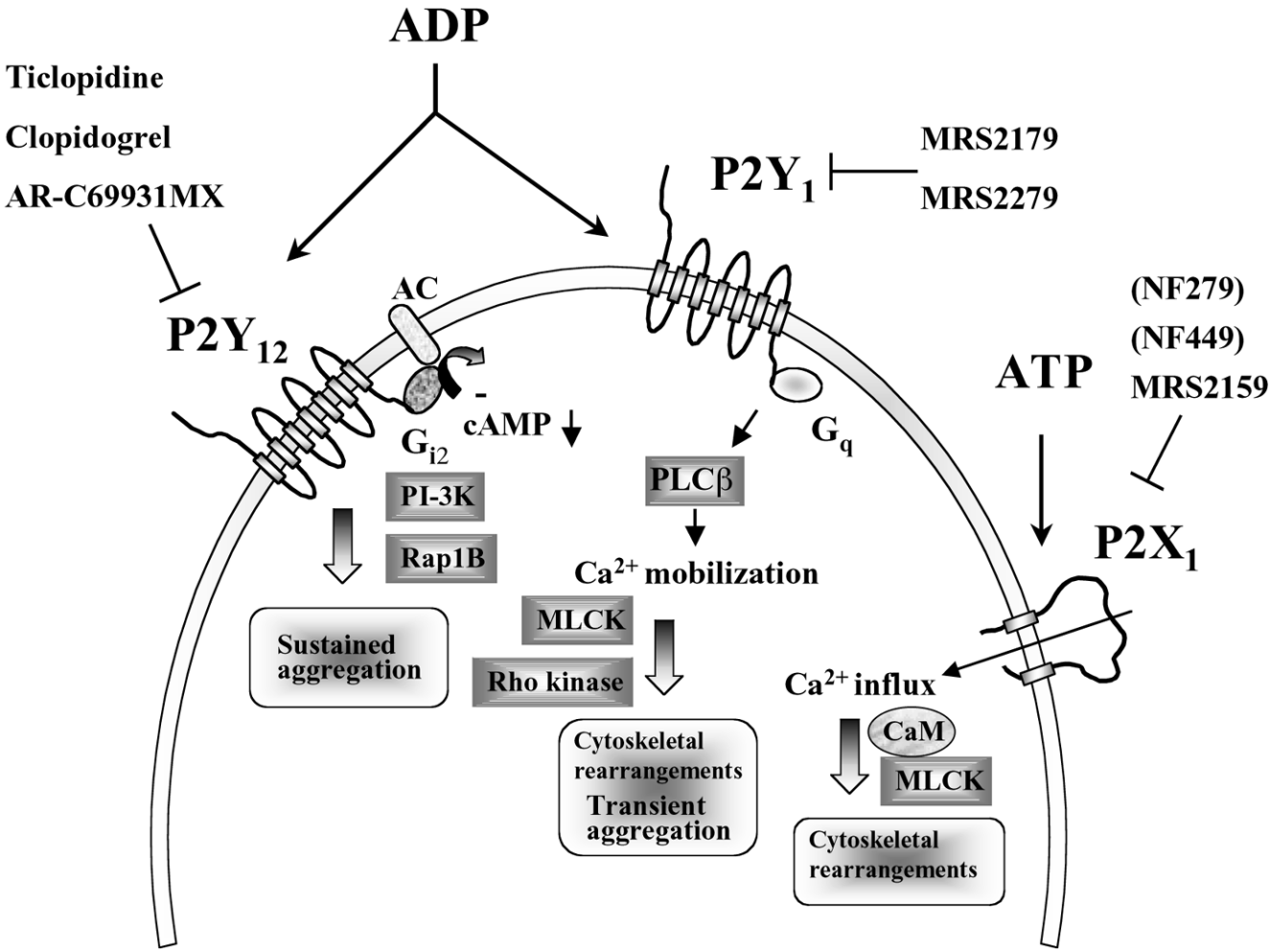

Fig. (1). Current model of platelet purinergic receptors. Two receptors for ADP, $P2Y_1$ coupled to G_q and $P2Y_{12}$ coupled to G_i, and one receptor for ATP, the $P2X_1$ ion channel, contribute to platelet activation. Inhibitors or antagonists of $P2Y_{12}$ are useful antithrombotic drugs. Selective $P2Y_1$ antagonists that inhibit platelet function *in vitro* also exist. Compounds acting as selective and highly potent antagonists at the recombinant $P2X_1$ receptor are shown. Whether these compounds are able to inhibit $P2X_1$–dependent events in platelets largely remains to be investigated. Nothing is known on the ability of NF279 and NF449 to antagonize the platelet $P2X_1$ receptor.

where the ribose moiety was substituted by methanocarba pseudosugars in either Northern (N) or Southern (S) conformations – to reduce the flexibility of the sugar spacer and enhance ligand docking. The resultant MRS 2279 represents the most potent and selective compound of this series of analogues, as reported by Boyer *et al.* [52]. Full understanding of the pharmacology of MRS 2279 will depend on its commercial availability. As yet, MRS 2279 has not been tested on recombinant P2X receptors. Also, no mention is made of its (in)activity at adenosine receptors. The chemical structures of these $P2Y_1$ antagonists have been presented in a recent review [15].

The $P2Y_{12}$ Receptor

In contrast to the ubiquitous expression of $P2Y_1$ [62], $P2Y_{12}$ expression is restricted to platelets and the brain [27, 63]. The intronless genes for $P2Y_1$ and $P2Y_{12}$ receptors are co-localized on chromosome 3q21-q25 [64] and share 22% sequence identity [27, 62]. $P2Y_{12}$ is the therapeutic target of ticlopidine and clopidogrel, two platelet aggregation inhibitors used for prevention and treatment of arterial thrombosis [65, 66]. Clopidogrel (SR25990C, PLAVIX) is a potent antiaggregant and antithrombotic drug, as demonstrated in several experimental models of thrombosis [67]. The drug was launched on the market following a successful clinical evaluation and demonstration of superior efficacy versus aspirin in preventing thrombotic events (myocardial infarction, stroke, and vascular death) in high risk patients (CAPRIE Steering Committee, 1996). Clopidogrel administered *in vivo* selectively and irreversibly inhibits the binding of 2MeS-ADP to platelet $P2Y_{12}$ [68-70]. Clopidogrel is inactive *in vitro* and has to undergo metabolic activation by hepatic cytochrome P450-1A [71, 72]. A possible metabolic pathway leading to the formation of the active metabolite of clopidogrel was tentatively deduced [73]. In the liver, clopidogrel is metabolized into 2-oxo-clopidogrel. This intermediate is then hydrolyzed and generates the highly labile active metabolite, which reacts as a thiol reagent with $P2Y_{12}$ on platelets when they pass the liver. This *in situ* biological effect could account for the absence of an antiaggregating activity in the plasma. In a recent work [74], the occurence of an *in vitro* active metabolite was documented after incubation of 2-oxo-clopidogrel with human liver microsomes. Mass spectrometry results suggested that the active metabolite belongs to a family of eight stereoisomers with the following primary chemical structure: 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene} acetic acid. However, only one of the eight metabolites retained biological activity, underlining the critical importance of the associated absolute chemical configuration. The active metabolite would be of S configuration at C 7 and Z configuration at the C 3-C 16 double bond.

