Purinergic Signalling (2015) 11:411–434
DOI 10.1007/s11302-015-9462-7

REVIEW ARTICLE

# Blood cells: an historical account of the roles of purinergic signalling

**Geoffrey Burnstock** [1,2]

Received: 21 July 2015 / Accepted: 23 July 2015 / Published online: 11 August 2015


**Abstract** The involvement of purinergic signalling in the physiology of erythrocytes, platelets and leukocytes was recognised early. The release of ATP and the expression of purinoceptors and ectonucleotidases on erythrocytes in health and disease are reviewed. The release of ATP and ADP from platelets and the expression and roles of P1, $P2Y_1$, $P2Y_{12}$ and P2X1 receptors on platelets are described. $P2Y_1$ and P2X1 receptors mediate changes in platelet shape, while $P2Y_{12}$ receptors mediate platelet aggregation. The changes in the role of purinergic signalling in a variety of disease conditions are considered. The successful use of $P2Y_{12}$ receptor antagonists, such as clopidogrel and ticagrelor, for the treatment of thrombosis, myocardial infarction and stroke is discussed.

**Keywords** ADP · Adenosine · Erythrocytes · Platelets · $P2Y_1$ · $P2Y_{12}$ · P2X1 · Purinoceptor · Thrombosis · Clopidogrel



✉ Geoffrey Burnstock
g.burnstock@ucl.ac.uk

[1] Autonomic Neuroscience Centre, University College Medical School, Rowland Hill Street, London NW3 2PF, UK

[2] Department of Pharmacology and Therapeutics, The University of Melbourne, Melbourne, Australia

## Introduction

Purinergic signalling, ATP acting as an extracellular signalling molecule, was proposed in 1972 [1]. Separate families of receptors for adenosine (P1) and adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) (P2) were recognised in 1978 [2] and receptors for purines and pyrimidines cloned and characterised in the early 1990s (see [3]). Four P1 receptor subtypes ($A_1$, $A_{2A}$, $A_{2B}$ and $A_3$), seven P2X ion channel receptor subtypes (P2X1–7) and eight P2Y G protein-coupled receptors ($P2Y_{1, 2, 4, 6, 11, 12, 13 \text{ and } 14}$) have been identified (see [4]).

The involvement of purinergic signalling in the biology of erythrocytes, platelets and leukocytes was recognised early, and this review aims to present an historical account leading to our current understanding of the various roles played by purine nucleotides and nucleosides in health and disease. A valuable earlier review was published about the roles of nucleotide receptors in blood cells [5].

## Erythrocytes

### Extracellular actions of nucleotides

Early papers were concerned with intracellular ATP levels (estimated at 107–284 μg/ml in a very early paper [6]) in erythrocytes and their relation to the cell shape [7–9] and storage ability [10]. There were also early papers concerned with the ectoenzymes involved in the metabolism of external adenine nucleotides [11–14]. A decrease in intracellular red cell ATP levels during aging was reported [15]. Early papers also showed that extracellular ATP increased $Na^+$ and $K^+$ permeability and altered the physical properties of mammalian red blood cells [16–19]. Erythrocyte membrane preparations ('ghosts') were used in numerous investigations of the actions of ATP (e.g. [20, 21]) and ATPases [22]. Using RT-PCR of red blood cell progenitor cells, messenger RNA (mRNA) expression of P2X1, P2X4 and P2X7, as well as $P2Y_1$ receptors (but not for $P2Y_2$, $P2Y_4$ or $P2Y_6$) was reported [23]. The turkey erythrocyte has also been utilised as a model for studies of purinergic signalling [24]. For example, P2Y receptors were identified and the kinetics of activation of phospholipase (PL) C by P2Y receptor agonists examined [25, 26]. Phosphatidylinositol 4,5-bisphosphate hydrolysis was shown to be regulated by P2Y receptors in turkey erythrocytes [27]. Later, this P2Y receptor was identified as the $P2Y_1$ subtype [28, 29]. Extracellular ATP was reported to stimulate a volume decrease in red blood cells from *Necturus* [30] and activate a P2 receptor during hypotonic swelling [31].

Human erythrocytes were shown to express P2X7 receptors on all erythrocytes examined from eight subjects. P2X2 receptors were also identified, although they were at a far lower staining intensity in six of the eight subjects [32]. These studies also showed that purines increase cation fluxes in the potency order of 2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BzATP) > ATP > 2-methythioATP > adenosine-5′-(γ-thio)-triphosphate, while ADP and uridine 5′-triphosphate (UTP) had no effect. A $P2Y_4$-like receptor was claimed to increase $[Ca^{2+}]_i$ in red blood cells of the lizard [33]. Elevated intracellular $Ca^{2+}$ revealed a functional membrane nucleotide pool in intact human red blood cells [34]. P2X7 receptor activation caused phosphotidylserine exposure and cell shrinkage in human erythrocytes [35]. Erythrocytes are reservoirs of epoxyeicosatrienoic acids, which are vasodilators, anti-aggregatory and anti-inflammatory lipid mediators. Stimulation of rat erythrocyte P2X7 receptors induces the release of epoxyeicosatrienoic acids, arachidonic acid-derived lipid mediators that dilate arterioles [36, 37]. Canine erythrocytes express P2X7 receptors, which mediate a massive increase in cation permeability compared to human erythrocytes [38, 39]. 5-Nucleotidase activities were reported in human erythrocytes [40]. Activation of $P2Y_1$ receptors triggers two calcium signalling pathways in bone marrow erythrocytes [41].

Extracellular adenosine was shown to significantly enhance glucose consumption and lactate production in washed human red blood cells [42]. The adenosine receptor, present on turkey erythrocytes, was shown to be coupled to adenylate cyclase [43]. Adenosine is rapidly taken up by erythrocytes [44, 45], which is critical since adenosine deaminase is localised in the plasma membranes of erythrocytes [46]. $A_2$ receptors are present in embryonic red blood cells, but their numbers were reduced in later development [47]. Suicidal death of erythrocytes or eryptosis is characterised by cell shrinkage and cell membrane scrambling, and adenosine was shown to inhibit eryptosis [48]. It was reported that $A_{2B}$ receptors mediate regulatory volume decrease in mature human erythrocytes [49].

The level of intracellular ATP is crucial for maintaining the function and structural integrity of circulating red blood cells [50]. Elevated levels of ATP in red blood cells of patients with renal failure was reported, 4.88 μmol/gHb compared to control 3.64 μmol/gHb [51]. The loss of adenosine 5′-monophosphate deaminase activity in senescent erythrocytes may explain elevated ATP levels [52].

Ticagrelor, a $P2Y_{12}$ receptor antagonist, reportedly inhibits adenosine uptake leading to augmentation of cardiac blood flow in a canine model of reactive hypoxia [53]. The authors suggest that ticagrelor may have additional benefits in patients with acute coronary syndrome beyond inhibition of platelet aggregation including the induction of ATP release, which was shown to occur in studies of human red blood cells [54]. The ticagrelor-induced adenosine increase may be beneficial by improving peripheral endothelial function [55] and also be cardioprotective by reducing myocardial infarct size [56].

Damage to healthy tissue is a major limitation of radiotherapy treatment of cancer patients, and radiation-induced release of pro-inflammatory cytokines may be involved in the side effects. In whole blood studies, ATP inhibited radiation-induced tumour necrosis factor-α release and increased interleukin (IL)-10 release, perhaps via $P2Y_{11}$ receptors, and it was concluded that ATP alleviates radiation toxicity, mainly by inhibiting radiation-induced inflammation and DNA damage [57]. The ATP released from erythrocytes is anti-adhesive, and storage-induced deficiency in ATP release from transfused erythrocytes may promote microvascular pathophysiology in lung endothelial cells possibly via increased cell adhesion [58].

### ATP release

Human erythrocytes release ATP upon exposure to mechanical deformation, β-adrenoceptor agonists, prostacyclin analogues, reduced $O_2$ tension, acidosis or swelling [59]. Release of ATP from erythrocytes exposed to hypertonic solutions was described by Deyrup in 1951 [60], and aging ATP-depleted

human erythrocytes were later shown to release vesicles [61, 62]. The release of ATP from human erythrocytes was shown to occur in response to a brief period of hypoxia in the presence of hypercapnia, such as would be found in exercising muscle [63] and was later demonstrated to similarly occur in response to low $O_2$ in the absence of hypercapnia [64]. It has been proposed that red blood cells are not only $O_2$ carriers but via ATP release have a direct role in regulation of vascular tone leading to the appropriate distribution of microvascular perfusion [64–67].

Erythrocytes release ATP in response to mechanical deformation as might occur when red cells are squeezed through small vessels or deformed in areas of high velocity [68–71] (Fig. 1). Increases in perfusate flow rate were a sufficient mechanical stimulus for ATP release from red blood cells in isolated rabbit lungs [72]. Studies with erythrocytes from individuals with cystic fibrosis suggested that this release required cystic fibrosis transmembrane conductance regulator (CFTR) [73] although they considered that it was unlikely that this is the channel by which ATP exits the red blood cell. Recent papers have reported that ATP release in response to reduced oxygen tension occurs partly via the hemichannel pannexin 1, which may also be the channel involved in ATP release in response to mechanical deformation above a specific stress threshold [74–76] (Fig. 2).

