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ORIGINAL ARTICLE

The P2X1 receptor and platelet function

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Abstract Extracellular nucleotides are ubiquitous signalling molecules, acting via the P2 class of surface receptors. Platelets express three P2 receptor subtypes, ADP-dependent P2Y1 and P2Y12 G-protein-coupled receptors and the ATP-gated P2X1 non-selective cation channel. Platelet P2X1 receptors can generate significant increases in intracellular Ca^{2+}, leading to shape change, movement of secretory granules and low levels of $\alpha_{IIb}\beta_3$ integrin activation. P2X1 can also synergise with several other receptors to amplify signalling and functional events in the platelet. In particular, activation of P2X1 receptors by ATP released from dense granules amplifies the aggregation responses to low levels of the major agonists, collagen and thrombin. In vivo studies using transgenic murine models show that P2X1 receptors amplify localised thrombosis following damage of small arteries and arterioles and also contribute to thromboembolism induced by intravenous co-injection of collagen and adrenaline. In vitro, under flow conditions, P2X1 receptors contribute more to aggregate formation on collagen-coated surfaces as the shear rate is increased, which may explain their greater contribution to localised thrombosis in arterioles compared to venules within in vivo models. Since shear increases substantially near sites of stenosis, anti-P2X1 therapy represents a potential means of reducing thrombotic events at atherosclerotic plaques.

Keywords Platelets · P2X1 · Thrombosis · Shear · ATP · Thromboembolism

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Introduction

In addition to its role as an energy source, ATP is an important extracellular signalling molecule, acting via the P2 family of surface receptors. These membrane-spanning proteins are divided into P2X receptors, which are ligand-gated non-selective cation channels, and P2Y receptors, which couple to signalling pathways principally through heterotrimeric G-proteins. The platelet is known to express three P2 receptor subtypes, P2X1, P2Y1 and P2Y12. Although ATP can activate P2Y1 and P2Y12 receptors when expressed at high density [1–3], it is a weak, partial agonist and unable to generate G-protein-coupled receptor (GPCR)-evoked responses in the platelet due to the low levels of P2Y receptor expression [4–6]. Therefore, the large amounts of ATP released from cells following vascular injury will directly stimulate platelet responses only through P2X1 receptors. Work from a number of laboratories, both in vivo and in vitro, has demonstrated a widespread ability for P2X1-evoked Ca^{2+} influx to contribute to platelet function. In this review, we discuss the biophysical properties of P2X1 and their signalling mechanisms. We also examine the evidence that this ligand-gated ion channel influences platelet function, both alone and in tandem with other receptor pathways. Finally, we discuss how platelet P2X1 receptors are activated in the circulation in health and disease, and thus when their inhibition could result in greatest therapeutic benefit.

Biophysical properties and regulation of P2X1 receptors

Seven P2X receptor subunit isoforms have been identified, classified as P2X1 to P2X7 by their chronological order of discovery [7, 8]. They comprise a novel family of ligand-

gated cation channels; each subunit having two transmembrane domains, intracellular amino and carboxy termini and a large extracellular ligand-binding loop. P2X channels form as either homotrimers or heterotrimers, which vary in their kinetics of desensitisation and pharmacology, although all are activated by the physiological ligand ATP [9]. The publication of a crystal structure for the zebra fish P2X4 receptor in an agonist-free state was a major advance in the understanding of the structural basis of P2X receptor properties [10]. The structure confirmed the predicted trimeric nature of the receptor and that the second transmembrane domain lines the ion conducting pore [11–13]. Mutagenesis has identified a role for conserved amino acids in ligand action at P2X receptors and mapping of the crystal structure shows that these residues are clustered at the interface between two adjacent receptor subunits, consistent with pre-crystalisation predictions [14–16]. The phosphate tail of ATP has been predicted to be co-ordinated by three lysine residues (K68 and K70 from one subunit and K309 from the adjacent subunit, P2X1 receptor numbering) with the adenine ring bound to a conserved NFR motif (290–292 on the same subunit as K309, P2X1 receptor numbering) deeper within the binding groove formed between the subunits [16]. This mode of ATP binding places the terminal phosphate facing out towards the surface of the receptor. This orientation also allows additional groups to be accommodated onto the terminal phosphate, consistent with the agonist action of dinucleotide polyphosphates (see later). Residues associated with sensitivity to the antagonists suramin and PPADs are also located around the predicted ATP binding pocket [10, 16].

P2X1 receptor subunits can form homomeric ion channels, but can also interact with other P2X subunits, e.g. P2X5, to form heteromeric ion channels with distinct properties [17, 18]. However, degenerate P2X primers detected only P2X1 mRNA in human platelets and related cell lines [19], and a quantitative PCR screen of all P2X receptors in highly purified human platelets found only P2X1 at significant levels [20]. Moreover, murine megakaryocytes from P2X1-deficient mice show no ATP-gated P2X currents in whole-cell patch clamp recordings [21]. Thus, only homomeric P2X1 receptors form ATP-gated ion channels in the platelet and megakaryocyte.

P2X1 receptors show appreciable permeability to Ca^{2+} (the relative permeability of Ca^{2+} compared to Na^{+}, i.e. PCa/PNa, is 3.9) [22, 23] and under physiological conditions ≈10% of current flow through the receptor is mediated by Ca^{2+} [24, 25]. These ion channels can therefore provide a significant source of direct Ca^{2+} influx into the cell following activation, as well as causing membrane depolarization that has been demonstrated to enhance signalling through P2Y1 and other Gq-coupled receptors [26, 27]. The time course of ATP-evoked P2X1 receptor-mediated currents is concentration-dependent with low concentrations (e.g. <100 nM, see [28]) taking several seconds to reach a peak response, which can be sustained for >30 s. This property may contribute to the ability of P2X1 to sustain Ca^{2+} signals following ATP secretion during stimulation by key platelet agonists [29]. In contrast, at maximal agonist concentrations, P2X1 receptor currents peak within tens of milliseconds and desensitise completely within seconds [28, 30, 31].