The active metabolite of clopidogrel, containing a free thiol group, inactivates $P2Y_{12}$ through formation of a disulfide bridge with extracellular cysteine residues [69, 73, 75]. Another antiplatelet drug, CS-747, neutralizes $P2Y_{12}$ by the same mechanism [76, 77]. $P2Y_{12}$ also is irreversibly antagonized by the thiol reagent pCMBS [69, 78]. By using site-directed mutagenesis analysis, Ding *et al*. [79] have identified two free cysteine residues in the $P2Y_{12}$ extracellular domain (C17 and C270) to be the targets for thiol reagents. They proposed that the lack of these free cysteines in the extracellular domain of $P2Y_1$ explains why this receptor is not inhibited by thiol reagents and by the active metabolites of clopidogrel or by CS-747.

Since ticlopidine and clopidogrel are prodrugs requiring metabolization, inhibition of platelet aggregation by these drugs is delayed until 24-48 h after administration, with maximal inhibition achieved after 3-5 days [65]. Recovery of platelet function after drug withdrawal is slow (7-14 days). Ticlopidine and clopidogrel are effective in preventing atherothrombotic events in cardiovascular, cerebrovascular and peripheral vascular disease. However, neutropenia and thrombotic thrombocytopenic purpura are significant and occasionally fatal adverse effects of ticlopidine. Clopidogrel appears to offer several advantages over ticlopidine: a more rapid onset of action and lower incidence of the adverse effects. A combination of clopidogrel and aspirin has become standard antithrombotic therapy in a few cardiovascular conditions (e.g. following stent placement). The pharmacology and clinical developments of the thienopyridine derivatives have been recently reviewed [65].

Competitive $P2Y_{12}$ antagonists are also being developed [80, 81]. Modification of the polyphosphate side chain of ATP to prevent breakdown to ADP and substitution of the adenine moiety to enhance affinity and selectivity for the $P2Y_{12}$ receptor led to the identification of 2-propylthio-β, γ-dichloromethylene ATP (AR-C67085MX) [80], having an IC_{50} of 2.5 nM against ADP-induced aggregation of human platelets. This compound was the first very potent competitive antagonist of the $P2Y_{12}$ receptor, with a selectivity for that subtype of the P2 receptor family of > 1000-fold. Further modification of the structure produced N^6-(2-methylthioethyl)-2-(3, 3, 3-trifluoropropylthio)-β, γ-dichloromethylene ATP (AR-C69931MX) with an IC_{50} of 0.4 nM [80]. In a canine model of arterial thrombosis, AR-C69931MX was found to antagonize the *ex vivo* and *in vivo* aggregatory actions of ADP, and to display a rapid onset and offset of action with the ability to prevent occlusive arterial thrombus formation [82]. This compound also sustains canine coronary artery recanalization and improves myocardial tissue perfusion [83]. It therefore may be suited for the management of patients who require short-term modulation of platelet function. AR-C69931MX currently is in phase II clinical trials for the treatment of acute coronary syndromes.

Chemical stuctures of the $P2Y_{12}$ antagonists and inhibitors are depicted in the recent review by Gachet [15].

Congenital Defects of $P2Y_{12}$

Congenital deficiency of $P2Y_{12}$ results in a bleeding disorder. Four patients with abnormalities of platelet function due to a severe defect of $P2Y_{12}$ have been described so far [29, 42, 84, 85]. In common, these patients have a lifelong history of mucosal bleeding, easy bruising and/or excessive post-operative bleeding, mildly to severely prolonged bleeding times, weak and rapidly reversible primary wave of aggregation induced by ADP and abnormalities of platelet aggregation induced by collagen, arachidonate and thromboxane A_2 analogues, but normal aggregation induced by high concentrations of thrombin. Platelets deficient for this receptor exhibit normal ADP-induced shape change but ADP

fails to inhibit the rise in cAMP levels after stimulation with prostaglandin E_1 [86]. The $P2Y_{12}$ defect is inherited as a autosomal recessive trait [86], and heterozygous patients display a mild abnormality in platelet function similar to that seen in the relatively common primary secretion defects.

The genetics of $P2Y_{12}$ defects have been investigated. In two instances, different homozygous frameshift mutations were found to cause premature termination of translation. In another case, one allele presented a reading frame shift caused by the deletion of two nucleotides, whereas the other case had a normal coding sequence but a reduced expression, possibly resulting from another mutation in a regulatory region of the gene [27]. In a recent report [85], the patient was a compound heterozygote for two distinct amino acid substitutions that independently impair all the responses mediated by $P2Y_{12}$ without affecting ADP binding.

Role of the ADP Receptors in Coagulation

While the ADP pathway is recognized to enhance thrombus formation by recruiting platelets and leukocytes to the primary layer of collagen-adhering platelets, its role for the initiation of coagulation has not been revealed.

The platelet $P2Y_1$ and $P2Y_{12}$ receptors both contribute to thrombosis and thrombin formation in an *in vivo* model of TF-induced thromboembolism [51]. The $P2Y_{12}$ receptor, but not $P2Y_1$, was also found to be involved in thrombin-induced exposure of phosphatidylserine on isolated platelets and in TF-induced thrombin formation in platelet-rich-plasma [87]. Both receptors contribute to the interaction between platelets and leukocytes mediated by P-selectin exposure, and resulting TF exposure at the surface of leukocytes [87].

A recent study [88] shows that ex vivo inhibition of the $P2Y_{12}$ ADP receptor by clopidogrel administration diminished the rapid exposure of TF in conjugates of platelets with leukocytes established by the contact of whole blood with fibrillar collagen. In *in vitro* conditions, the $P2Y_{12}$ and $P2Y_1$ ADP receptors were both found to be implicated in the exposure of TF in collagen-activated whole blood. Immunoelectron-microscopy revealed that collagen elicited the release of TF from its storage pools within the platelets. Functional activation of the intravascular TF was reduced by inhibition of the ADP receptors, partially due to the disruption of the platelet-neutrophil adhesions. Injection of collagen into the venous circulation of mice increased the number of thrombin-antithrombin complexes, indicative for the formation of thrombin *in vivo*. In $P2Y_1$-deficient mice, the ability of collagen to enhance the generation of thrombin was impaired. This study indicate that the platelet ADP pathway supports the initiation of intravascular coagulation, which is likely to contribute to the concomitant formation of fibrin at the site of the growing thrombus.

THE PLATELET $P2X_1$ RECEPTOR FOR ATP

The Family of P2X Receptors

Seven distinct P2X subunits ($P2X_1$-$P2X_7$) assemble to form homo- or heteromeric non-selective ATP-gated cation channels composed of at least 3 monomers; these have been cloned from mammalian cell types and characterized pharmacologically by patch clamp electrophysiological measurements in heterologous expression systems [reviewed in 89, 90]. Their overall characteristics and functional properties closely resemble those in native tissues.

The pharmacological and biophysical properties allow the receptors to be classified into three groups, which correspond in broad terms to the three phenotypes described for the native receptors.