The release of ATP from erythrocytes in response to both low oxygen tension and mechanical deformation has been shown to require signal transduction pathways involving activation of pathway-specific membrane-bound adenylyl cyclase, cyclic adenosine monophosphate (cAMP), protein kinase (PK) A and CFTR; in addition, the direct stimulation of the G protein $G_i$ also results in the release of ATP [77–80]. Although not conclusively established, evidence suggests that the release of ATP from erythrocytes in response to reduced oxygen tension is linked to the oxygenation state of the haemoglobin molecule via alterations in its confirmation [81, 82]. The release of ATP from erythrocytes in response to low $O_2$ tension was demonstrated to occur in milliseconds making it a physiologically relevant mediator of microvascular blood flow [83]. A computational model to measure the dynamics of $O_2$-dependent ATP release from erythrocytes confirmed this time course [84]. Data was subsequently presented from studies of the simultaneous effect of hypoxia and deformation on ATP release from erythrocytes to suggest that at an oxygen saturation point of around 25 % deformation contributes to ATP release, but beyond this saturation point, ATP release is largely due to hypoxia [85]. Data from in vivo and in vitro studies showed that significant amounts of ATP were released from erythrocytes on exposure to hypoxia and shear stress at the same time [86].

In addition to mechanical deformation and low oxygen tension, erythrocytes release ATP in response to prostacyclin analogs and β-adrenergic agonists. Although the physiological impact of the latter is unclear, prostacyclin is released from endothelial cells in response to shear stress and, although it clearly has direct vasodilatory effects, its capacity to release ATP would enhance its effectiveness. The release of ATP by prostacyclin analogs involves a distinct signal transduction pathway which is initiated by activation of $G_s$ and involves distinct pools of cAMP which are regulated by pathway-specific phosphodiesterases [87]. Prostacyclin receptor-induced ATP release occurs via the voltage-dependent anion channel, suggesting the presence of yet another channel for ATP release from erythrocytes [88].

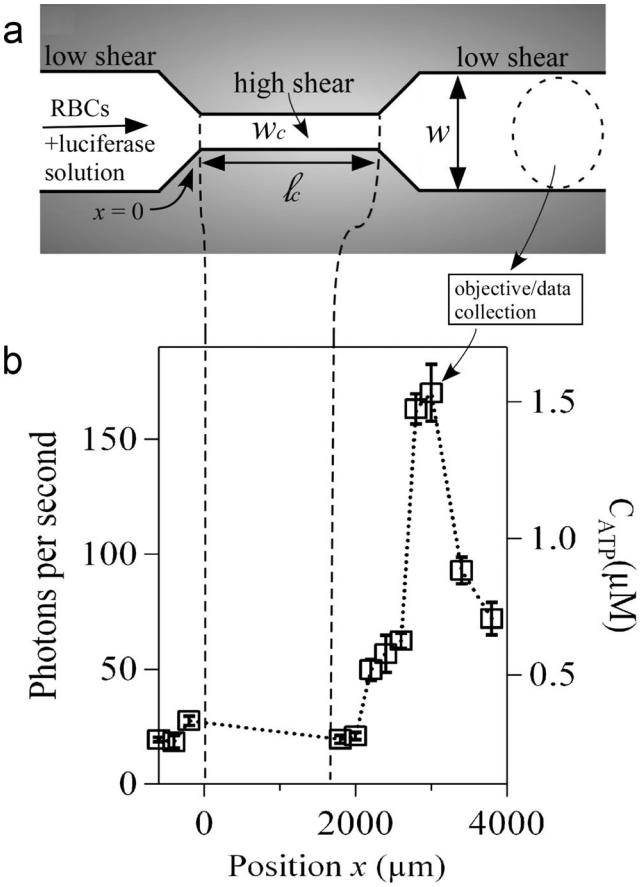


**Fig. 1** Microfluidic approach for shear-triggered release of ATP. **a** Schematic of the experimental apparatus (not to scale). A mixture of red blood cells (RBCs) and luciferase/luciferin solution are pumped through a microfluidic constriction. **b** Representative experimental measurements of the photon emission rate resulting from the reaction between luciferase/luciferin and ATP, measured versus position along the channel ($\ell_c$=1600 μm and $w_c$=20 μm). The position $x$=0 is defined as where the entrance to the constriction is located. The approximate ATP concentration ($C_{ATP}$) converted from the calibration curve is shown on the *right axis*. We focus here only on light collected outside of the constriction; no appreciable signal was measured inside the constriction. The *error bars* are reported as the standard error of the mean ($n$=5 different measurements). Note that the photon emission rate increases far downstream from the constriction. (Reproduced from [103] with permission.) (Copyright note: Wan, J., Ristenpart, W.D. & Stone, H.A. (2008) Dynamics of shear-induced ATP release from red blood cells. *Proc. Natl. Acad. Sci. U. S. A*, **105**, 16432-16437. Copyright (2008) National Academy of Sciences, USA)

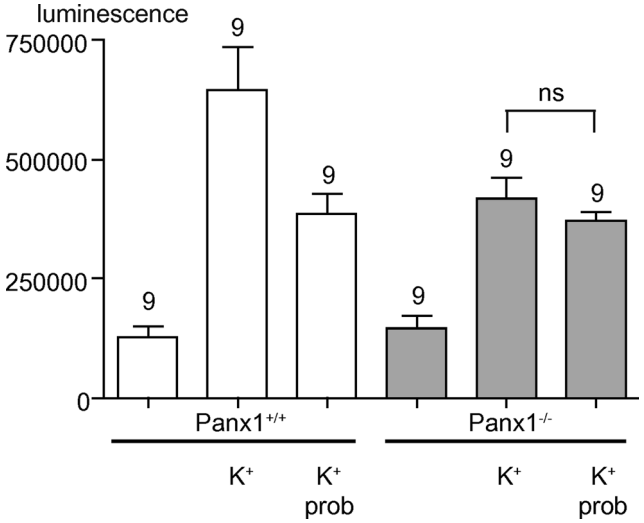


**Fig. 2** ATP release from pannexin-1 wildtype ($Panx1^{+/+}$) (*white bars*) and knockout ($Panx1^{-/-}$) (*grey bars*) erythrocytes. ATP release, as determined with a luciferase assay, is stimulated by hypotonic $K^+$ solution ($K^+$) more profoundly in $Panx1^{+/+}$ erythrocytes than in $Panx1^{-/-}$ erythrocytes. The Panx1 channel inhibitor probenecid (prob, 1 mM) attenuated ATP release in $Panx1^{+/+}$ cells but not significantly ($P>0.05$) in $Panx1^{-/-}$ cells. (Reproduced from [76] with permission from Elsevier.)

A number of factors affect erythrocyte ATP release. Nitric oxide (NO) was shown to inhibit the signal transduction pathway for ATP release from erythrocytes via its action on heteromeric G protein, $G_i$ [89]. Statins increase erythrocyte deformability and reduce low $O_2$-induced ATP release [90]. ATP was released in the presence of cell-free haemoglobin [91]. Fluoride causes ATP depletion and oxidative stress in rat erythrocytes in vitro [92]. Insulin inhibits low oxygen-induced ATP release from human erythrocytes [93, 94].

The ATP degradation product, ADP, inhibits ATP release by a negative feedback pathway mediated by $P2Y_{13}$ receptors on human red blood cells [95]. Caffeine enhances ATP release from erythrocytes, most likely due to its effect on levels of cAMP [96], while lactate, in the absence of changes in pH, interferes with ATP release [97]. High blood lactate is a dangerous metabolic consequence of several common diseases, including septic shock and malaria. It has been proposed that nitrite-induced vasodilation is due to nitrite enhancement of release of ATP from erythrocytes, which then acts as a vasodilator [98, 99].

Human limb muscle and skin blood flow increases significantly with elevations of temperature. Erythrocytes from rabbits release ATP and dilate skeletal muscle arterioles in the presence of reduced oxygen tension [100]. Erythrocyte ATP release is sensitive to physiological increases in temperature, possibly via activation of CFTR channels [101]. The authors suggest that this raises the possibility of treatment of patients with peripheral vascular disease, by using local heating to stimulate erythrocyte ATP release to increase flow and oxygen to limbs.

It has been suggested that the shape changes of erythrocytes related to intracellular ATP concentrations can be explained in terms of ATP-induced cytoskeletal changes involved in binding of actin to spectrin filaments [102]. In a more recent paper, data was presented to suggest a model wherein the retraction of the spectrin-actin cytoskeleton network triggers the mechanosensitive release of ATP, while a shear-dependent membrane viscosity controls the rate of release [103].