P2X1 receptor-mediated currents can be potentiated following the activation of Gαq-coupled GPCRs (GqPCRs) in a protein kinase C-dependent manner [32, 33]. This regulation is thought to occur through the phosphorylation of a protein that interacts with the intracellular amino terminus of the receptor [34]. Amongst the GqPCRs shown to potentiate P2X1 receptor currents in recombinant systems are P2Y receptors [32], suggesting that platelet P2Y1 receptor activation may sensitize subsequent responses to ATP. A second regulatory mechanism for P2X1 receptors is their association with cholesterol-rich lipid rafts [35] that could act to establish a pre-formed signalling complex in the cells. In both recombinant systems and platelets, disruption of lipid rafts results in >80% reduction in the amplitude of P2X1 receptor responses [35, 36]. In platelets, >80% of P2X1 receptors reside outside lipid rafts [36], raising the possibility that these constitute a reserve pool of receptors that can become active on recruitment into the rafts. These studies also raise the possibility that platelet P2X1 responses may be modulated by diet or by cholesterol-lowering statins. Finally, trafficking of P2X1 receptors can play an important role in their regulation. Recent studies have shown that green fluorescence protein-tagged P2X1 receptors are highly mobile in the membrane with movement of ≈75% of receptors into/out of a given area of membrane within 5 min [37]. This work has also shown that following receptor activation, internalization and recycling plays a key role in recovery of responses from desensitisation [37]. P2X1 receptor-mediated currents are therefore subject to regulation by a range of mechanisms giving rise to the ability to fine-tune P2X1 receptor functional responsiveness in platelets.

Desensitisation and recovery of P2X1 activity

In order to study P2X1 receptor function in platelets in vitro, it is essential to reduce the desensitisation that occurs due to spontaneously secreted ATP [38]. To achieve this, most studies have used apyrase (EC 3.6.1.5) derived from potato, which has both ADPase and ATPase activity. A higher apyrase concentration is required to protect P2X1 receptors from desensitisation compared to that needed to limit P2Y1 receptor desensitisation; ≈0.3–1 U/ml apyrase is

used in many P2X1 studies, whilst 0.02 U/ml allows P2Y1 receptors to function with negligible P2X1 responses [29, 38–42]. Further increases to 5 U/ml have been shown to amplify α,β-meATP-evoked shape change [43]; however, even in the presence of high apyrase levels, P2X1 activity still declines [38, 44], either due to insufficient speed of apyrase action or release of non-metabolised nucleotide polyphosphates. This raises the possibility that in vitro studies have underestimated the maximal effects of P2X1 receptors on platelet function. P2X1 receptor currents in human platelets and mouse megakaryocytes [31, 45] (in studies where the active agonist ATP was added as a contaminant in commercial samples of ADP) recover almost completely from an agonist-induced desensitised state over a period of ≈5 min. Platelet P2X1 receptor activity in plasma also shows recovery from a desensitised state due to endogenous ectonucleotidases [46]. Thus, in an in vivo environment where platelets are surrounded by substantial ectonucleotidase activity [47, 48], platelet P2X1 receptors will be expected to recover from activation within minutes after degradation of agonist and be available for stimulation multiple times during the lifespan of a platelet.

Tools to activate and inhibit P2X1 function

ATP is the physiological agonist at P2X1 receptors; however, the hydrolysis-resistant analogues α,β-methyleneATP (α,β-meATP) and β,γ-methylene ATP (β,γ-meATP) are commonly used to activate platelet P2X1 responses due to the requirement for ectonucleotidase activity to prevent desensitisation. α,β-meATP displays a similar potency to ATP (EC_{50} of ≈1 μM), although some studies show it is a partial agonist in expression systems (maximal response ≈60–70% of that observed with ATP [28] (Allsop and Evans, unpublished observations). β,γ-meATP has been used preferentially in some studies of platelet P2X1 receptors [49], as a consequence of its >30-fold selectivity for P2X1 over P2X3 receptors, in contrast to the similar EC_{50} for α,β-meATP at these two desensitising P2X receptors [49, 50]. However, β,γ-meATP is far less potent at P2X1 receptors, with a maximal response ≈40% of that observed with ATP and displaying a 10-fold higher EC_{50} [50]. Thus, it is possible that the maximal contribution of P2X1 receptors to platelet function may have been underestimated in some studies using non-hydrolyseable analogues.

Despite the availability of high affinity P2X1 antagonists based on suramin and PPADS [51–53], caution must still be taken to achieve selective inhibition of P2X1 without also affecting P2Y receptors, particularly P2Y1 [29, 41, 42, 54]. A further complication is the significantly lower potency of these antagonists at P2X1 receptors in native compared to recombinant systems [29, 41, 42, 51–57]. In human platelets, the suramin derivative NF449 shows good (≈72-fold) ability to discriminate between P2X1 and P2Y1 receptors, and only starts to influence P2Y12 receptors at very high concentrations [42, 54]. Thus, 0.3–1 μM NF449 has been used to achieve almost complete block of human P2X1-evoked responses with no observable reduction in P2Y-mediated effects [29, 41, 42, 54]. In contrast, MRS 2159 shows far less ability to discriminate between platelet P2X1 and P2Y1 receptors, with only a 6-fold difference in potency at Ca^{2+} responses [29, 54], thus selective block of P2X1 responses with this compound is difficult. Other P2X1 antagonists used in platelet studies are the PPADS derivative PPNDS [58] and the suramin derivative NF279 [59, 60]; however, their ability to discriminate between P2 receptors in the platelet has not been fully examined and non-specific actions have been described, including block of ectonucleotidases for NF279 [61] and modulation of AMPA receptors (which also exist in platelets [62]) for PPNDS [63]. ADP is not an agonist at P2X1 receptors [64] and has been used by one group to block α,β-meATP-evoked platelet P2X1 responses at equimolar concentrations [43, 65]. However, this antagonism has not been reported by other groups and is at odds with early patch clamp studies using commercial ADP samples, where an estimated 0.5–1 μM ATP could activate human platelet P2X1 receptors in the presence of ≈50-fold higher ADP concentrations [31]. One established approach to selectively abolish P2X1 activity is to desensitise the receptor with α,β-meATP or β,γ-meATP, particularly if this can be achieved prior to addition of external Ca^{2+}, thereby avoiding functional responses [29, 38]. α,β-meATP is far preferable as a desensitising agent since it is without effect on P2Y1 receptor-evoked Ca^{2+} increases, compared to weak competitive inhibition by β,γ-meATP [5, 66, 67].