The first group comprises the $P2X_1$ and $P2X_3$ receptors; they are both highly responsive to the specific, non-hydrolyzable, stable P2X analogue, α,β−meATP as well as to ATP, and their desensitization is rapid ($P2X_1$ has a time constant of about 100 to 300 ms, $P2X_3$ desensitization is best fit by two exponentials of about 50 ms and 1 s).

The second group comprises the $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ receptors. In all these cases, α,β−meATP is ineffective and the receptor desensitization rate is slow. These receptors can be further subdivided according to their antagonist sensitivity: $P2X_2$ and $P2X_5$ receptors are reversibly inhibited by PPADS and suramin (<30 μM) (Fig. **2**), while $P2X_4$ and $P2X_6$ receptors are not blocked by this concentration of PPADS and suramin.

The third distinct type of functional response is exhibited by the $P2X_7$ receptor. It has a 240 amino acid longer intracellular C-terminus than other P2X receptors. $P2X_7$, cloned from rat macrophages and brain, is the cytolytic P2 receptor previously described in mast cells, fibroblasts and macrophages. Receptor distribution is generally limited to hemopoietic bone marrow cells including granulocytes, monocytes, macrophages, B-lymphocytes, erythrocytes and erythroleukemia cells. The unique feature of cloned and endogenous $P2X_7$-like receptors is that, whereas under physiological conditions they are selectively permeable only to small cations, in the continued presence of ATP and when divalent cation levels are low, the cation channel can convert to a pore, that is permeable to larger molecules upto 900 daltons in addition to ions. This effect is associated with cytotoxicity. Currents evoked at recombinant $P2X_7$ and endogenous $P2X_7$-like receptors do not readily desensitize.

Subunit Topology

Although the three-dimensional structure of the P2X subunits is not known, the absence of a leader sequence and their hydrophobicity pattern predict that they have two transmembrane domains, placing a large protein loop outside the cell [91] (Fig. **1**).

The different P2X subunits vary between 379 and 595 amino acids in length and share an overall sequence identity ranging from 35% to 48%. The extracellular loop of P2X channels is highly glycosylated; in the $P2X_2$ channel, glycosylation occurs at three asparagine residues (N182, N239 and N298) and deletion of any two out of the three leads to non-functional channels [92].

Based on structural similarities with the mechanosensitive channels of the MscL class, the amino- and carboxy-terminal tails of P2X subunits might be close to each other, sitting beneath the channel pore in a closed state. This stoichiometry may explain how truncations, deletions, mutations and splicing in these regions greatly affect the kinetics, permeation and desensitization of the channels [90]. In

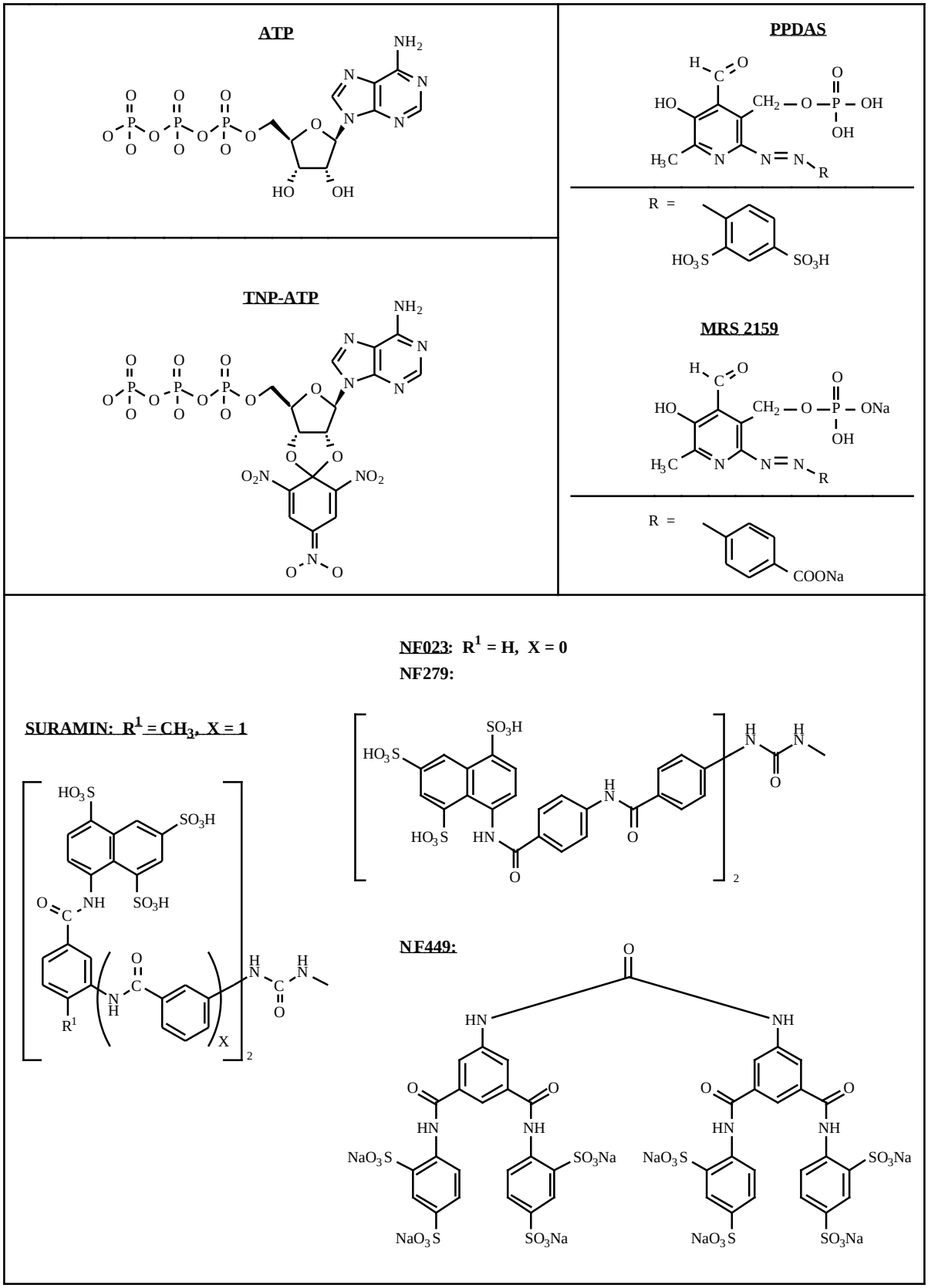

Fig. (2). Chemical structures of selective antagonists of the recombinant $P2X_1$ receptor.

addition, these intracellular regions are clearly important, but not the sole determinants of receptor function, as expected for allosteric proteins. The possibility that the long carboxy tail of P2X receptors interacts with other proteins has been suggested; interaction of $P2X_7$ channels with several cytoskeletal proteins has been demonstrated [93].

Using disulfide bond formation between engineered cysteines, a recent study demonstrated close proximity between the outer ends of the first transmembrane domain of one subunit and the second transmembrane domain of another [94]. In the hetero-oligomeric $P2X_2/P2X_3$ receptor, the coexpression in HEK293 cells of doubly substituted subunits with wild-type partners revealed that the hetero-oligomeric channel contains adjacent $P2X_3$ subunits but does not contain adjacent $P2X_2$ subunits. This study suggests a "head-to-tail" subunit arragement in the quaternary structure of P2X receptors and show that a trimeric $P2X_2/P2X_3$ receptor would have the composition $P2X_2(P2X_3)_2$.