Treatment of erythrocytes with diamide, a compound that decreases erythrocyte deformity, inhibits low $O_2$ tension-induced ATP release [82, 104]. Hydroxyurea, a substance that affects erythrocyte deformability, stimulates the release of ATP from rabbit erythrocytes through an increase in calcium and NO production [105]. Reducing erythrocyte membrane cholesterol and simvastatin both increase cell deformability and therefore ATP release [106]. Hypoxia-induced ATP release from human erythrocytes is triggered through mechanisms involving haemoglobin [107]. Erythrocytes from older healthy humans fail to release ATP during haemoglobin deoxygenation [108]. Exchange proteins activated by cAMP inhibit ATP release via activation of PKC [109]. ATP release following complement receptor 1 ligation increased the mobility of the lipid fraction of erythrocyte membranes and had a stimulatory effect on phagocytosis of immune-adherent immune complexes [110].

It has been suggested that sensing of low blood $O_2$ content may involve ATP release from red blood cells, leading to stimulation of sensory aortic body neurons via P2X2/3 receptors [111]. ATP released from erythrocytes incubated with hydroxyurea resulted in increased endothelium-derived NO production [112].

## Pathology

An important role of ATP release from erythrocytes in vascular regulation has been suggested to have predictive value in disease processes. Infection with the malaria protozoan parasite, *Plasmodium falciparum*, induces osmolyte and anion channels in the host erythrocyte membranes involving ATP release and autocrine purinergic signalling [113]. Purinergic receptors are expressed in *P. falciparum*, where occupation by ATP triggers increase in $[Ca^{2+}]_i$, which is essential for the invasion of erythrocytes [114]. Hydrolysis of ATP with apyrase drastically reduced erythrocyte infection by the parasite. The effect of parasite infection on the kinetics of extracellular ATP accumulation was studied, using analysis of the rates of ATP release and extracellular hydrolysis at different stages of the infection cycle [115]. ATP depletion of erythrocytes stimulates the phenotype associated with pyruvate kinase deficiency and confers protection against *Plasmodium* in vitro [116]. Extracellular ATP did not induce osmolyte permeability in non-infected human erythrocytes but induced osmolyte

permeability in malaria-infected erythrocytes [117]. They showed further that induction of osmolyte permeability in *Plasmodium*-infected erythrocytes involved autocrine purinoceptor signalling. In mouse erythrocytes harbouring the malaria parasite, *P. yoelii*, nucleoside transport had abnormally low sensitivity to nitrobenzylthioinosine [118]. In a more recent study, it was suggested that ATP released by the rupture of erythrocytes during the blood stage of *Paramecium chabaudi* malaria induced an increase in the expression P2X7 receptors in $CD4^+$ T cells [119]. A review has been published concerned with malaria-infected erythrocytes and purinergic signalling [120].

Leukotoxin is a virulence factor secreted by some bacteria, which can cause localised aggressive periodontitis. Leukotoxin-mediated haemolysis is significantly potentiated by ATP release and P2X receptor activation of human erythrocytes [121] (Fig. 3). The bacterium *Escherichia coli* can produce virulence factors such as the exotoxin α-haemolysin (HlyA). HlyA is a protein that induces haemolysis by creating large pores in erythrocyte membranes, increasing permeability thereby producing cell swelling, which finally ruptures the erythrocyte. A study shows that this pore formation triggers purinergic receptor activation to mediate the full haemolytic action [122]. They showed that antagonists to P2X1 and P2X7 receptors and apyrase inhibited HlyA-induced lysis of erythrocytes and concluded that selective P2X receptor antagonists may ameliorate symptoms during sepsis with haemolytic bacteria. *E. coli* HlyA evoked ATP release and P2 receptor-mediated $Ca^{2+}$ influx in human erythrocytes through the toxin pore [123, 124]. Another recent study proposed that erythrocytes damaged by HlyA insertion are effectively cleared from the blood stream, reducing the risk of intravascular haemolysis [125]. It was reported that, similar to haemolysis produced by HlyA, leukotoxin and α-toxin complement-induced haemolysis is amplified through ATP release and activation of P2 receptors [126]. Adenosine deaminase activity was altered in erythrocytes of dogs infected with *Rangelia vitalii* as well as the serum concentration of adenosine [127]. It was suggested that these changes may contribute to the pathogenesis of anaemia and immune response in infected dogs.

Isoproterenol substantially altered cardiovascular haemodynamics and induced breakdown of ATP in erythrocytes to ADP and AMP, particularly in dying rats [128]. It was suggested that the relative concentrations of ATP, ADP and AMP in red blood cells may be used as a predictive biomarker for cardiovascular mortality. There is impaired release of ATP from red blood cells of humans with primary pulmonary hypertension [129]. Prostacyclin analogs and phosphodiesterase inhibitors had synergistic effects on ATP release from human erythrocytes, and it was suggested that that could influence the development of new therapeutic approaches for the treatment of pulmonary arterial hypertension [130].

It was proposed that reduced ATP release from erythrocytes contributes to vascular disease in type 2 diabetes [131, 132]. In type 2 diabetes, erythrocytes are under high oxidative stress and considered to be less deformable leading to lowered levels of deformation-induced ATP release [133]. The selective phosphodiesterase 3 inhibitor, cilostazol, facilitates $PO_2$-induced ATP release from erythrocytes of humans with type 2 diabetes [134]. C-peptide and insulin were shown to have synergistic effects on low $O_2$-induced ATP release from human erythrocytes, suggesting that administration of a combination of C-peptide and insulin could help in the prevention and treatment of peripheral vascular disease associated with diabetes [135, 136].

It has been suggested that adenosine is a potentially important therapeutic target for the treatment and prevention of sickle cell disease, a debilitating haemolytic genetic disorder where an abnormal type of haemoglobin precipitates in erythrocytes when blood is deprived of oxygen forming crystals that distort the cell (sickling) resulting in anaemia and jaundice [137–139]. However,

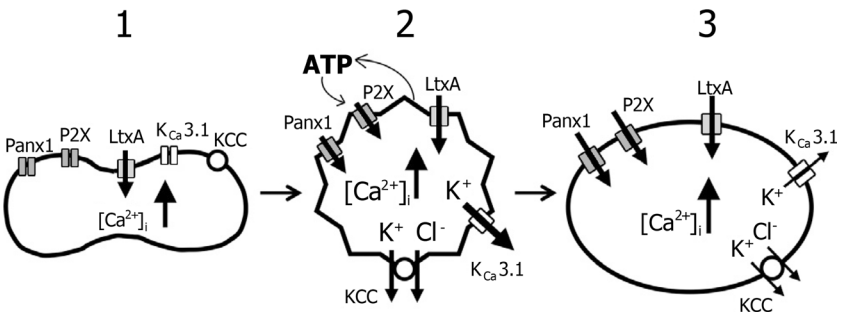


**Fig. 3** Model for leukotoxin from *Aggregatibacter* (LtxA)-induced haemolysis. *1* Interaction between LtxA and the erythrocyte membrane leads to an influx of ions and increase in $[Ca^{2+}]_i$. *2* The increase in $[Ca^{2+}]_i$ stimulates a $Ca^{2+}$-activated $K^+$ efflux mediated by the $Ca^{2+}$-activated $K^+$ channel, KCa3.1. $K^+$–$Cl^-$ co-transporters (KCCs) contribute to the $K^+$ efflux. Initially, the $K^+$ efflux exceeds the influx of ions leading to osmotically obliged $H_2O$ efflux and volume reduction. ATP is released from the cell through a yet unknown pathway and activates P2X receptors on the erythrocyte membrane, which is required for the full haemolytic effect of LtxA. In addition, activation of pannexin channels is also necessary for LtxA-induced haemolysis. *3* Later, the influx of ions exceeds the efflux of $K^+$ resulting in osmotically obliged $H_2O$ influx and cell swelling. Finally, the erythrocyte lyses. (Reproduced from [121] with permission from John Wiley and Sons.)

complicating this approach, adenosine signalling also induces haemoglobin S polymerization, promoting sickling, vasoocclusion, haemolysis and organ damage [140, 141] (Fig. 4). Amyloid β peptide inhibits ATP release from deoxygenated erythrocytes by activating red cell caspase 3, suggesting a pathophysiologic role for vascular amyloid peptide in Alzheimer's disease [142].

## Platelets

### Introduction

Platelets express $P2Y_1$, $P2Y_{12}$ and P2X1 receptor subtypes involved in platelet aggregation (see Fig. 5).

Review articles have been published on various aspects of purinergic signalling in platelets, including

- Platelet adenosine receptors [143–145]
- ATP and platelet dense bodies [146–148]
- Platelet P2X1 receptors [149–151]
- Platelets, P2Y receptors and pharmacology [152–167]
- Purinoceptor-evoked calcium signalling in human platelets [168]
- Ectonucleotidases and platelets [169, 170]
- Thrombosis—clopidogrel/ticagrelor [160, 171–179]
- Platelets and inflammation [180, 181]
- P2Y receptor polymorphisms and disease [182]

### $P2Y_1$ and $P2Y_{12}$ receptors

In 1956, it was shown that platelets contain very high concentrations of ATP [183] and that extracellular ATP is rapidly broken down to ADP [184]. It was shown by Hellem and coworkers that a factor derived from red blood cells was responsible for the adhesiveness of platelets to glass beads [185]. This factor was identified as ADP, and the ability of ADP to produce platelet aggregation was recognised early in two Nature papers ([186, 187] and see [188, 189]). Later, the possible mechanisms underlying ADP-induced platelet aggregation were explored [190–193]. ATP itself did not induce platelet aggregation but inhibited aggregation produced by ADP and platelet shape change [194, 195].