Functional effects of platelet P2X1 receptor activation reported in vitro

Effects of selective P2X1 receptor activation It is likely that desensitisation by spontaneously released nucleotides accounts for the lack of P2X1-dependent functional responses reported by early in vitro studies [68–70]. Selective activation of P2X1 receptors is now known to stimulate a rapid and reversible shape change, peaking within a few seconds and returning to a resting state after ≈1 to 2 min [38, 43, 71, 72] (see Fig. 1a). This receptor also stimulates transient granule centralization and low levels of inside-out activation of $\alpha_{IIb}\beta_3$ integrin leading to weak, transient aggregation [39, 73]. Quantification of scanning electron micrographs [72] indicated that only 40% of platelets altered their shape in salines with 1 mM Ca^{2+} in response to a maximal α,β-meATP concentration; however, it is likely that all platelets possess functional P2X1

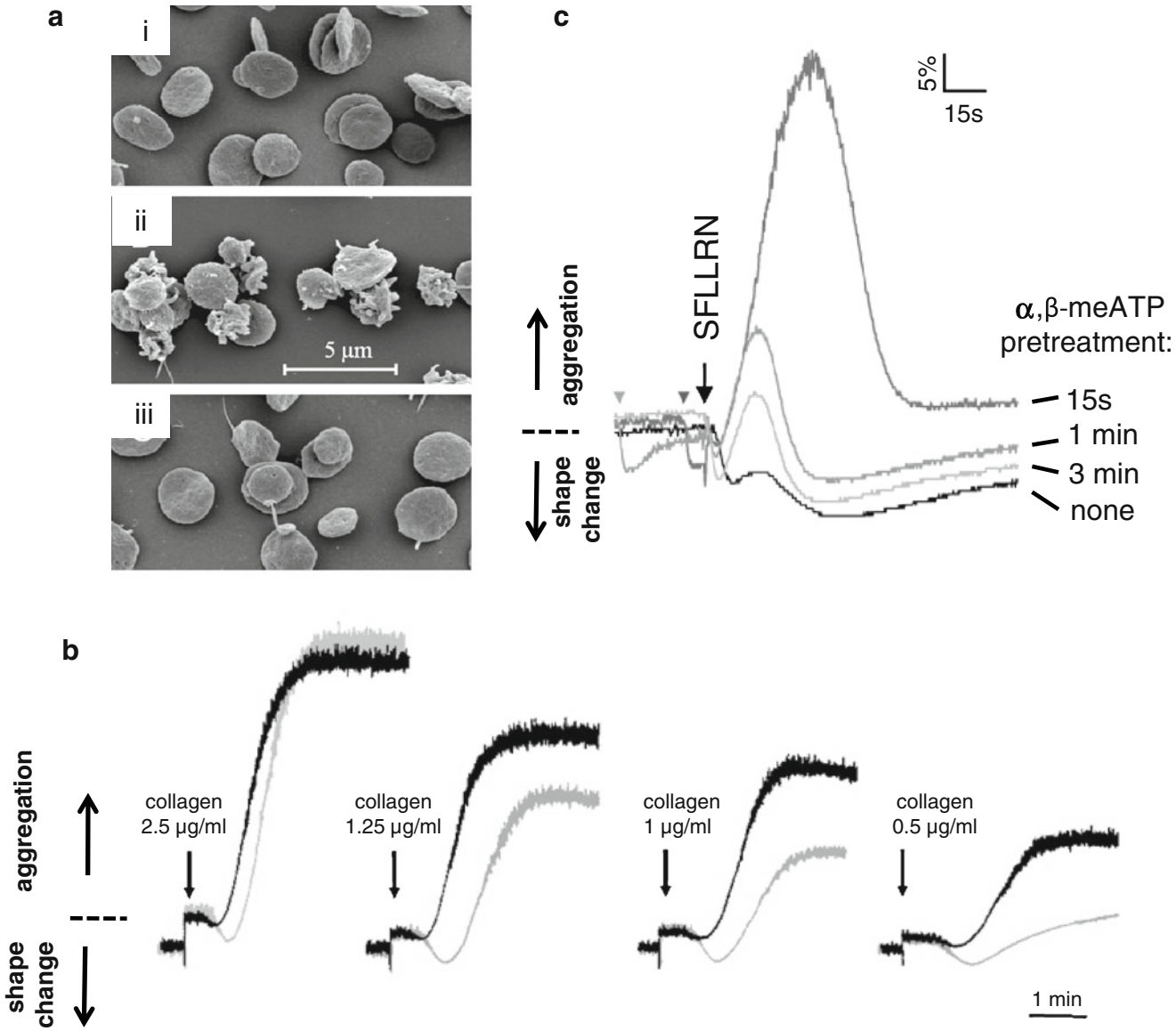

Fig. 1 Effects of P2X1 receptors on platelet activation studied in vitro. **a** Reversible shape change of human platelets following selective activation of P2X1 receptors with α,β-meATP. Scanning electron micrographs of washed platelets before stimulation (*i*), or 10 s (*ii*) and 60 s (*iii*) after addition of 10 μM α,β-meATP in the presence of 1 mM extracellular Ca^{2+}. **b** Contribution of P2X1 receptors to aggregation at different collagen concentrations demonstrated using platelets from wild-type (*black traces*) and P2X1-deficient (*grey traces*) mice. **c** Enhancement of PAR1-dependent aggregation in human platelets by P2X1 receptor activation. The amplification of TRAP1 (SFLLRN)-induced aggregation is reduced as the time between P2X1 stimulation with α,β-meATP (1 μM) and TRAP (SFLLRN, threshold concentration) is increased from 0.25 to 3 min; additions at 15 s and 1 min are shown by the *arrow heads*, while the addition at 3 min is not shown. Figures reproduced from [38, 40, 71], with permission. The *vertical axes* in **b** and **c** have units of % light transmission; an *upward deflection* represents an increase in transmission and thus an aggregation. *Traces* in **c** have been inverted from the original reference to be consistent with those in **b**

receptors since increasing Ca^{2+} entry by elevating external Ca^{2+} induced a shape change in virtually all platelets [72]. Nevertheless, this does imply an inter-platelet heterogeneity either in the amplitude of the P2X1 response in vivo or the speed of desensitisation in vitro. Evidence for both mechanisms exists. For example, Wang et al. [20] show a far greater mRNA degradation rate in vitro of P2X1 compared to P2Y1 and P2Y12 receptors and, given reports that platelet mRNA is transcriptionally active [74], suggest that P2X1 receptor levels may decrease with the age of the platelet. We have also observed a significant inter-donor variability in the rate at which P2X1 receptor Ca^{2+} responses decline in vitro, with between 0% and >90% of the response to 10 μM α,β-meATP remaining after 1 h [38, 44].