The ATP-binding site of P2X receptors is thought to be located within the cysteine-rich extracellular loop, because ATP gates P2X receptors when applied extracellularly. In addition, purified extracellular loops obtained from $P2X_2$ subunits bind ATP [95]. P2X receptors do not contain consensus motifs common to other ATP-binding proteins. Interestingly, alterations in ATP potency were easily produced by mutagenesis of many residues [90]. Systematic analyses of the extracellular loop indicate that positively charged residues (K68 and K70, R292 and K309) in this region form part of the channel vestibule and contribute to the binding of the terminal phosphate of ATP in P2X subunits [96]. Accordingly, a recent study in Xenopus oocytes revealed that among the 20 conserved aromatic amino acids located in the extracellular ligand binding loop of $P2X_1$, residues K68, F185, F291, R292 and K309 contribute to ligand binding, with F185 and F291 co-ordinating the binding of the adenine ring of ATP [97].

Combined mutagenesis and pharmacological studies have suggested that the non-specific P2 antagonist PPADS binds to the same site as ATP, whereas other classes of P2 antagonists, like suramin, have a different binding site within the extracellular loop [98, 99] (Fig. **2**). These observations indicate that other binding sites in addition to the ATP site exist, modulate receptor function and probably might be targeted selectively with different drugs.

Desensitization of P2X Receptors

P2X receptors were divided into two broad groups according to current desensitization i.e. returning to zero rapidly (within 100 to 300 ms), or slowly if at all. The rapid desensitization of the ion currents should not be confused with receptor "desensitization", which occurs over a few seconds and reflects the inability of the receptor to respond to subsequent agonist application. Desensitization clearly serves to terminate the purinergic response even though ATP still is present in the environment.

The molecular mechanism of P2X receptor desensitization is not well understood. For the rapidly desensitizing $P2X_1$ receptor, this may involve the hydrophobic domains of the receptor because in $P2X_1$-$P2X_2$ chimeras, transfer of the $P2X_1$ hydrophobic domains, but not the extracellular loop of the $P2X_1$ receptor changes the phenotype of the $P2X_2$ receptor from non-desensitizing to rapidly-desensitizing [100]. Accordingly, a $P2X_1$-$P2X_2$ chimera containing the entire $P2X_1$ extracellular domain shows the non-desensitizing property of $P2X_2$ [101]. The N-terminal region of the $P2X_3$ receptor has been suggested to be important in the desensitization process. Desensitization of the $P2X_3$ receptor seems to involve the activation of calcineurin through the entry of extracellular calcium [102].

Regulation of Channel Function

Similarly to other neurotransmitter-gated channels, prolonged agonist stimulation results in changes in the surface localization of P2X receptors. $P2X_1$ receptors tagged with green fluorescent protein are rapidly internalized following agonist stimulation [103]; native $P2X_1$ receptors were also internalized in smooth muscle [104]. However, this phenomenon, probably involving cytosolic factors, does not explain the rapidly desensitizing phenotype of the $P2X_1$-mediated currents. It remains to be determined whether other P2X receptors are similarly internalized and how this mechanism affects the regulation of P2X receptor-mediated responses in native cells.

All P2X subunits contain a consensus protein kinase C (PKC) phosphorylation site (Thr-X-Lys/Arg) in the amino-terminal tail. PKC-mediated threonine phosphorylation was shown to preserve the slowly desensitising property of the $P2X_2$ channel [105]. Disruption of this residue leads to absent phosphorylation of the protein and simultaneously converts the ion current to the rapidly desensitizing phenotype. The same Thr residue is essential in $P2X_3$ channel function since, when replaced by Ala, the channel became inactive. Moreover, this site has been shown to be responsible for the PKC-mediated potentiating effect of inflammatory mediators on $P2X_3$ channel function, probably explaining the ability of these mediators to modulate nociception [106]. In transfected HEK293 cells, the absence of $P2X_1$ phosphorylation on residue T18 abolishes the ATP-induced inward current. Moreover, mutating residue R20 into T20 leads to absence of phosphorylation on residue T18 [107]. A recent study reported that in the slowly-desensitizing $P2X_7$ channel, phosphorylation of a single tyrosine residue at position 343 is required to maintain the run-down of the current as well as to sustain the interaction of $P2X_7$ with several, mainly cytoskeletal proteins, resulting in formation of a signaling complex [94]. These observations suggest that phosphorylation of the conserved PKC site serves as effective regulator of the channel function.

Using site-directed mutagenesis, we have investigated the function of the four highly conserved intracellular tyrosine residues of the $P2X_1$ ion channel [108] (Fig. **3**). Simultaneous electrophysiological and calcium measurements in transfected human embryonic kidney (HEK293) cells indicated that Y362F and Y370F mutants were non-functional, despite their proper plasma membrane expression. The Y16F and Y363F mutants retained 2.2 % and 26 % of the wild-type $P2X_1$ activity, respectively. However, no tyrosine phosphorylation was detected on Western blots of $P2X_1$ immunoprecipitates derived either from HEK293 cell lysates or from

Fig. (3). Sequence alignment of human P2X receptors. Conserved tyrosine residues are boxed with solid lines and partially conserved residues with dashed lines. $P2X_1$ transmembrane domains (TM1 and TM2) are overlined with a bar.

human platelets, with or without stimulation of the receptor. Thus, we concluded that Y16, Y362, Y363, and Y370 are required for the appropriate three-dimensional structure and function of the intracellular $P2X_1$ domains, independently of their phosphorylation.

Extracellular cations modulate ATP-activated currents in cloned and endogenous P2X receptors. Mg^{2+} and Ca^{2+} generally inhibit P2X receptor currents, probably by decreasing the affinity of the ATP binding site *via* an allosteric modulation in the receptor. Interestingly, the recombinant $P2X_1$ receptor seems to be less susceptible to inhibition by increases in extracellular Ca^{2+} [109]; Zn^{2+} potentiates the cation conductance induced by ATP at most P2X receptors, including recombinant $P2X_1$.

Modulation of the affinity of the ATP-binding site also occurs by extracellular protons; acid pH causes an increase, and alkaline pH causes a decrease in currents [110]. This may be particularly significant for P2X receptor-mediated signaling in pathophysiological conditions where injury or inflammation can alter the extracellular pH.

The $P2X_1$ Receptor

The $P2X_1$ receptor has been first cloned from rat vas deferens and human and mouse urinary bladder [111, 112]. The $P2X_1$ receptor encoding gene contains 12 exons spanning 20 kilobases on chromosome 17p13.2. $P2X_1$ receptor mRNA is expressed in urinary bladder, vas deferens, smooth muscle layers of small arteries and arterioles, with lower levels in spinal cord and rat brain, in lung and spleen and finally in dorsal root, trigeminal and coeliac ganglia [90, 111]. There is a prominent variation in the size of transcripts expressed in different tissues. The regulatory processes underlying tissue specific expression of the $P2X_1$ gene are unknown except for an E box response element that binds a βhelix-loop-helix protein and contributes to smooth muscle specific transcriptional regulation [113].