ADP was shown to be a potent inhibitor of human platelet plasma membrane adenylate cyclase [196], in retrospect an early indication that ADP was acting via G protein-coupled receptors, later identified as $P2Y_1$ and $P2Y_{12}$ receptors. ADP induced binding of von Willebrand factor to human platelets [197]. ATP analogues produced greater inhibition of aggregation induced by ADP than did AMP analogues [198], which in retrospect indicated inhibition via P2, rather than P1 receptors. ATP, UTP, guanosine-5′-triphosphate (GTP) and cytidine triphosphate inhibited platelet aggregation induced by collagen and epinephrine by acting as antagonists of the $P2Y_{12}$ receptor [199, 200]. ADP produces an increase in $[Ca^{2+}]_i$ in platelets [201]. Potentiation of ADP-induced platelet aggregation in platelet-rich plasma by 5-hydroxytryptamine (5-HT) and adrenaline was shown [202]. Diadenosine tetraphosphate ($Ap_4A$) had anti-platelet aggregation activity [203]. ADP induced platelet α-granule release [204].

The non-selective P2 receptor antagonist, suramin, inhibited platelet aggregation induced by ADP [205]. It was claimed in 1993 that ADP-induced increase in $[Ca^{2+}]_i$ in platelets was mediated by the P2T receptor (later identified as the $P2Y_{12}$ receptor) [206, 207]. ADP-induced platelet aggregation was inhibited by the P2T receptor antagonists FPL 66096 [208] and FPL 67085 (also known as ARC 67085), both ATP variants [209]. The human platelet ADP receptor activates $G_{i2}$ proteins [210], another indication that $P2Y_{12}$ receptors were involved. A radiolabelled selective antagonist, [$^3$H]PSB-0413, was shown to be a tool for radioligand binding studies aimed at quantifying $P2Y_{12}$ receptors to identify patients with $P2Y_{12}$ receptor deficiencies and to quantify the effect of $P2Y_{12}$ targeting drugs [211].

ADP inhibited 5-HT uptake into human platelets [212]. The aggregation behaviour of post-mortem platelets has been claimed to be a tool for estimating time of death [213]. The $P2Y_1$ receptor was also shown to be expressed by platelets and megakaryocyte cell lines; it was antagonised by ATP [214, 215] and coupled to $G_q$ [216]. Platelet shape change was identified as the main role of $P2Y_1$ receptors [217, 218], although it also contributes to platelet aggregation [219]. Evidence for three P2 receptors on platelets was presented [220, 221]. The cloning of P2X1-specific complementary DNA (cDNA) from human platelets was achieved in 1998 [222]. BzATP was claimed to be an antagonist of rat and human $P2Y_1$ receptors and of platelet aggregation [223]. It was confirmed that ADP can induce aggregation of human platelets via both $P2Y_1$ and P2T ($P2Y_{12}$) receptors [224]. The P2T receptor was identified as a $P2Y_{12}$ receptor in 2001 (see [225–227]). Combinations of antagonists of $P2Y_1$ and $P2Y_{12}$ receptors were effective inhibitors of direct shear-induced platelet aggregation [228].

The chemokines, macrophage-derived chemokine, thymus activation-regulated chemokine and stromal cell-derived factor one, which may be produced during inflammatory responses, coupled with low levels of ADP or thrombin to serve as stimuli for activating platelet formation [229]. Evidence was presented to show that collagen required not only the thromboxane $A_2$ ($TxA_2$) receptor Tpα but also $P2Y_1$ receptors, to induce platelet shape change [230]. Stimulation of the $P2Y_{12}$ receptor is involved in platelet activation initiated by the binding of von Willebrand factor to platelet receptor protein GP Ibα induced by a high shear rate [231]. Quantitative RT-PCR studies showed that the order of expression of P2Y receptor mRNA was $P2Y_{12} \gg P2X1 > P2Y_1$ [232]. Depending

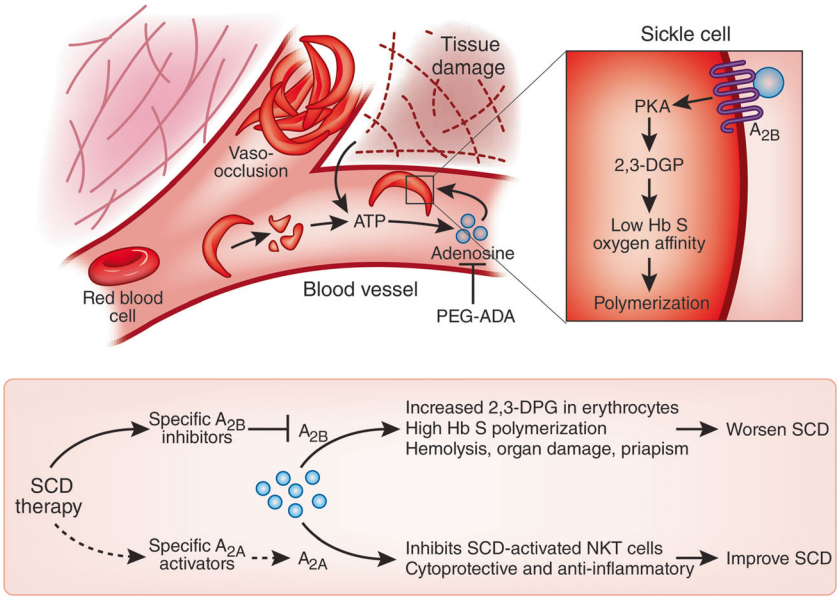


**Fig. 4** Adenosine worsens sickle cell disease (SCD) by increasing 2,3-diphosphoglycerate (2,3-DPG) in red blood cells through the $A_{2B}$ receptor. Increased amounts of ATP in circulation owing to chronic sickle red blood cell hemolysis and tissue damage from vasoocclusion are rapidly converted to adenosine. Activation of the $A_{2A}$ receptor on natural killer T (NKT) cells suppresses the innate immune response and limits inflammation and cellular injury during ischemia and reperfusion injury. Top, in contrast, Zhang et al. [140] show that activation of the $A_{2B}$ receptor by adenosine on erythrocytes increases 2,3-DPG levels through cAMP-dependent protein kinase A (PKA) activation, which reduces haemoglobin S (Hb S) oxygen affinity and promotes its polymerization and red blood cell sickling. Bottom, 'crossroads' of adenosine signaling determine positive or negative effects. The adenosine antagonist polyethylene glycol-modified adenosine deaminase (PEG-ADA) may be used to block adenosine signalling as a therapy for SCD; however, the development of specific agonists and inhibitors of these receptors may allow for selective inhibition of red blood cell $A_{2B}$-dependent 2,3-DPG production and activation of $A_{2A}$-dependent immune modulation to ease the disease more effectively. (Reproduced from [141] with permission from The Nature Publishing Group.)

on the experimental conditions, signalling from both fibrinogen and $P2Y_1$ and $P2Y_{12}$ receptors are necessary for $PLA_2$ activation, resulting in arachidonic acid liberation and $TxA_2$ generation [233]. Using a high-resolution channelyzer, it was concluded that $P2Y_{12}$, as well as $P2Y_1$ receptors, play a role in controlling shape change in human platelets [234], although this is controversial. Evidence has been presented to support the view that for thrombin-induced human platelet activation, the $P2Y_{12}$ receptor is the drug target compared to the $P2Y_1$ receptor [235]. However, a synergistic interaction was reported between

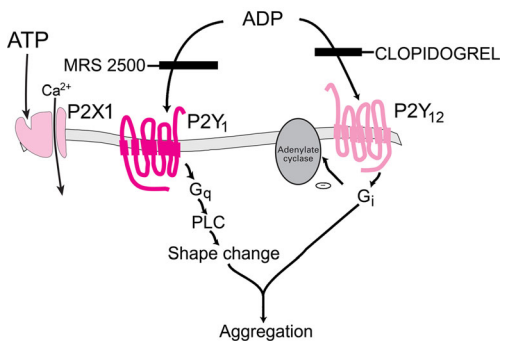


**Fig. 5** Three P2 receptor subtypes, P2X1, $P2Y_1$ and $P2Y_{12}$, are involved in ADP-induced platelet activation. Clopidogrel is a $P2Y_{12}$ receptor blocker that inhibits platelet aggregation and is in highly successful use for the treatment of thrombosis and stroke. A $P2Y_1$ receptor antagonist, MRS 2500, inhibits shape change. (Modified from [403] with permission from Elsevier.)

antagonists of $P2Y_1$- and $P2Y_{12}$-mediated inhibition of ADP- and thrombin-induced human platelet activation [236].

In healthy subjects, it has been claimed that ADP-induced platelet aggregation is associated with a haplotype polymorphism of the $P2Y_{12}$ receptor gene [237, 238]. Homozygosity for the $P2Y_1$ 1622G allele is associated with increased receptor signalling and platelet aggregation [239]. However, this $P2Y_{12}$ receptor gene H2 haplotype was shown not to be associated with increased ADP-induced platelet aggregation in a separate study [240]. Furthermore, this genetic haplotype was not associated with the risk of myocardial infarction in a large study with more than 3000 patients [241]. Mutational analysis of the residues important for ligand interaction with the human $P2Y_{12}$ receptor is available [242]. Interestingly, all cells contain an endogenous $P2Y_{12}$ antagonist, farnesyl pyrophosphate, which acts as a traditional competitive antagonist to ADP [243].