Role of P2X1 receptors during activation by collagen The greatest influence of P2X1 receptors on platelet function reported during in vitro studies with stirred platelet-rich plasma (PRP) or washed suspensions is an enhancement of aggregation mediated by low to intermediate (0.5–1.25 μg ml^{-1}) concentrations of collagen [43, 71, 75] (Fig. 1b). This may be a consequence of the significant contribution of P2X1 receptors to intracellular Ca^{2+} responses (up to 90% of the peak) generated by low concentrations of collagen [29, 76]. P2X1 receptors also enhance dense granule secretion responses to collagen [71], although whether this contributes to, or is a consequence of, the increased aggregation has not been investigated. At higher collagen concentrations (2.5 μg ml^{-1}), P2X1 has no essential involvement in the aggregation response (Fig.1b). In studies of stirred platelets, collagen stimulates ATP release and thus P2X1 activation through the glycoprotein VI receptor, since the effects can be mimicked by stimulation of human platelets with collagen-related peptide or stimulation of P2X1 over-expressing platelets with the snake venom convulxin [29, 75].

The role of P2X1 receptors in collagen-evoked platelet activation has also been studied under conditions of flow, where blood is perfused through a narrow chamber across a surface coated with collagen. The flow rate (and in some cases, the chamber lumen) is varied to mimic the wall shear rates experienced by blood cells in different parts of the circulation. Studies with blood from $P2X1^{-/-}$ mice show little role for this receptor at a shear rate of 800 s^{-1}, but that it amplifies thrombus formation on the collagen surface as the shear is increased, becoming highly significant at levels experienced in small arteries and arterioles (6,000 s^{-1}) [71]. Over-expression of P2X1 also allows this receptor to contribute to aggregate formation and phosphatidylserine exposure when the platelets are perfused over a collagen surface at low shear (1,000 s^{-1}) [75].

Role of P2X1 receptors during activation by thrombin Hechler and colleagues [41, 71] observed no significant role for P2X1 receptors in protease-activated receptor (PAR)-evoked aggregation for either murine or human platelets stimulated by thrombin or human platelets stimulated by PAR1 thrombin receptor activating peptide (TRAP1). Over-expression of P2X1 in murine platelets also had no detectable effect on thrombin-evoked aggregation [75]. In contrast, in a study by Erhardt et al. [40], P2X1 substantially amplifies aggregation induced by TRAP4 in murine platelets or TRAP1 in human platelets. P2X1 contributes only at threshold concentrations of the peptides, which for humans varied between donors, thus it is possible that the thrombin or TRAP1 levels used in the work of Hechler et al. [41, 71] and Oury et al. [75] represent a supra-threshold stimulus. In support of this explanation, secondary activation of P2X1 has been shown to amplify thrombin-evoked Ca^{2+} increases mainly at low to medium thrombin concentrations [29]. Erhardt et al. [40] also observed synergy between P2X1 and PAR1 when α,β-meATP was added prior to TRAP1 in human platelets [40]. As expected from the transient time course of P2X1-evoked Ca^{2+} and cytoskeletal responses, the synergy decreased as the interval between addition of α,β-meATP and TRAP1 was increased, although was still detectable for up to 3 min (see Fig. 1c). This effect could be important in the context of thrombus formation since release of ATP from damaged cells will stimulate P2X1 receptors very quickly following vascular injury compared to the generation of thrombin [77]. Grenegard et al. [78] have also observed small levels of aggregation induced by α,β-meATP after a subthreshold thrombin concentration.

Interactions between P2X1 and adrenaline receptor pathways Adrenaline activates platelets via α_{2A} receptors which, like P2Y12 receptors, couple through the G_i family of G-proteins and strongly synergise with G_q-coupled receptors to stimulate aggregation [79–84]. Activation of P2X1 receptors in hirudinated PRP synergizes with subthreshold concentrations of adrenaline to generate small, but significant levels of aggregation [39]. Surprisingly, this synergy was not observed between P2Y12 and P2X1 receptors [72], which may result from different Gαi-coupled pathways downstream of P2Y12 compared to α_{2A} [85–89], or the fact that the P2Y12 study was conducted in washed platelets since adrenaline displays a greater ability to stimulate aggregation in plasma compared to washed suspensions [79–84]. In a study of synergy between PAR and α_{2A} receptors in aspirin-treated human platelets, both MRS 2159 and NF449 (P2X1 antagonists), but not MRS 2179 or cangrelor (P2Y1 and P2Y12 antagonists, respectively), abolish the aggregation that results when adrenaline is added after subthreshold concentrations of thrombin, suggesting a major role for ATP secretion and activation of P2X1 receptors [78].

Role of P2X1 receptors during activation by thromboxane A_2 In human platelets, $[Ca^{2+}]_i$ increases stimulated by low-intermediate (0.5–1 μM) concentrations of the stable thromboxane A_2 analogue, U46619, are enhanced by release of ATP and secondary activation of P2X1 receptors [29]. However, no amplification of thromboxane A_2-evoked aggregation was observed by one group in human or murine platelets at a slightly higher concentration of U46619 (2–2.5 μM), which induced secretion of nucleotides from dense granules [41, 71]. Human P2X1 receptor over-expression in murine platelets markedly amplifies U46619-induced aggregation at 1–1.75 μM U46619, although surprisingly the synergy was not observed at only

slightly higher concentrations (2 μM) of this compound [75]. Thus, the apparent discrepancy between different reports may result either from varying P2X1 receptor response magnitudes or a role for P2X1 over only a narrow concentration range of U46619. The underlying mechanism for the synergy may involve P2Y receptor stimulation since P2X1 can enhance U46619-induced dense granule secretion [75].

P2X1:P2Y receptor interactions To study interactions between P2Y and P2X1 receptors, the ADP used to stimulate P2Y1 and P2Y12 receptors must be purified by HPLC or treated with hexokinase in the presence of glucose to remove the small but significant contaminating levels of ATP [31, 64, 90–94]. The level of ATP contamination can be easily determined using a luciferin–luciferase assay [29]. Despite the fact that P2Y receptors induce only a weak secretion response, secondary activation of P2X1 receptors by secreted ATP can significantly enhance the peak Ca^{2+} increase, albeit briefly compared to the effect after collagen and thrombin stimulation [29]. This amplification varies between donors, which may be due to natural variations known to exist in the amplitude of P2Y receptor responses [95, 96] and/or variable P2X1 responses (see earlier). The consequence of this P2Y-evoked ATP release and P2X1 activation is unclear, since deletion, pharmacological block or upregulation of P2X1 in murine platelets has no effect on ADP-evoked aggregation [41, 71, 75, 96]. ATP and ADP are both released from dense granules, therefore, it is more relevant to consider the consequences of co-stimulation of P2X1 and P2Y receptors. Thus, it is interesting to note that co-addition of α,β-meATP and purified ADP accelerates and enhances the peak Ca^{2+} response compared to that expected for summation of individual responses [21]. In the arterial circulation, platelets that initially attach to a damaged vessel wall or ruptured plaque will be swept away unless sufficiently activated within a narrow time-window. The synergy between P2X1 and P2Y1 may increase the chances of this occurring and thus contribute to the involvement of P2X1 in arterial thrombosis.