Pharmacology

The recombinant receptor is activated by 2MeSATP ≥ ATP > α,β−meATP, and inward currents evoked by these compounds are reversibly blocked by suramin, PPADS [110] and are very sensitive to inhibition by TNP-ATP (IC_{50} about 1 nM) [114] (Fig. **2**). Suramin analogues showing selectivity for $P2X_1$ receptors have been synthetized and characterized pharmacologically: one such compound is the symmetrical 3'-urea of 8-(benzamido)naphtalene-1, 3, 5-trisulfonate (NF023) [115] (Fig. **2**). NF023 is wholy selective for $P2X_1$ receptors in studies with the cloned receptor expressed in *Xenopus* oocytes, being weak at recombinant $P2Y_1$ (pIC_{50}= 4.6). A series of second generation suramin analogues with 4'-aminobenzoyl-linkages of one or two phenyl rings have been evaluated. One of these compounds, NF279, was the subject of a more detailed pharmacological investigation [116]. In prostatic segments of the rat vas deferens, NF279 (0.3-3 μM) inhibited the contractions evoked by α,β-meATP. A physiological role for $P2X_1$ receptors in renal microvascular autoregulatory behavior has been described [117]. Afferent arterioles from rats and $P2X_1$-deficient mice (see below) were examined using the juxtamedullary nephron technique. Pressure-mediated vasoconstriction were measured before or during $P2X_1$ receptor blockade with

NF279. This compound as well as deletion of $P2X_1$ receptors attenuated the autoregulatory response induced by an increase in renal perfusion pressure. NF279 was also a highly potent $P2X_1$-selective antagonist (IC_{50}=0.02-0.05 μM) in studies with cloned receptors expressed in *Xenopus* oocytes [118]. This compound was found to be weak at recombinant $P2Y_1$ (pIC_{50}=4.6) and inactive (up to 100 μM) at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.

The antagonistic effects of the novel suramin analogue 4, 4', 4'', 4'''-(carbonylbis(imino-5, 1, 3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1, 3-disulfonic acid (NF449) [119] (Fig. **2**) were analyzed at homomeric human $P2X_1$ and $P2X_7$ receptor subtypes ($hP2X_1$ and h $P2X_7$) heterologously expressed in Xenopus oocytes using the two-microelectrode voltage-clamp technique. At activating ATP concentrations of 1 microM ($hP2X_1$) and 100 microM ($hP2X_7$), IC_{50} values of 0.05 nM and 40 μM were found for $hP2X_1$ and $hP2X_7$ receptors, respectively. The Schild analysis revealed a pA_2 of 10.7 at $hP2X_1$. Wash-in and wash-out of 10 nM NF449 were nearly complete within 16 s and 4 min, respectively, at the $hP2X_1$ receptor. An increase in the activating ATP concentration to 100 microM shifted the NF449 concentration-inhibition curve rightwards for the $hP2X_1$ receptor. NF449 decelerated activation as well as desensitization of $hP2X_1$. It is concluded that NF449 acts as a reversible competitive antagonist at the $hP2X_1$ with much higher potency at $hP2X_1$ than at $hP2X_7$ receptors. NF449 may hence be excellently suited to discriminate between both receptors in native human tissues.

Novel analogues of PPADS have also been developed and characterized as antagonists at recombinant $P2X_1$, $P2X_2$, and $P2X_3$ receptors in *Xenopus* oocytes and turkey erythrocyte $P2Y_1$ receptors [120]. From these studies, the compound MRS2159 was found to be a very potent and selective $P2X_1$ antagonist (IC_{50}=10 nM) (Fig. **2**).

L-β,γ¬meATP proved to be a useful agonist to distinguish $P2X_1$ from other P2X receptors. It is a potent agonist at recombinant $P2X_1$ (EC_{50}=1.9 μM) [121] and induces contraction of a variety of smooth muscle preparations *via* $P2X_1$-like receptors [122]. In contrast, L- β,γ¬meATP has been found to be ineffective at recombinant $P2X_2$ [121] and $P2X_4$ receptors and at native P2X receptors in α,β−meATP-responding and non-responding neurons. Furthermore, L-β,γ¬meATP is inactive on P2Y receptors mediating relaxant responses in guinea-pig taenia coli and on native and recombinant P2Y receptors from chick brain. It is also resistant to degradation by ecto-nucleotidases, and its potential breakdown product, L-adenosine, is inactive at P1 receptors.

Activation and desensitization kinetics of the rat $P2X_1$ receptor at nanomolar ATP concentrations were further studied in Xenopus oocytes using two-electrode voltage-clamp recording [123]. The authors propose that these low ATP concentrations obscure $P2X_1$ receptor responses by driving a significant fraction of the receptor pool into a long-lasting refractory closed state. The $K_{1/2}$ of 3.2 nM for receptor desensitization would reflect the nanomolar ATP affinity of the receptor found by others in agonist binding experiments. The high EC_{50} value of 0.7 μM for receptor activation is a consequence of fast desensitization combined with non-steady-state conditions during recording of peak currents, which are the basis of the dose-response curve.

In order to study the intrinsic $P2X_1$ receptor kinetics, the same authors have used a non-desensitizing $P2X_1/P2X_2$ chimera comprising the entire $P2X_1$ ectodomain [118]. This chimera showed a 200- and 7000-fold higher ATP potency than observed for parent $P2X_1$ and $P2X_2$(A) receptors, respectively. Upon washout, the chimera deactivated slowly with a time constant t inversely related to the EC_{50} value of the corresponding agonist tested, suggesting that deactivation time courses reflect unbinding rates. The chimera also possesses virtually identical sensitivity to NF279. Thus, in agreement with the previous work, this study shows that the $P2X_1$ ectodomain confers nanomolar ATP sensitivity, which within the wild type receptor is obscured by desensitization such that only a micromolar ATP potency can deduced from peak current measurements, representing an amalgam of activation and desensitization.

$P2X_1$-Null Mice

The gene for mouse $P2X_1$ was cloned and its genomic structure defined by sequencing [124]. The gene spans about 10 kb and consists of 12 exons. All splice sites conformed to the GT-AG motif and the exon-intron boundaries were largely conserved with other members of the P2X gene family so far cloned. A single transcription-starting site was identified by 5' RACE analysis, 233 bp upstream of the translation start site. The $P2X_1$ gene maps to the central region of mouse chromosome 11.

$P2X_1$-deficient mice show reduced male fertility to approximately 90% [125]. Male mice copulate normally reduced fertility results from a reduction of sperm in the ejaculate and not from sperm dysfunction. In these mice, contraction of the vas deferens to sympathetic nerve stimulation is reduced by up to 60% and responses to P2X receptor agonists are abolished. These results show that $P2X_1$ receptors are essential for normal male reproductive function.