The $P2Y_1$ receptor antagonist, MRS2500, was shown to be the most potent inhibitor of $P2Y_1$ receptor-mediated platelet shape change and aggregation [244]. There is a complex signalling interaction between $P2Y_1$ and $P2Y_{12}$ receptors; $P2Y_{12}$ receptors positively regulate $P2Y_1$ action, while $P2Y_1$ receptors negatively regulate the action of $P2Y_{12}$ receptors [245].

ADP caused desensitization of the $P2Y_1$ receptor-driven calcium signal, but $P2Y_{12}$ receptor-mediated inhibition of cAMP formation was not affected [246]. It was suggested that the absence of desensitization of the $P2Y_{12}$ receptor-mediated

platelet response could represent a mechanism to preserve the haemostatic properties of unresponsive platelets. In another paper, it was claimed that both $P2Y_1$- and $P2Y_{12}$-mediated platelet responses desensitise rapidly, but by different kinase-dependent mechanisms [247]. The desensitization of the P2Y receptor on platelets requires receptor internalization, and it was claimed that the GTP-binding protein ADP ribosylation factor 6 is required for P2Y receptor internalization [248].

ADP was shown to play a key role in irreversible platelet aggregation through the activation of phosphoinositide 3-kinase [249]. Platelet integrin $\alpha_{IIb}\beta_3$ plays a crucial role in platelet aggregation, and it was claimed that phosphatidylinositol 3-kinase is essential for ADP-stimulated $\alpha_{IIb}\beta_3$-mediated platelet activation and calcium oscillations [250]. Continuous interaction between ADP and $P2Y_{12}$ receptors is critical for the maintenance of $\alpha_{IIb}\beta_3$ activation [251–253]. Evidence was presented that $P2Y_{12}$ receptors potentiate platelet shape change induced by $P2Y_1$ receptor activation by a Rho kinase-dependent mechanism [254]. The residual arachidonic acid-induced platelet activation in aspirin-treated patients is mediated in part by ADP-induced platelet activation [255]. It was suggested that the interaction of calmodulin with the $P2Y_1$ receptor C-terminal tail may regulate $P2Y_1$-dependent platelet aggregation [256].

$P2Y_{14}$ receptor mRNA and protein were shown to be expressed by platelets, although the functional role of this receptor is not yet known [257, 258]. Involvement of basic amino acid residues in transmembrane regions 6 and 7 in agonist and antagonist recognition of the human platelet $P2Y_{12}$ receptor has been reported [259]. A novel $P2Y_1$ receptor radioligand has been synthesised, which is valuable for examining the expression of $P2Y_1$ receptors on human and mouse platelets [260]. Resolvin E1, generated during acute inflammation, regulates ADP activation of human platelets [261]. It was suggested that cAMP regulates ADP-stimulated platelet activation due to inhibition of heat shock protein (HSP) 27 phosphorylation via p38 mitogen-activated protein MAP kinase [262]. 5-HT reuptake inhibitors reduce $P2Y_{12}$ receptor-mediated amplification of platelet aggregation [263]. Inhibition of $P2Y_{12}$ receptors potentiated the anti-platelet effect of prostacyclin [264]. Circulating platelets are exposed to NO released from endothelial cells, and NO reduces platelet aggregation and thrombus formation. Blockade of $P2Y_{12}$ receptors significantly increased the platelet inhibitory actions of NO [265]. Platelet $P2Y_1$ and $P2Y_{12}$ and arachidonic acid receptor inhibition is a prominent early feature of coagulopathy in traumatic brain injury [266].

## P2X1 receptors

ATP inhibited both collagen- and a thromboxane mimetic (U46619)-induced platelet aggregations via a P2X-like receptor [267], in retrospect by P2X1 receptors, since it was blocked by α,β-methylene ATP (α,β-meATP). It was suggested that human platelets express a P2X1 receptor, which mediates rapid $Ca^{2+}$ entry, in contrast to the P2Y receptors which evoke release of calcium from intracellular stores [268]. Clopidogrel did not affect the binding of α,β-meATP to platelet P2X1 receptors [269]. The P2X1 receptor cDNA and protein was identified on human platelets, but not leukocytes [270–272]. It was reported that P2X1 receptors did not play a significant role in ADP-induced platelet shape change and aggregation [273]. ATP, but not ADP, is an agonist at P2X1 receptors on human platelets [274]. However, a later paper claimed that novel structurally altered P2X1 receptors on platelets and megakaryocytic cells were preferentially activated by ADP [275].

The $P2Y_1$ receptor antagonist, adenosine-2′,5′-diphosphate, non-selectively antagonised the platelet P2X1 ion channel [276]. ATP, acting on P2X1 receptors, was claimed to contribute to platelet activation in addition to the earlier suggestion that ATP activation of P2X1 receptors had an inhibitory action at metabotropic platelet receptors [277]. During collagen-initiated platelet activation, the early secretion of ATP resulted in P2X1-mediated stimulation, which played a role as a positive regulator of further platelet responses [278]. From studies of P2X1 receptor knockout (KO) mice, it was shown that accumulation of P2X1 KO platelets on a collagen-coated surface was greatly reduced compared to wild-type (WT) platelets, suggesting a role of P2X1 receptors in platelet interaction with collagen [279].

The role of P2X1 receptors expressed by platelets has been difficult to assess, due to its rapid desensitization. However, P2X1 and $P2Y_1$ receptor synergy was claimed in both murine megakaryocytes and human platelets [280]. From a study of P2X1 KO and WT mouse platelets treated with apyrase to prevent desensitization, it was shown that collagen-induced aggregation and secretion of P2X1-deficient platelets was decreased, as well as adhesion and thrombus growth on a collagen-coated surface [281]. The mortality of P2X1 KO mice in a model of systemic thromboembolism was reduced, and it was concluded that P2X1 receptors contribute to the formation of platelet thrombi, particularly in arteries in which shear forces are high [281]. In contrast, over-expression of P2X1 receptors in transgenic mice led to enhancement of platelet dense granule secretion and aggregation evoked by collagen or the $TxA_2$ mimetic U46619; it also enhanced platelet responses under shear stress, but the responses to ADP or thrombin were normal [282]. The authors concluded that over-expression of P2X1 receptors on platelets generated a novel prothrombotic phenotype. It was reported that ADP did not contribute to the rapid ionotropic P2X1 receptor-mediated response in platelets but suggested that ATP plays a role during haemostasis and thrombosis [283]. Pharmacological inhibition of the P2X1 receptor using NF449 also resulted in thrombosis inhibition in vivo [284].

In an authoritative review of the emerging roles for P2X1 receptors in platelet activation, it was concluded that P2X1 receptors can mediate transient shape change and granule release, important early events in platelet activation, and that ATP acting on P2X1 receptors can synergise with ADP acting on platelet P2Y receptors to potentiate functional events, particularly under conditions of shear stress [149]. A detailed description of the intracellular pathways involved in P2X receptor stimulation is shown in Fig. 6. Subsequently, it was shown that ATP augments von Willebrand factor-dependent shear-induced platelet aggregation through $Ca^{2+}$-calmodulin and myosin light chain kinase activation [285]. A major role for P2X1 receptors in early collagen-evoked intracellular $Ca^{2+}$ responses of human platelets was reported, contributing to arterial thrombosis [286]. NF864, claimed to be the most potent platelet P2X1 receptor antagonist, blocked α,β-meATP-induced $[Ca^{2+}]_i$ increases and shape change [287].

Evidence was presented to suggest that lipid rafts play a significant role in the regulation of P2X1, but not $P2Y_1$ receptors in human platelets [288]. Another study concluded that ATP should be considered alongside ADP and $TxA_2$ as a significant secondary platelet agonist [289]. Activation of P2X1 receptors with ATP had a dual effect, causing a significant concentration-dependent increase in platelet NO production and causing aggregation and adhesion, although platelet aggregation was initially decreased [290]. $Ap_4A$, a constituent of platelet dense granules, is an antagonist of platelet $P2Y_1$ receptors where it inhibits the effects of ADP and an agonist of platelet P2X1 and $P2Y_{12}$ receptors [291]. Margatoxin, a voltage-dependent $K^+$ channel inhibitor, reduced the P2X1- and $TxA_2$ receptor-evoked $[Ca^{2+}]_i$ increases [292]. P2X1 receptors are constitutively regulated by HSP90, and inhibitors of HSP90 reduce trafficking of ATP-gated P2X1 receptors and human platelet responsiveness [293].

## P1 (adenosine) receptors

Adenosine was shown to be a competitive inhibitor of platelet aggregation by ADP [294]. A receptor for adenosine on platelets that mediated inhibition of platelet function via activation of adenylate cyclase was recognised early [295–297]. Aggregation of human platelets induced by ADP was inhibited by 2-azidoadenosine, a photolysable analogue of adenosine, and deamination of adenosine by adenosine deaminase was inhibited by 2-azidoadenosine [298]. Adenosine is taken up and deaminated by platelets [299]. Dipyridamole inhibited adenosine uptake into platelets [300] and potentiated the anti-platelet action of adenosine [301, 302].