Role of P2X1 during Toll receptor activation Platelets are known to contribute to immune responses, particularly by linking the innate and adaptive immune systems [97]. Toll-like receptors (TLR) 1, 2, 4, 6 and 9 are expressed on platelets and recognise a number of molecules derived from microbes (e.g. lipopolysaccharide, peptidoglycan) [97–99]. A specific agonist of TLR2/1 receptors stimulates aggregation and secretion in human platelets that can reach approximately 50% and 100%, respectively of those induced by 1 μg ml^{-1} collagen [100]. Of particular note, TLR2/1-induces an intracellular Ca^{2+} increase that is totally blocked by 10 μM MRS 2159, and although this concentration will start to inhibit P2Y1 receptors (see above), MRS 2179 on its own had no effect [100]. P2X1 receptors can also contribute to TLR2/1-evoked functional responses, since MRS 2159 reduced the aggregation response to a TLR2/1-specific ligand by 50%. A low concentration of MRS 2159 (1 μM) was used in this experiment, thus the maximal contribution of P2X1 may be greater. However, the relative importance of P2X1 as a secondary receptor is unclear since antagonists of P2Y1, P2Y12 and thromboxane A_2 generation also have substantial inhibitory effects on TLR2/1-dependent aggregation. Nevertheless, these data suggest that P2X1 may have a role in immune responses, and thereby may play a role in the development of atherosclerosis, a chronic inflammatory disease. P2X1 responses have also been implicated in the platelet antimicrobial responses to *Staphylococcus aureus*, but based upon a high concentration (300 μM) of the less well-characterized P2X1 receptor inhibitor PPNDS [101].

Role of P2X1 receptors in shear-induced platelet aggregation The relative contribution of different surface receptors and agonists to platelet responses varies over the range of shear levels experienced within the circulation (reviewed in [102, 103]). As the shear rate increases, the adhesive ligand von Willebrand Factor (vWF) plays an increasingly important role in the tethering of platelets and subsequent aggregation. vWF binds to both GPIb and $\alpha_{IIb}\beta_3$, although these receptors directly generate only weak intracellular signals, such that additional responses evoked by collagen through GPVI and by soluble agonists are required for sufficient activation of $\alpha_{IIb}\beta_3$ to form a secure thrombus (reviewed in [102, 103]). The importance of P2X1 at high shear has been studied by flow over collagen surfaces (see above section). In addition, shear-induced platelet aggregation (SIPA) has been studied in a cone and plate viscometer, at levels where aggregation is dependent upon the presence of vWF and involves both GPIb and $\alpha_{IIb}\beta_3$ [41, 49]. The shear stress used in these studies stimulates release of dense granules, and the major role of released ADP and P2Y receptors in the subsequent aggregation is well established [104, 105]. In the study of Hechler et al. [41], addition of 0.5 U/ml apyrase increased SIPA, an effect antagonized by low levels of NF449 or preincibation with α,β-meATP, thus suggesting involvement of P2X1 receptors. In contrast, Oury et al. [49] observed a reduced SIPA with the same concentration of apyrase and MRS 2159 reduced SIPA even in the absence of apyrase, therefore altered P2Y receptor responses may be responsible for these effects. However, their study does support a role for P2X1 receptors in responses to shear since β,γ-meATP enhanced SIPA in normal apyrase-treated platelets and was also able to induce SIPA in platelets from a patient with an almost total absence of granule secretion (Hermansky–Pudlak syndrome) [49].

Other synergistic effects of P2X1 P2X1 receptors also show mild synergy with the weak platelet agonist thrombopoietin, resulting in enhanced aggregation, albeit still a minor response [39]. The underlying mechanism is not known but does not involve an enhancement of Ca^{2+} mobilisation [39]. Platelets prepared using heparin as an anticoagulant also show potentiated P2X1 receptor responses in plasma such that α,β-meATP can induce significant aggregation responses, compared to only shape change when hirudin is the anticoagulant [39]. Again, the underlying mechanism was not studied but it is well-known that heparin enhances the activation of platelets in plasma in response to a number of agonists [106, 107].

Signalling through the P2X1 receptor in the platelet

Activation of P2X1 receptors leads to influx of both Ca^{2+} and Na^{+} [30, 38, 71, 72, 93], and may also depolarize the platelet membrane potential from its resting level of −50 to −60 mV [108–110]. Although depolarization and increased cytosolic Na^{+} are putative contributors to signalling [26, 93, 111], all functional events reported to date following activation of P2X1 receptors depend upon Ca^{2+} influx [38, 39, 71–73]. The Ca^{2+} increase activates calmodulin-dependent myosin light chain kinase (MLCK) resulting in myosin light chain phosphorylation (MLC-P) and thereby generating the cytoskeletal events that underlie shape change and granule localization [38, 73]. P2X1 does not stimulate shape change through Rho-kinase [73], the pathway used dichotomously by certain receptors (e.g. P2Y1) to generate cytoskeletal events [112–115]. Oury et al. [65] also show that selective P2X1 activation causes transient phosphorylation of ERK2, a member of the mitogen-activated protein kinase family. Based upon experiments with extracellular EGTA, the broad spectrum PKC inhibitor GF109203-X and the calmodulin inhibitor W-7, P2X1-evoked ERK2 phosphorylation requires extracellular calcium and activation of both calmodulin and PKC [65, 73]. However, P2X1-evoked ERK2 phosphorylation is slow compared to the shape change, reaching maximal phosphorylation at 2 min and disappearing after 10 min [65]. Thus, not surprisingly, inhibition of ERK2 phosphorylation with the MEK1/2 antagonist U0126 does not alter αβ-meATP-evoked shape change [73]. The main role of ERK2 activation by P2X1 is proposed to be the enhancement of aggregation during low to intermediate levels of collagen [65, 73, 75]. Following stimulation by ≤1 μg ml^{-1} collagen, desensitisation of P2X1 reduces ERK2 phosphorylation; furthermore, block of ERK2 phosphorylation with U0126 impairs the aggregation and major dense granule secretion at this low level of GPVI stimulation [43]. However, at high collagen concentrations, aggregation and secretion do not depend upon P2X1 or ERK2 activation. Collagen causes an early, minor dense granule secretion that does not involve ERK2 and P2X1 activation, although it has not been directly investigated how dependent the subsequent P2X1-induced ERK2 phosphorylation is upon this early ATP release [65]. Studies with transgenic mice over-expressing the human P2X1 receptor in the megakaryocytic cell lineage support the involvement of ERK2 in P2X1-induced potentiation of collagen responses [75]. ERK2 phosphorylation, secretion and aggregation in response to threshold collagen concentrations are all enhanced in platelets from these mice, and these increased responses are all abolished by prior P2X1 desensitisation [75]. A number of outstanding questions remain regarding the role of the reported P2X1-induced ERK2 activation in platelet function, including its relative importance in the potentiation of responses to agonists other than collagen. Furthermore, it is unclear why ADP (up to 20 μM) was not able to activate ERK2 phosphorylation [65], since P2Y receptors have been shown to generate Ca^{2+} responses that can exceed and outlast those generated by P2X1 receptors in physiological salines [65, 72, 73].