P2X receptor mediated inward currents and contractions are abolished in bladder smooth muscle from $P2X_1$-deficient mice [126]. In normal bladder nerve stimulation evoked contractions with P2X and muscarinic acetylcholine (mACh) receptor mediated components. In bladder from the $P2X_1$-deficient mouse the contraction was mediated solely by mACh receptors. These results indicate that homomeric $P2X_1$ receptors underlie the bladder smooth muscle P2X receptor phenotype. This animal model also revealed that homomeric $P2X_1$ receptors underlie the artery smooth muscle P2X receptor phenotype and contribute approximately 50% to sympathetic neurogenic vasoconstriction of mouse mesenteric arteries [127]. However, from a study examining the distribution, ontogeny and role of $P2X_1$ receptors in the smooth muscle of the mouse intestine, bladder, and male and female reproductive tracts, it appeared that $P2X_1$ receptors are not expressed throughout all smooth muscles and that their expression is developmentally regulated [128]. So, α,β-meATP (100 microM) failed to evoke contractions of the epididymis, or seminal vesicle and $P2X_1$ receptors did not contribute to the control of uterine smooth muscle. In contrast, in the ileum, α,β-meATP (100 microM) evoked a tran-

sient relaxation followed by a contraction. These responses were abolished by the P2 receptor antagonist iso-pyridoxalphosphate-6-azophenyl-2'-5'-disulphonate (iso-PPADS) (30 microM). Accordingly, contractile responses were reduced by > 80% in the ileum from P2x1 receptor-deficient mice.

The use of $P2X_1$-null mice also showed that $P2X_1$ subunits contribute to the properties of heteromeric P2X receptors in sympathetic neurons [129]. Finally, tubuloglomerular feedback signals are coupled to autoregulatory preglomerular vasoconstriction through ATP-mediated activation of $P2X_1$ receptors [117].

The Platelet $P2X_1$ Receptor for ATP

Following molecular identification of $P2X_1$ as the first known P2X subunit from rat vas deferens [111], the $P2X_1$ cDNA was also cloned from human platelets and megakaryoblastic cell lines [130]. The use of P2X-based degenerated primers led to the amplification of a single band of cDNA from human platelet RNA preparations as well as from three megakaryoblastic cell lines (Meg-01, K562, and CMK11-5). Subsequent DNA sequencing confirmed that this fragment was identical to a region of cloned human urinary bladder smooth muscle $P2X_1$ receptor. Importantly, no additional homologous clones were obtained, indicating that in platelets only $P2X_1$ subunits are expressed, forming rapidly desensitizing non-selective homomeric cation channels. Western blotting of platelet lysates separated by SDS-PAGE and probed with anti-$P2X_1$ IgG reveals the expected protein with a molecular mass of 60 kDa under reducing or non-reducing conditions. Endoglycosidase treatment results in altered migration, the reduced protein weight of 45 kDa confirms the highly glycosylated nature of the mature protein [131].

Interestingly, even before molecular cloning of the receptor, its existence in platelets had been anticipated by patch clamp techniques and intracellular calcium measurements. Several studies indicated that the extremely rapid kinetics of ADP- and ATP-mediated increase in intracellular Ca^{2+} concentration were consistent with direct opening of a plasma membrane ion channel in human platelets [132, 133]. More recently, it was specified that ADP/ATP activates a non-selective inward cation current, which does not involve a diffusible second messenger. It had also been demonstrated that the ADP/ATP-gated non-selective cation channel resembles a P2X-type purinoceptor, since the selective P2X-agonist α,β−meATP activated rapidly desensitizing, non-selective cation currents in human platelets. Similarly to platelets, in rat megakaryocytes ATP and the stable, non-selective ATP-analogue ATPγS activated a rapid, nonselective cation channel, consistent with P2X-type responses [134, 135]. Interestingly, spontaneously activated single channel currents in platelets were similar to P2X currents, suggesting both to be caused by autocrine activation following release of endogenous nucleotides. The transient, non-selective P2X-type current under physiological ionic conditions seemed to be carried mostly by Na^+, although $P2X_1$ receptors exhibit five fold selectivity for Ca^{2+} over Na^+ [111, 135].

The $P2X_1$ receptor was initially thought to be an ADP/ATP-activated ion channel and therefore to contribute to platelet responses to ADP. However, this proposal did not fit with the findings that P2Y-mediated G_q and G_i protein activation were sufficient to support ADP-induced platelet aggregation, and that α,β−meATP did not interfere with this platelet response, which brought doubts on the role of $P2X_1$ in platelet function. In view of the possible pitfalls and artefactual results arising from contaminating tri-phosphate nucleotides in commercially available di-phosphate nucleotide samples, the $P2X_1$ agonist profile was re-examined using HPLC-purified compounds. It appeared that $P2X_1$ receptor-triggered ion currents in megakaryocytes were activated by α,β−meATP and by commercially available ADP, while HPLC-purified ADP was inactive [136]. This observation has motivated further analyses of the platelet $P2X_1$ receptor as an ATP receptor, while ADP would act exclusively at $P2Y_1$ and $P2Y_{12}$ receptors. Using HPLC-purified nucleotides, we have further confirmed that ATP is the agonist of platelet $P2X_1$ receptors at which ADP acts as a weak antagonist [137]. Even though the issue of ADP versus ATP as a $P2X_1$-specific ligand was "reactivated" by the identification of an alternate $P2X_1$ receptor termed $P2X_{1del}$ (lacking 17 amino acids in the extracellular domain) [138], clear and definitive evidence now exist that the $P2X_{1del}$ receptor fails to form functional ion channels and is below the limit of detection in human platelets and that ATP is the sole $P2X_1$ agonist [139, 140].

Estimations based on transient whole-cell currents indicated a channel density of only 14-34 per platelet [141]. Nevertheless, due to rapid desensitization, a proper evaluation of functional $P2X_1$ receptors is complicated. Immunoblot analysis suggests significant, high expression of the $P2X_1$ protein, strongly maintained during megakaryocytic differentiation and platelet formation [131]. Study of [^{3}H]α,β−meATP binding on platelets showed only one type of binding site, its affinity being in the same order of magnitude compared to that reported for ATP receptors in rat bladder and vas deferens [142]. Scatchard analysis of the saturation binding data indicated that [^{3}H]α,β−meATP bound with high affinity (K_d = 23.6 nM). Compared to the binding of [^{3}H]2-MeS-ATP, the number of $P2X_1$ receptors detected with [^{3}H]α,β−meATP was 4-fold higher, being evaluated at 4190 ± 150 sites per platelet. 2-MeS-ADP and ADP competitively inhibited the specific binding of [^{3}H]α,β−meATP with IC_{50} values of 103 and 1120 nM, respectively.

Using $P2X_1$-deficient mouse platelets and megakaryocytes [143], the lack of α,β−meATP-induced Ca^{2+} responses was demonstrated, further confirming that the P2X receptor phenotype in megakaryocytes and in platelets is due to expression of homomeric $P2X_1$ receptors. Interestingly, co-application in wild-type megakaryocytes of α,β−meATP and ADP resulted in acceleration and amplification of the peak Ca^{2+} response, implying a priming role for P2X receptors in the subsequent activation of metabotropic P2Y receptors during platelet stimulation.