5′-*N*-ethylcarboxamidoadenosine was shown to be a potent inhibitor of human platelet aggregation [303]. A xanthine amine congener was introduced as a radioligand for $A_2$ receptors of human platelets [304]. Prostacyclin analogues diminished $A_2$ receptor responsiveness of platelets [305]. The effects of adenosine derivatives confirmed that $A_2$ receptors mediate inhibition of human and rabbit platelet aggregation [306], later identified as $A_{2A}$ receptors [307–309]. Synergistic inhibition of thrombin-induced platelet aggregation by an NO donor and adenosine was reported [310]. It was claimed that there was $A_2$ receptor-mediated inhibition of platelet aggregation in humans, but not in canine models [311].

Treatment of mouse and human blood with 5′-nucleotidase (which led to increased extracellular adenosine) inhibited platelet aggregation [312]. Gene expression profiling led to

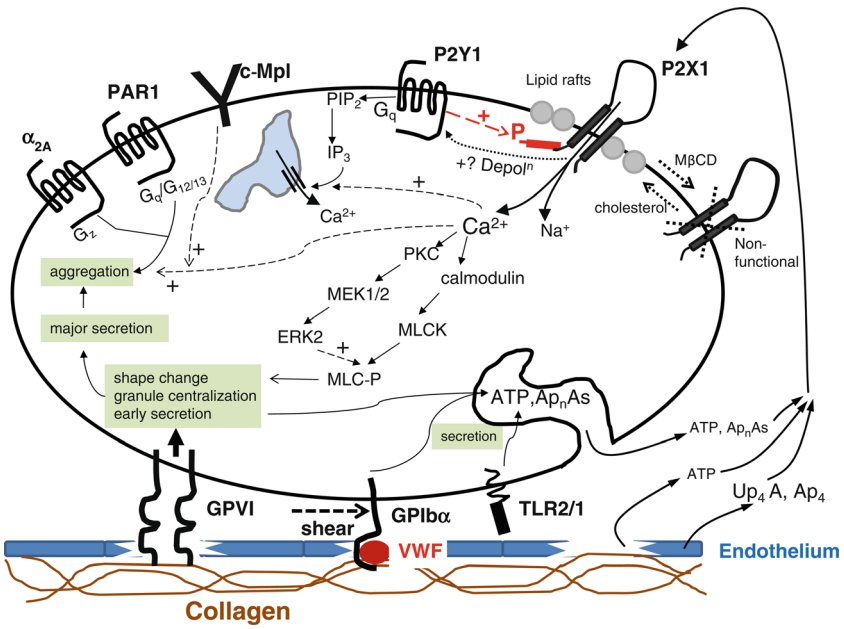


**Fig. 6** P2X1 receptor signalling and regulation in the platelet. Summary of the pathways whereby P2X1 receptors have been proposed to couple to functional responses in platelets, together with the mechanisms that regulate these ion channels. (Reproduced from [150] with permission from Springer.)

the identification of functional $A_{2B}$ receptors on human platelets [313]. It was later shown that $A_{2B}$ receptors on mouse platelets were upregulated under stress in vivo and played a significant role in regulating ADP receptor expression [314]. ADP inhibited platelet aggregation in the presence of $P2Y_{12}$ receptor antagonists due to its conversion to adenosine [315]. It has been claimed recently that adenosine may be the major active ingredient for anti-platelet activity of black soybean, used for the treatment of cardiovascular diseases [316].

In summary, platelets express pro-aggregatory $P2Y_1$ and $P2Y_{12}$ receptors, anti-aggregatory $A_{2A}$ and $A_{2B}$ receptors, as well as P2X1 receptors, which appear to have synergistic actions with the P2Y receptors.

### ATP release

Upon stimulation, platelets secrete ATP and ADP, which evoke platelet aggregation (see [159]). ATP release from activated platelets was shown using cell surface-attached firefly luciferase [317]. Later, lumi-aggregometers were used as an ATP release assay for the assessment of platelet function disorders [318–320]. An HPLC assay has also been used to determine ATP and ADP secretion [321]. The vesicular nucleotide transporter, VNUT, has been claimed to be responsible for vesicular storage and release of nucleotides from platelets [322, 323] (Fig. 7).

### Ectonucleotidases

It was suggested that ADPase may act as a platelet aggregation inhibitor in the placental and foetal circulation [324]. The ecto-ATPase present on human platelets, responsible for breaking down ATP to ADP, was examined, and a direct role of ecto-ATPase activity on platelet aggregation was shown to be relatively small [325]. ATP diphosphohydrolase (apyrase) was later identified on rat platelets to hydrolyse ATP to ADP [326–328]. ATPDase/CD39 expression was described in human platelets and endothelial cells [329] to modulate platelet activation and thrombus formation [330]. Endothelial cells contribute to control of platelet reactivity via endothelial ectoNTPDase-1/CD39 [331, 332] by rapidly metabolizing ADP released from platelets, thereby preventing further platelet activation or recruitment. Acetylsalicylic acid inhibited ATP diphosphohydrolase activity by platelets from adult rats [333]. Extracellular hydrolysis of ATP by intact rat blood platelets is achieved by NTPDase 3 and 5′-nucleotidase, resulting in the production of adenosine [334]. Ebselen, which exhibits anti-oxidant, anti-inflammatory, anti-atherosclerotic and cytoprotective properties, inhibited the extracellular hydrolysis of ATP [334]. The possibility of inhibiting platelet P2X1 receptors or elevating CD39/NTPDase1 activity as novel therapeutic approaches to reduce platelet reactivity and recruitment of platelets at prothrombotic locations has been discussed [335]. Reduced degradation of ADP by ectonucleotidases contributes to the amplification of ADP-evoked aggregation [336].

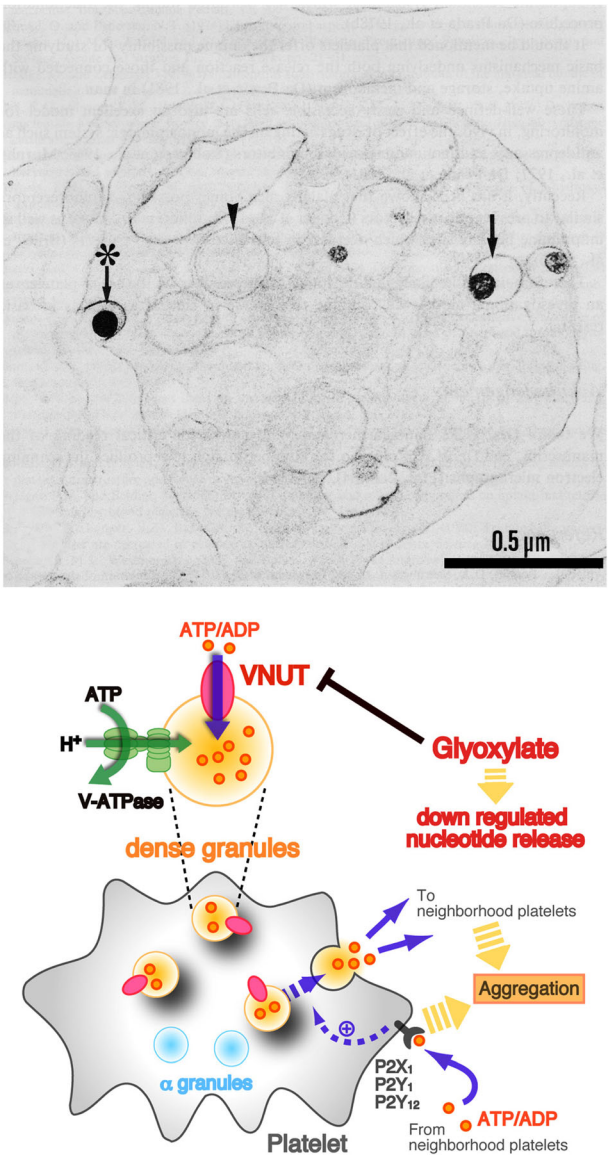


**Fig. 7** **a** Transmission electron micrograph of an untreated rabbit platelet after the uranaffin reaction. Four 5-HT organelles (→) are selectively stained. One of these (*asterisk*) has apparently fused with the platelet membrane to release its contents (5-HT and ATP), possibly by the process of exocytosis. Note the difference in staining between the membrane of this organelle and the platelet plasma membrane. α-Granules (*arrowhead*). (Reproduced from [322] with permission; Prada, M., Lorez, H.P. & Richards, J.G. (1982) Platelet granules. In Poisner, A. M. & Trifaro, J. M. (eds), *The Secretory Granule*. Elsevier Biomedical, Amsterdam, pp. 279-316, Copyright Elsevier.) **b** Schematic diagram of nucleotide storage and release in platelets. Vesicular nucleotide transporter (VNUT) is associated with dense granules and transports nucleotides into granule using $\Delta\psi$ that is established by V-ATPase. VNUT is thus involved in nucleotide release and that is inhibited by glyoxylate, a VNUT inhibitor. (Reproduced from [323] with permission from Wiley Periodicals, Inc.)