The mechanisms by which P2X1 receptors contribute to SIPA appear to be distinct from those involved in amplification of the collagen responses. Oury et al. [49] demonstrated that the P2X1-evoked component of SIPA was dependent on calmodulin and MLCK activation. However, ERK2 phosphorylation was primarily coupled directly to GPIb and not P2X1, consequently, the reduction in SIPA observed with U0126 was independent of P2X1 contributions [59]. Furthermore, shear-induced dense granule secretion and aggregation were independent of all of the known conventional, novel and atypical PKC isoforms [49].

Activation of P2X1 following secretion of ATP from dense granules contributes to the Ca^{2+} increase induced by all major platelet agonists, including collagen, thrombin, thromboxane A_2 and ADP [29, 76]. Patch clamp studies in the megakaryocyte show that autocrine activation of P2X1 occurs as a series of discrete events with a quantal amplitude distribution, suggesting that granular ATP release stimulates groups of P2X1 receptors in a multiple, focal manner [111, 116, 117]. It has been suggested that this may result from a close proximity of release sites to P2X1 receptors or be due to compartmentalization of ATP release by the open canalicular system/demarcation membrane system [111]. Either way, ATP release very efficiently activates P2X1-dependent Ca^{2+} influx [29]. The central role of Ca^{2+} in platelet activation is demonstrated by the ability of cytosolic BAPTA (a Ca^{2+} chelator) to block or markedly inhibit a number of mainstream platelet responses, including inside-out activation of $\alpha_{IIb}\beta_3$, procoagulant activity and arachidonate production [118–120]. However, intracellular Ca^{2+} normally operates in tandem with other signalling

pathways to synergistically stimulate functional events [118–122], which probably explains the ability of P2X1 receptors to enhance aggregation mediated by a number of other agonists, particularly collagen and thrombin (see previous section).

Precisely how P2X1-dependent signals couple to inside-out activation of $\alpha_{IIb}\beta_3$ alone, or during co-stimulation of other platelet receptors, remains unclear. A major candidate is Ca^{2+} and diacylglycerol (DAG)-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) [123]. CalDAG-GEFI is an intracellular signalling molecule involved in the activation of small GTPases of the Ras family [124]. It contains binding sites for Ca^{2+} and DAG and a guanine nucleotide exchange factor (GEF) responsible for catalyzing the activation of Rap1 and Rap2. The expression of CalDAG-GEFI is restricted to only a few cell types namely neurons, neutrophils and platelets/megakaryocytes [124, 125]. Several studies on platelets and other cell types have demonstrated only a very weak affinity of the C1 domain for DAG suggesting that CalDAG-GEFI is predominantly regulated by Ca^{2+}. For example, studies performed in CalDAG-GEFI-deficient platelets have demonstrated reduced activation of integrin $\alpha_{IIb}\beta_3$, in addition to impaired granule release and reduced TXA_2 production [125–127]. Convulxin (100 ng/ml)-stimulated TXB_2 generation was completely abolished in CalDAG-GEFI-deficient platelets and reduced in response to higher convulxin concentrations (500 ng/ml); similarly, ERK phosphorylation and Rap1 activation were abolished in CalDAG-GEFI-deficient platelets at low dose convulxin and delayed and reduced at higher doses [126].

The signalling pathways and regulation of platelet P2X1 receptors is summarized in Fig. 2.

In vivo studies of platelet P2X1 receptor function

Genetic deletion or selective pharmacological inhibition of P2X1 receptors in mice has only mild effects on haemostasis, with most $P2X1^{-/-}$ animals displaying no change in tail bleed time [41, 71]. Furthermore, P2X1 receptor-deficient mice have normal platelet counts, unaltered levels of major adhesion receptors and normal megakaryocyte development, indicating no major role for this ATP-gated channel in platelet production [21, 71]. In contrast, in a platelet-dependent model of acute vascular occlusion where animals are challenged by co-injection of adrenaline and collagen, loss of P2X1 receptors reduced thrombus formation and halved the number of mice that normally die as a consequence of blocked pulmonary circulation [71] (Fig. 3a). Furthermore, loss of P2X1 responses reduced the size of a non-occlusive thrombus induced by laser injury of small arteries or arterioles in both cremaster muscle and mesentery [40, 71] (Fig. 3b). Whilst the role for P2X1 receptors in thrombus development in mesenteric arteries has been attributed to its interaction with exposed collagen combined with the effect of high shear, synergistic interactions with PAR receptors were suggested to account for the effect in cremaster muscle. The suggested role for thrombin:P2X1 interactions in the latter study is based upon the view that laser-induced thrombus formation in cremaster muscle is largely thrombin-dependent [128], combined with the observation that P2X1 deletion had no effect in another thrombosis model suggested to be GPVI/collagen-dependent (occlusive thrombus formation following injury of carotid arteries with $FeCl_3$) [129]. More work is required to clarify the underlying mechanisms of P2X1 involvement, which could vary depending upon the vascular bed, the extent of damage, or the stimulus (chemical versus laser) [129, 130]. Nevertheless, P2X1 clearly represents a potentially important target for antithrombotic therapy, as shown by treating mice with low concentrations of NF449 [41, 59]. Greater antithrombotic effects are observed with P2X1 blockers in arteriolar compared to venular models of localised thrombosis [59], which is consistent with an increased contribution of this cation channel to platelet adhesion as the shear level increases [49, 71, 75]. In addition, low levels of NF449 significantly reduced the levels of systemic thrombosis resulting from co-injection of collagen and adrenaline [41], as predicted from the ability of P2X1 to enhance collagen-induced aggregation in vitro [43, 71], although the role of shear stress in this model is unclear. Importantly, as shown in one comparative study of $P2X1^{-/-}$, $P2Y1^{-/-}$ and $P2Y12^{-/-}$ mice [40], the reduction in thrombus area following loss of P2X1 (reduction to ≈23% of control) [40] was as large as that observed following loss of either P2Y1 or P2Y12 (Fig. 3b (ii)). Thus, considering the lower risk of bleeding [41, 71, 83, 84, 131], therapeutic targeting of P2X1 receptors may have greater advantages compared to P2Y1 or P2Y12. Transgenic mice over-expressing human P2X1 receptors in their platelets show enhanced thrombosis in intestinal arterioles induced by release of reactive oxygen species from the photosensitive dye Rose Bengal, and also accelerated death following intravenous injection of collagen and adrenaline [59, 75]. This murine model raises the possibility that increased platelet P2X1 receptor responses could elevate the risk of thrombosis, although no disease phenotype has yet been shown to enhance P2X1 expression. Interestingly, one study has reported far greater P2X1-evoked shape change responses in human compared to murine platelets ex vivo [40]. It is possible that this reflects greater levels of desensitisation during platelet separation, as higher relative centrifugal forces are often used to prepare mouse platelets. However, an alternative explanation is that human platelets possess a higher density of P2X1 receptors, which could translate into greater antithrombotic benefits of blocking this receptor compared to those reported in murine studies.