We have identified a clonal mutation in the platelet $P2X_1$ receptor of a patient showing a severe bleeding disorder [144]. This mutation results in deletion of one leucine residue contained in the second transmembrane (TM2) domain of this receptor. Voltage-clamped HEK293 cells

expressing mutated $P2X_1$ channels failed to develop an ATP-induced current. Furthermore, when co-expressed with the wild type receptor in *Xenopus* oocytes, the mutated protein exhibited a dose-dependent dominant negative effect on the normal ATP-induced $P2X_1$ channel activity. These data indicate that deletion of a single apolar amino acid residue at the inner border of the $P2X_1$ TM2 generates a non-functional channel. The platelet defect of this patient was characterized by defective platelet secretion and aggregation induced by low or intermediate doses of collagen, thromboxane A_2 mimetic, and thrombin-related peptide; the ADP-induced platelet aggregation were also strongly impaired when analyzed in the presence of high extracellular Ca^{2+} concentrations (hirudinized-PRP). Despite of this, the $P2Y_1$ and $P2Y_{12}$ receptors were found to be normal. The molecular basis of the platelet defect and the causative link between the $P2X_1$ mutation and the bleeding phenotype are still unclear.

The two stable ATP analogs α, β-meATP and L-β,γ¬meATP produce a $P2X_1$-mediated extracellular Ca^{2+}-dependent quickly reversible platelet shape change [137, 145]. Recently, it was shown that, by increasing the extracellular Ca^{2+} concentration, the $P2X_1$-evoked light transmission decrease (a measure of shape change) reached 82 % of that obtained *via* $P2Y_1$ receptors [146]. Despite of this, following blockade of $P2Y_1$ receptors with A3P5PS, co-stimulation with α, β-meATP and ADP failed to induce aggregation. Therefore, it appears that the early Ca^{2+} influx *via* $P2X_1$ does not readily synergise with $P2Y_{12}$ to support aggregation. However, it has been shown that the $P2X_1$ agonist α,β−meATP potentiates platelet microaggregate formation when added in conjunction with other weak platelet agonists such as epinephrine and thrombopoietin [147].

By using a selective $P2X_1$ desensitization strategy, we found that $P2X_1$ contributes to platelet aggregation induced by collagen [137]. The ionotropic P2X receptors regulate intracellular calcium levels through the ligand-stimulated increase in calcium permeability. However, how these receptors are linked to intracellular signaling pathways subserving their biological actions still is poorly understood. We have described that stable $P2X_1$ agonists elicit reversible extracellular signal-regulated kinase 2 (ERK2) phosphorylation through a Ca^{2+}-and protein kinase C-dependent pathway [148] (Fig. **4**). Moreover, we have shown that ERK2 activation caused by low concentrations of collagen exclusively depends on the $P2X_1$-mediated Ca^{2+}-influx through early ATP secretion evoked by this agonist. The $P2X_1$-PKC-ERK2 pathway plays a role in amplifying dense granule release, an event needed for completion of platelet aggregation upon mild stimulation with collagen. Furthermore, we found that α,β-meATP causes rapid, transient (2-5 s), and dose-dependent myosin light chain (MLC) phosphorylation [149]. Phosphorylation was inhibited by the calmodulin (CaM) inhibitor W-7, but not by the Rho kinase inhibitor HA-1077, i.e. it is exclusively regulated by Ca^{2+}/CaM-dependent MLC kinase. Correspondingly, the $P2X_1$-induced platelet shape change was inhibited by W-7 and by the MLC kinase inhibitor ML-7 but not by HA-1077. W-7, ML-7, the protein kinase C inhibitor GF109203-X, and the Src family kinase inhibitor PP1 inhibited the collagen and convulxin-induced early platelet degranulation, shape change, and subsequent aggregation, indicating a role for Ca^{2+}/CaM and MLC kinase in these glycoprotein VI-related platelet responses. The secreted ATP-mediated $P2X_1$-dependent ERK2 activation induced by low collagen concentrations contributes to MLC kinase activation since $P2X_1$ desensitization or blockade of ERK2 phosphorylation by U0126 strongly attenuated MLC phosphorylation, degranulation, and aggregation. We therefore conclude that at low doses of collagen, glycoprotein VI activation leads to early protein kinase C- and MLC kinase-dependent degranulation. Rapidly released ATP triggers $P2X_1$–mediated Ca2+ influx, activating ERK2, in turn amplifying platelet secretion by reinforcing the early MLC kinase phosphorylation. Hence, the $P2X_1$-ERK2-MLC axis contributes to collagen-induced platelet activation by enhancing platelet degranulation (Fig. **4**).

In order to investigate further the role of $P2X_1$ and the coupled ERK2 in hemostasis and thrombosis, we have generated transgenic mice overexpressing the human $P2X_1$ receptor in the megakaryocytic cell lineage [150]. Platelets from transgenic mice exhibited a gain of $P2X_1$ ionotropic activity as determined by more prominent $P2X_1$-mediated Ca^{2+} influx and platelet shape change. $P2X_1$ overexpression enhanced platelet secretion and aggregation evoked by low doses of collagen, convulxin, or the thromboxane A_2 mimetic U46619. In contrast, transgenic platelet responses to ADP or thrombin were normal. Perfusing whole blood from transgenic mice over collagen fibers at a shear rate of 1000 s^{-1} resulted in increased $P2X_1$-dependent aggregate formation and phosphatidylserine exposure. Platelet hyperreactivity to collagen was correlated with upregulated extracellular signal-regulated kinase 2 (ERK2) phosphorylation. Accordingly, the MEK1/2 inhibitor U0126 potently inhibited the collagen-induced aggregation of transgenic platelets, either stirred or when perfused over a collagen surface. In a viscometer, shear stress caused potent aggregation of transgenic platelets in conditions where wild-type platelets did not aggregate. In an *in vivo* model of thromboembolism consisting of intravenous injection of a low dose of collagen plus adrenaline, transgenic mice died more readily than wild-type mice. Preinjection of U0126 not only fully protected transgenic mice against thrombosis, but also enhanced the survival of wild-type mice injected with a higher collagen dose.

In agreement with our findings, the analysis of $P2X_1$-deficient mouse platelets [151] showed decreased collagen-induced aggregation and secretion; adhesion and thrombus growth on a collagen-coated surface was also reduced, particularly when the wall shear rate was elevated. The mortality of $P2X_1$-deficient mice in a model of systemic thromboembolism was reduced and the size of mural thrombi formed after a laser-induced vessel wall injury was decreased as compared with normal mice, whereas the time for complete thrombus removal was shortened.

Both studies ($P2X_1$ overxpressing mice and $P2X_1$-deficient mice) thus indicate that the $P2X_1$ receptor contributes to the formation of platelet thrombi, particularly in conditions in which shear forces are high.