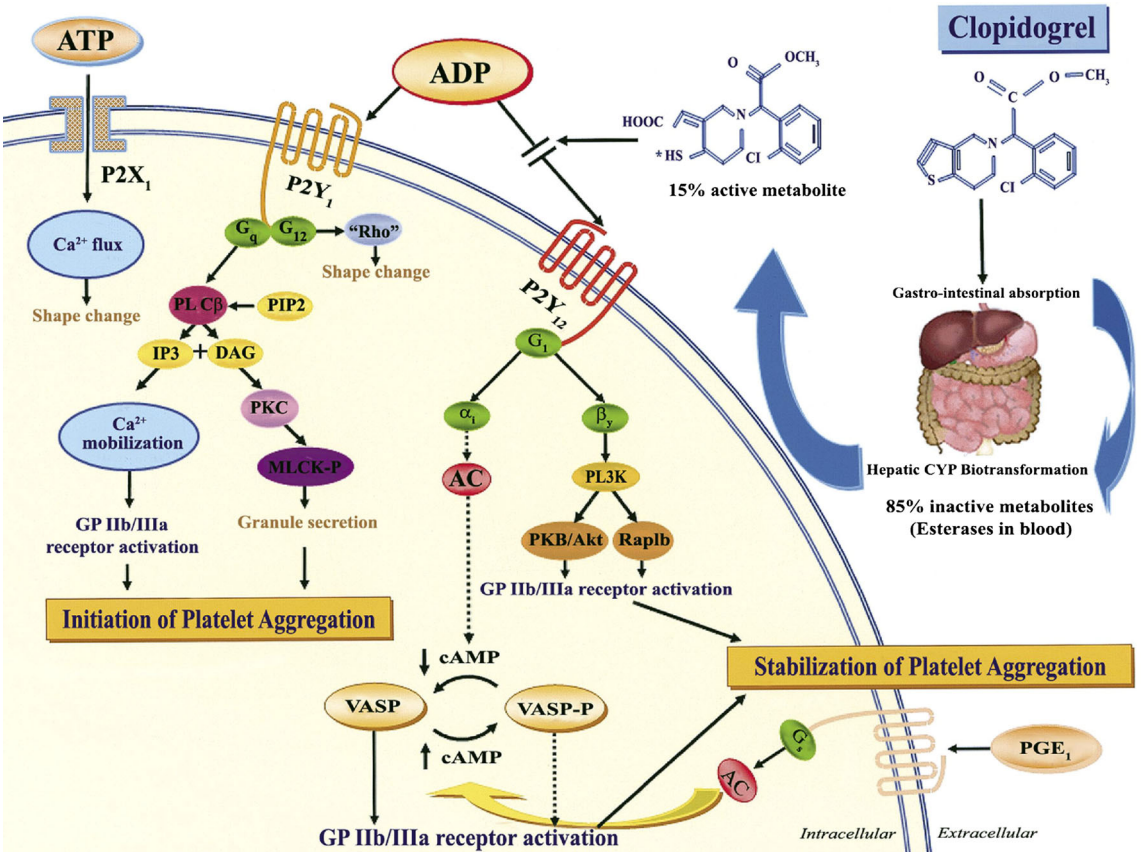


**Fig. 8** Purinergic receptors and mechanism of action of clopidogrel. Clopidogrel is a pro-drug of which approximately 85 % is hydrolysed by esterases in the blood to inactive metabolites, and only 15 % is metabolised by the cytochrome P450 (CYP) system in the liver into an active metabolite. The active metabolite irreversibly inhibits the adenosine diphosphate (ADP) $P2Y_{12}$ receptor. The $P2X_1$ receptor, which uses adenosine triphosphate (ATP) as an agonist, is involved in platelet shape change through extracellular calcium influx and helps to amplify platelet responses mediated by other agonists. Activation of the $P2Y_1$ receptor leads to alteration in shape and initiates a weak and transient phase of platelet aggregation. The binding of ADP to the $G_q$-coupled $P2Y_1$ receptor activates phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol bisphosphate (PIP2). Diacylglycerol activates protein kinase C (PKC) leading to phosphorylation of myosin light chain kinase (MLCK-P), and IP3 leads to mobilization of intracellular calcium. The $P2Y_1$ receptor is coupled to another G-protein, $G_{12}$, which activates the "Rho" protein and leads to the change in platelet shape. The binding of ADP to the $G_i$-coupled $P2Y_{12}$ receptor liberates the $G_i$ protein subunits $\alpha_i$ and $b_\gamma$, resulting in stabilization of platelet aggregation. The $\alpha_1$ subunit inhibits adenylyl cyclase (AC) and, thus, reduces cyclic adenosine monophosphate (cAMP) levels, which diminishes cAMP-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP-P). The status of VASP-P modulates glycoprotein (GP) IIb/IIIa receptor activation. The subunit $\beta_\gamma$ activates the phosphatidylinositol 3-kinase (PI3K), which leads to GP IIb/IIIa receptor activation through activation of a serine-threonine protein kinase B (PKB/Akt) and of Rap1b GTP binding proteins. Prostaglandin $E_1$ ($PGE_1$) activates AC, which increases cAMP levels and status of VASP-P. *Solid arrows* indicate activation; *dotted arrows* indicate inhibition. (Reproduced from [404] with permission from Elsevier.)

After ovariectomy, there was a decrease in the ecto-hydrolysis of ATP by platelet NTPDase and 5′-nucleotidase, indicating that hormonal deprivation affects platelet aggregation [337]. Distinct roles for PKC isoforms have been described in the regulation of platelet P2Y receptor function and trafficking [338]. Plasma ectonucleotidases prevent desensitization of purinergic receptors on platelets [339]. Enhanced NTPDase and 5′-nucleotidase activities in platelets in human pregnancy suggests that these enzymes are involved in thromboregulation in pregnancy [340]. Intravascular ADP augments platelet activity during strenuous exercise, and these prothrombotic responses are counteracted by concurrent release of soluble nucleotide-inactivating enzymes [341].

## Thrombosis

Antagonists to P2T ($P2Y_{12}$) receptors were reported for use against ADP-induced arterial thrombosis [154]. Non-peptide glycoprotein inhibitors were reported to show anti-thrombotic efficacy against ADP-induced platelet aggregation [342]. An analogue of ATP, AR-C67085MX, was identified as a very potent antagonist at P2T platelet receptors [343]. Defective platelet aggregation and increased resistance to thrombosis were reported in $P2Y_1$ receptor KO mice [344].

Percutaneous coronary interventions with metal stents initially had serious problems causing life-threatening stent thrombosis. It was not until the addition of a $P2Y_{12}$ receptor

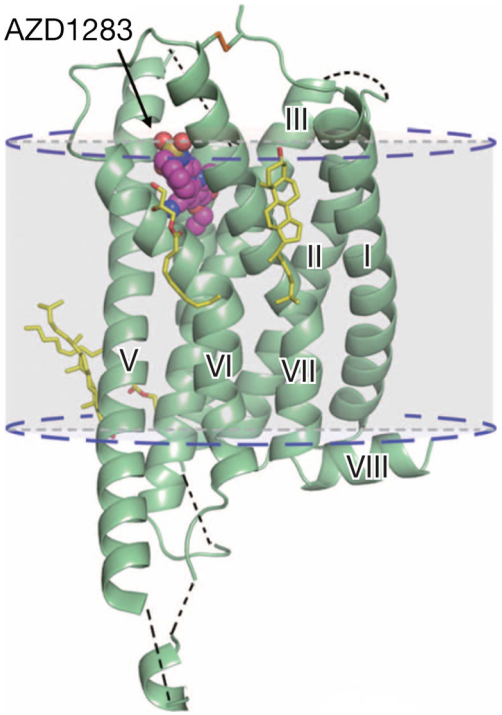


**Fig. 9** Cartoon representation of the $P2Y_{12}$ receptor–AZD1283 complex structure. The $P2Y_{12}$ receptor is *coloured green* and AZD1283 is shown as *magenta spheres*. Cholesterol and lipids have *yellow carbons*. The disulphide bridge is shown as *lime sticks*. Missing loops and membrane boundaries are indicated as *black* and *blue dashed lines*, respectively. (Reproduced from [377] with permission from The Nature Publishing Group.)