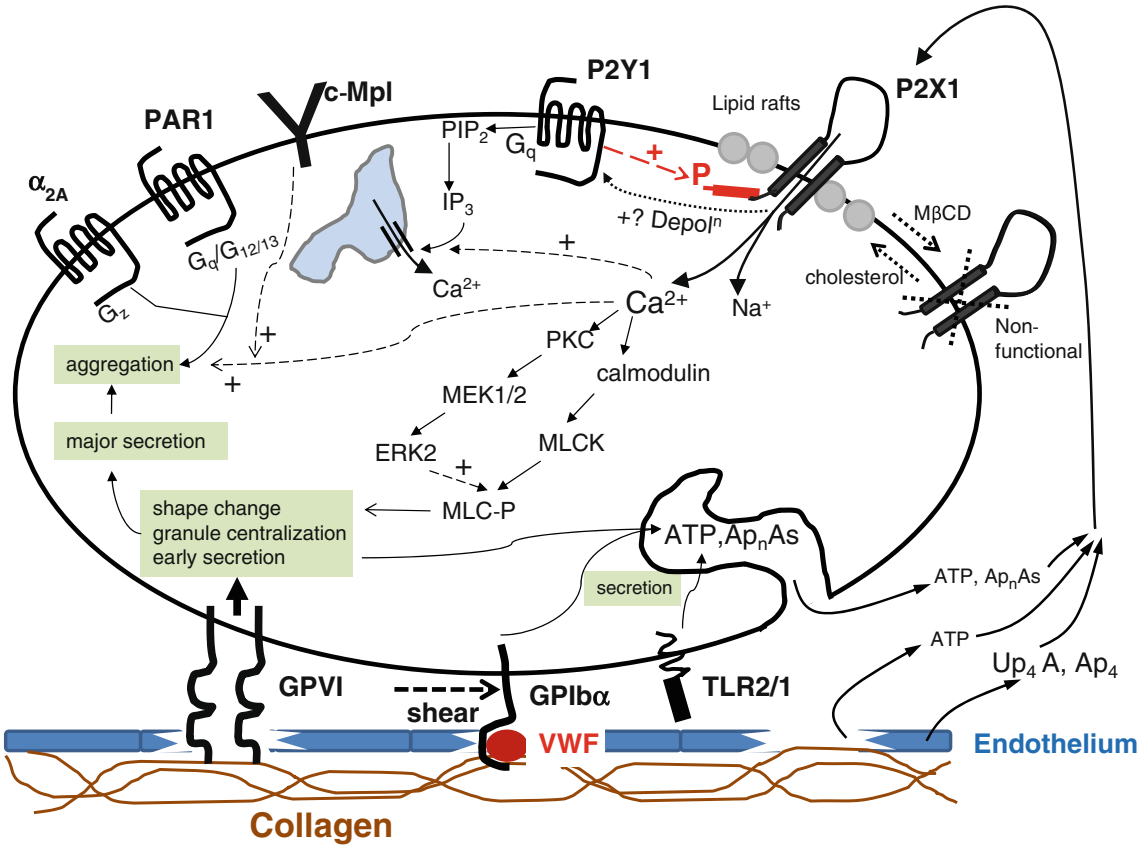

Fig. 2 P2X1 receptor signalling and regulation in the platelet. Summary of the pathways whereby P2X1 receptors have been proposed to couple to functional responses in platelets, together with the mechanisms that regulate these ion channels. See text for explanation

It is worth noting that all in vivo studies of platelet P2X1 activation have used relatively young animals and yet atherosclerosis is a disease that develops with age. Considering that collagen is a component of atherosclerotic plaques and shear is considerably enhanced at sites of stenosis caused by atheromas [132–134], P2X1 may play more crucial roles in acute thrombotic events following plaque rupture than suggested from the thrombosis models used so far.

How are platelet P2X1 receptors stimulated and modulated in the circulation?

To date, P2X1 receptor activation has only clearly been demonstrated following exposure to an extracellularly applied ligand. Thus, no study has reported a constitutively active P2X1 mutant, as shown for certain GPCRs, although this has been observed for a mutant of the non-desensitising P2X2 receptor [135, 136]. There is also no current evidence to suggest that the P2X1 receptor is directly mechanosensitive, thus the greater contribution of P2X1 receptors in the arterial circulation or under high shear may result from the relatively greater speed of activation of P2X1 compared to other platelet Ca^{2+} elevating receptors, or reflect shear-dependent secondary activation of P2X1 by ATP released from endothelial cells, platelets or other blood cells. ATP is present at millimolar concentrations in the cell cytoplasm, thus vascular damage leads to rapid and sustained intravascular concentrations of this nucleotide [137]. ATP is also stored at ≈0.5 M in the platelet dense granules and will reach concentrations at the platelet surface membrane well in excess of the level needed to activate P2X1 receptors [138]. Indeed, P2X1 is efficiently activated at very low concentrations of some agonists, particularly collagen, and the levels of ATP measured in the bulk phase suggest that P2X1 receptors are mainly activated in an autocrine as opposed to paracrine manner [29]. Red blood cells release significant amounts of ATP in response to hypoxia and shear stress [139, 140], which may also activate platelet P2X1 receptors. As described in the "Introduction" section, ATP is unable to stimulate functional responses through platelet P2Y1 and P2Y12 receptors due to their low level of expression [1–6]. Consequently, ATP can act as a competitive antagonist of ADP-evoked platelet responses [66, 141, 142]. It is therefore interesting to speculate that this effect may increase the relative importance of P2X1 compared to P2Y receptors in platelet activation prior to conversion of released ATP to ADP by nucleotidases.