Owing to the lack of selective platelet $P2X_1$ antagonists and because of the rapid desensitization of this receptor during platelet preparation, it had been difficult to analyze the

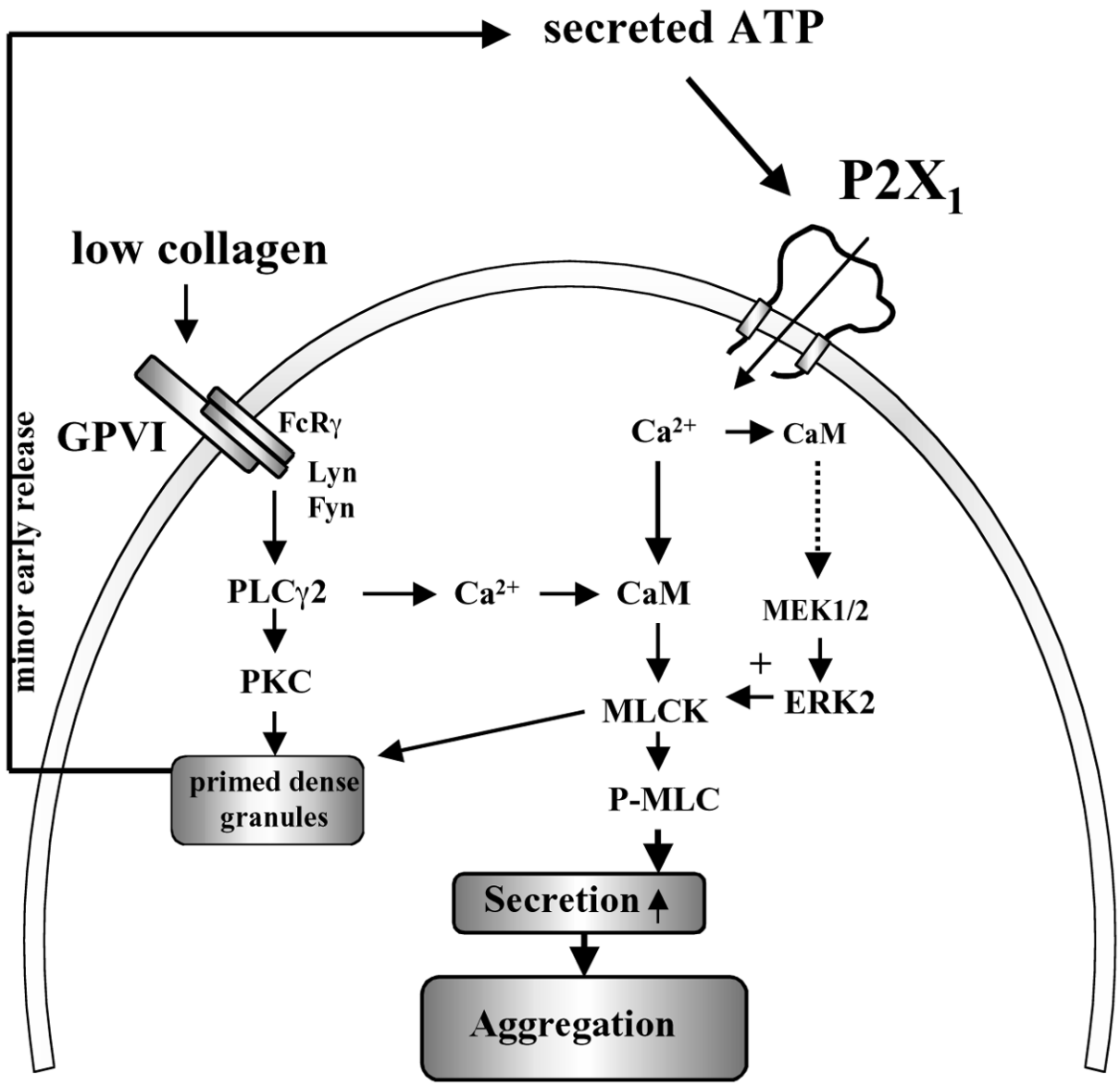

Fig. (4). Model depicting the role of $P2X_1$ during platelet activation by low doses of collagen. Glycoprotein VI activation by collagen leads to early protein kinase C- and MLC kinase-dependent degranulation. Rapidly released ATP triggers $P2X_1$–mediated Ca2+ influx, activating ERK2, in turn amplifying platelet secretion by reinforcing the early MLC kinase activation leading to increased MLC phosphorylation, further granule release and platelet aggregation.

contribution of this receptor to platelet function. Hence, these animal models demonstrate *in vivo* a role for $P2X_1$ in hemostasis and thrombosis.

Mice overexpressing the platelet $P2Y_1$ receptor have been generated [152]. Platelets from these mice displayed hyper-reactivity *in vitro*, as reflected by increased aggregation and induction of the release reaction in response to ADP. Platelet hyper-reactivity was also apparent *in vivo*: bleeding times were shortened and platelet counts were decreased following infusion of collagen and adrenaline. The increased platelet reactivity of either $P2X_1$ or $P2Y_1$ overexpressing transgenic platelets highlights the positive contribution of both ATP and ADP to platelet activation. In addition, it implies that controlled expression of the $P2X_1$ and $P2Y_1$ receptor genes is important for normal platelet activation. Whether variable expression levels of these receptors exist in humans and whether this might play a role in thrombotic states remains to be assessed.

Low concentrations of apyrase (a soluble ATP-diphosphohydrolase (EC 3.6.1.5) catalysing the conversion of ATP into ADP, and ADP into AMP) [153] are widely used during the preparation of washed platelet samples, and it was found to be necessary to preserve $P2Y_1$ receptor functionality during blood handling. More importantly, due to rapid desensitization of $P2X_1$ by spontaneously released ATP, a high concentration of apyrase is often required to demonstrate $P2X_1$ function. Working with human and murine platelets, the presence of apyrase is necessary to detect α,β−meATP-induced Ca^{2+} influx and platelet shape change.

Besides this protective function, the presence of apyrase leads to simultaneous degradation of actively secreted ATP and ADP, which interferes with the amplification of ongoing platelet activation, explaining the well-described inhibitory effects of apyrase. Therefore, the use of apyrase for *in vitro* studies always constitutes a compromise between receptor protection and simultaneous ligand breakdown.

In vivo experiments in mouse models, where endogenous ecto-ATPases prevent receptor desensitization, are thus necessary to confirm the conclusions of the *in vitro* analyses and to demonstrate a physiological role for platelet P2 receptors.

This point is even more important if we consider that already nanomolar ATP concentrations can obscure $P2X_1$ receptor responses by driving a significant fraction of the receptor pool into a long-lasting refractory closed state [118, 123]. Thus, we must keep in mind that $P2X_1$-mediated responses studied *in vitro* may not reflect correctly the *in vivo* situation.

CONCLUSIONS

Over the last eight years, the identification and detailed characterization of the platelet P2Y receptors for ADP have revealed new targets for the development of antithrombotic drugs. Whether the $P2X_1$ receptor represents a potential target for antithrombotic therapy remains an open question. Even though compounds like MRS2159 has shown some efficacy to selectively antagonize $P2X_1$ in washed human platelet suspensions, the ability of most available P2X-subtype selective antagonists to inhibit platelet function has still to be defined.

Mice deficient for the three P2 receptors identified on platelets as well as transgenic mice overexpressing platelet $P2X_1$ and $P2Y_1$ receptors now exist. The combined analyses of these animal models should help to further understand how P2X and P2Y receptors contribute to platelet function *in vivo*, and should provide insights on the physiological action of ATP *versus* ADP at these receptors.

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ABBREVIATIONS

α,β-meATP	=	α,β-methylene ATP
β,γ-meATP	=	β,γ-methylene ATP
ERK	=	Extracellular signal-regulated kinase
MAPK	=	Mitogen-activated protein kinase
2MeS-ADP	=	2-methylthio-ADP
MLCK	=	Myosin light chain kinase
PI-3K	=	Phosphoinositide 3-kinase
PLC	=	Phospholipase C
PPADS	=	Pyridoxal-5'-phosphate 6-azophenyl-2', 5'-disulfonate
TF	=	Tissue factor
VWF	=	Von Willebrand factor

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