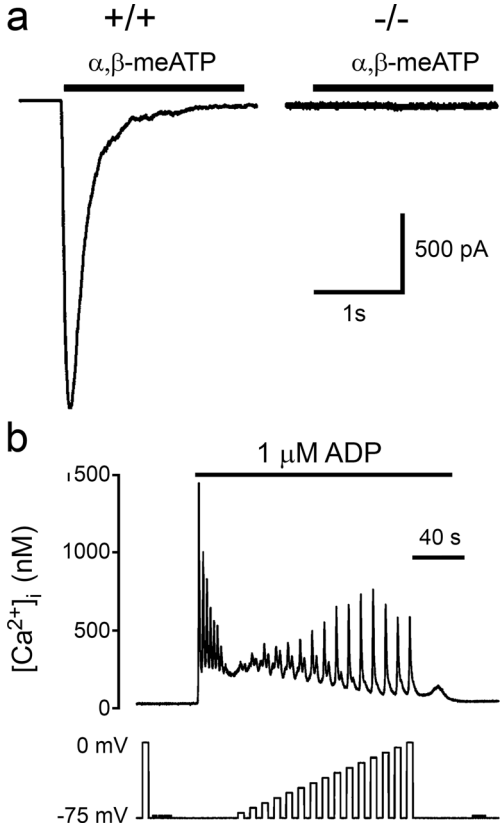


**Fig. 10** **a** P2X receptor-mediated inward currents are absent in P2X1 receptor-deficient mice megakaryocytes. α,β-Methylene ATP (α,β-meATP; 10 μM) evoked rapid transient inward currents in wild-type (+/+) megakaryocytes; these were absent in megakaryocytes from P2X1 receptor-deficient (−/−) mice. *Bar* indicates period of drug application. (Reproduced from [280] with permission from John Wiley and Sons.) **b** Effect of pulse amplitude on the depolarization-evoked $[Ca^{2+}]_i$ increase during stimulation of P2Y receptors. $[Ca^{2+}]_i$ responses of rat megakaryocytes to ADP (1 μM, *horizontal bar*) and step depolarizations from a holding potential of −75 mV. The effect of depolarization during ADP application was assessed after the agonist-evoked increase had settled to a raised plateau level. Effect of increasing the amplitude of the depolarizing step in 5 mV increments up to 75 mV. (Reproduced from [393] with permission from John Wiley and Sons.)

antagonist (first ticlopidine, later clopidogrel) to aspirin that stent thrombosis could be prevented. A randomised, blinded, clinical trial of clopidogrel, a thienopyridine like the later drug prasugrel, versus aspirin showed improved protection against thrombosis, ischaemic stroke and myocardial infarction [345]. The CURE study demonstrated that addition of clopidogrel to aspirin reduced the incidence of myocardial infarction, which led to the worldwide adoption of this dual anti-platelet therapy for patients with acute coronary syndromes [346]. Inhibition of ADP-induced platelet aggregation by clopidogrel was reported [171, 347], and it was shown to act as an antagonist to $P2Y_{AC}$ (later identified as $P2Y_{12}$), but not $P2Y_1$ and P2X1 receptors [348]. The anti-platelet activity of clopidogrel was shown early to be dependent on hepatic transformation to an active metabolite as an antagonist to $P2Y_{12}$ receptors ([349, 350] and see [351]) (Fig. 8). A subpopulation of patients are not responsive to clopidogrel, due to polymorphisms of either the cytochrome P450 isoenzyme, CYP2C19 [352] or the $P2Y_{12}$ receptor [353]. A new dysfunctional platelet $P2Y_{12}$ receptor variant associated with bleeding diathesis has been identified [252].

Apyrase, an ectoenzyme that metabolises ATP and ADP released from platelets and endothelial cells, reduces platelet activation and was recommended for the treatment of platelet-mediated thrombosis [354]. Reversible $P2Y_{12}$ receptor antagonists, BX 667, INS50589 and Arg256, were shown to be inhibitors of platelet aggregation and thrombus formation [355–357]. Unlike the irreversible action of the anti-thrombotic clopidogrel, an orally active, reversible direct $P2Y_{12}$ receptor antagonist, AZD 6140 (ticagrelor), was introduced for the prevention of myocardial infarction [358]. Ticagrelor and prasugrel are more potent $P2Y_{12}$ receptor inhibitors compared to clopidogrel and both have been shown to demonstrate improved clinical effects in preventing myocardial infarction [359, 360]. Additional inhibition by $P2Y_1$ receptor antagonists of platelet aggregation produced by $P2Y_{12}$ receptor antagonists has been considered [361, 362]. A complex of high molecular weight heparin and ATP prevented thrombus formation [363]. ADP-inducible platelet reactivity

increased with age [364]. Various side effects have been reported for both clopidogrel (bleeding, liver and bone injury, enhanced lipopolysaccharide-induced inflammation) [365, 366] and ticagrelor (bleeding, dyspnoea) [367]. In more recent reports, it was suggested that there may be a role for endogenous adenosine in ticagrelor-induced dyspnoea [368, 369].

As well as the anti-thrombotic use of clopidogrel, other actions have been identified. For example, clopidogrel has been used for the prevention of cardiac ischaemic complications in percutaneous coronary intervention [370–372]. Clopidogrel has also been reported to enhance periodontal repair in rats through decreased inflammation [373]. $P2Y_{12}$ receptor antagonists are widely used in combination with aspirin for the treatment of thrombosis, especially for patients with acute coronary syndrome and those undergoing percutaneous coronary intervention [374, 375]. Clopidogrel has also been reported to prevent endothelial dysfunction and vascular remodelling in aortas from hypertensive rats [376].

An important recent publication describes the crystal structure of the $P2Y_{12}$ receptor complex with a non-nucleotide reversible $P2Y_{12}$ receptor antagonist, AZD1283 [377] (Fig. 9). This will aid medicinal chemists to develop further new $P2Y_{12}$ receptor antagonists. $P2Y_{12}$ receptor antagonists are now one of the world's most used medications and have saved many lives by preventing myocardial infarction and stroke. Recently, a fast acting $P2Y_{12}$ receptor antagonist (Cangrelor), which is given as an infusion, was shown to prevent stent thrombosis and myocardial infarction. Since it is rapidly degraded, it reduces bleeding risk.

### Megakaryocytes

Megakaryocytes are platelet precursor cells in bone marrow. ADP, as for platelets, raised $[Ca^{2+}]_i$ and evoked release of granules from megakaryocytes and aggregation [378–380]. Release of ATP from megakaryocytes was reported [381]. Rat megakaryocytes responded to ATP, which mediated activation of $K^+$ channels and oscillations of cytoplasmic calcium concentrations [382]. Adenine enhanced the ATP-induced $Ca^{2+}$ oscillations [383] and suramin, and reactive blue 2 antagonised this action [384]. Patch clamp studies confirmed the presence of both ATP- and ADP-activated receptors in rat megakaryocytes [385]. A P2T ($P2Y_{12}$) receptor was identified on the human megakaryocyte cell line, Dami [386]. The authors reported that this cell line also responded to ATP and UTP, suggesting the presence of $P_{2U}$ receptors, later identified as $P2Y_2$ and/or $P2Y_4$ receptors. The presence of these receptors was also described for the human megakarioblastic Meg-01 cell line [387]. ADP induced rapid inward currents through $Ca^{2+}$ cation channels in mouse, rat and guinea pig megakaryocytes [388]. Functional expression of an ADP-activated receptor in *Xenopus* oocytes injected with megakaryocyte (CMK11-5) RNA was reported [389]. P2X1 receptor mRNA was identified in two megakarioblastic cell lines, Dami and CHRF-288 cells [270]. A study has characterised the functional P2X1 receptor in mouse megakaryocytes both pharmacologically and electrophysiologically [280, 390] (Fig. 10a). A $P2Y_1$ receptor was also shown to be expressed by megakarioblastic cells [214, 391, 392]. $Ca^{2+}$ signals evoked via $P2Y_1$ receptors can be markedly potentiated by depolarisation or inhibited by hyperpolarisation [393] (Fig. 10b). As platelets have no nucleus, the level of P2X1 receptor expression depends on transcriptional regulation in megakaryocytes, the platelet precursor cell. It was shown that Sp1/3 and NF-1 mediate transcription of the human P2X1 receptor gene in megakarioblastic Meg-01 cells [394]. Acetylsalicylic acid, a cyclooxygenase-1 inhibitor and anti-thrombotic agent, enhanced P2Y receptor-mediated outward current in rat megakaryocytes [395]. ADP released by megakaryocytes regulates pro-platelet formation by human megakaryocytes via $P2Y_{13}$ receptors [396, 397].

## Leukocytes

Leukocytes are white blood cells. They consist largely of immune cells, which have been reviewed in detail in an associated article (see [398]). The different immune cells are all involved in protecting the body from foreign substances and in antibody production. In disease, a variety of the cell types may appear in the blood, notably immature forms of the normal red and/or white bold cells.

Extracellular ATP and ADP at micromolar concentrations lead to impaired production of interferon-γ and IL-12 in leukocytes in the lipopolysaccharide-stimulated whole human blood model of sepsis [399]. ATP contributes to atherogenesis, via $P2Y_2$, $P2Y_6$, P2X4 and P2X7 receptors by inducing leukocyte recruitment in mice [400]. Abacavir is linked to cardiovascular disease, and ATP has been shown to play a role in leukocyte accumulation induced by abacavir, via P2X7 receptors [401]. P1 (adenosine) receptor mRNA expression has been described in human leukocytes of patients with valvular disease [402].

**Acknowledgments** The author thanks Professor Howard A. Stone, Professor David Erlinge, Professor Randy S. Sprague and Dr. Vera Ralevic for their valuable and constructive comments on this manuscript and Dr. Gillian E. Knight for her excellent editorial assistance.

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