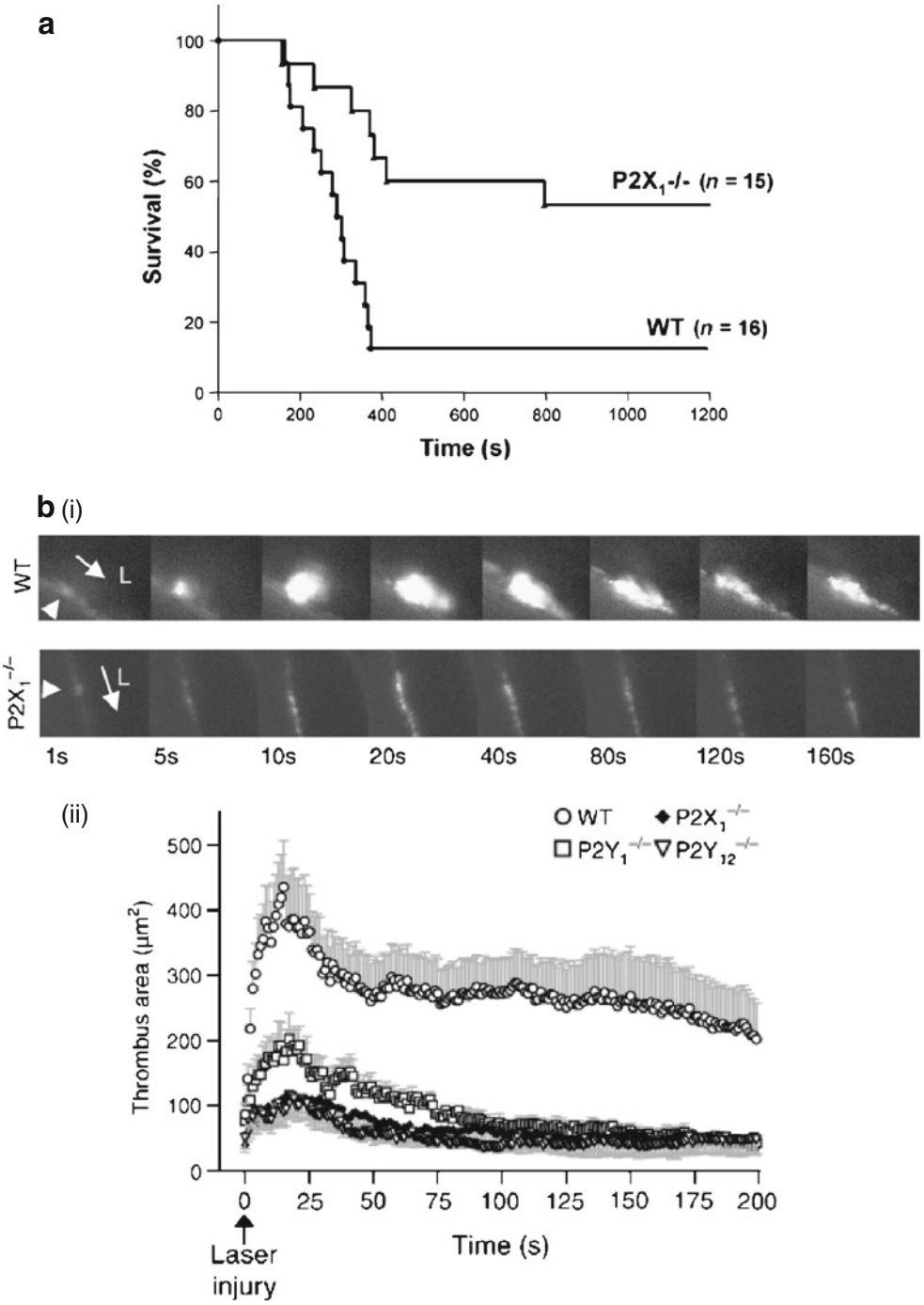

Fig. 3 In vivo evidence for a contribution of platelet P2X1 receptors to thromboembolism and thrombosis. **a** Improved survival of P2X1-deficient mice following collagen/adrenaline injection due to reduced pulmonary thromboembolism. **b** Laser-induced thrombus development in murine cremaster muscle arterioles; (*i*) images showing the deposition of fluorescently tagged platelets in wild-type (WT) and $P2X1^{-/-}$ mice following laser injury at the position marked by the *arrow head*; *L* indicates the arteriole lumen and the *arrow* shows the direction of blood flow (*ii*). Comparison of thrombus area development in WT, $P2X1^{-/-}$, $P2Y1^{-/-}$ and $P2Y12^{-/-}$. Reproduced from references [40, 71], with permission

Other than ATP, P2X1 receptors are also stimulated by several related compounds that have been shown to be released into the bloodstream. These include a number of diadenosine polyphosphates (Ap_nAs) and adenosine polyphosphoguanosines (Ap_nGs) [44, 143–148] that are stored and secreted from platelet dense granules. Of these compounds, Ap_4A and Ap_5A have received most attention as they are stored at the highest concentrations, although at levels many fold lower than ATP [149–151]. Furthermore, most Ap_nAs and Ap_nGs active at P2X1 receptors in rat mesenteric arteries are partial agonists and 10-fold-less potent than α,β-meATP or ATP [146, 152]. Since diadenosine polyphosphates are less rapidly degraded compared to ATP [153, 154], they may act as longer range P2X1 receptor stimuli. Uridine adenosine tetraphosphate (Up_4A) and adenosine tetraphosphate (Ap_4) are released from endothelial cells in response to agonists or mechanical shear and reach plasma concentrations that induce potent P2X1-dependent vasoconstriction in a rat perfused kidney model [155, 156]. Indeed, Ap_4 is the most

potent endothelial-derived vasoconstrictor reported to date using this assay. More work is required to confirm the true potency of these various compounds compared to ATP on platelet P2X1 receptors. One study reports an EC_{50} of 0.1 μM for Ap_4A at platelet P2X1 receptors [157], however this is much lower than concentrations reported previously to be effective for this receptor in human platelets (EC_{50} 23.5 μM) [44, 111] or rat mesenteric smooth muscle (no maximal response, but ≈50 μM required for 50% of the response to 10 μM αβ-meATP [146]). Although earlier data suggested that ADP is a weak agonist of wild-type P2X1 receptors [28, 31, 91, 92, 147] or a preferential agonist at a naturally occurring shortened variant [158], it is now firmly established that ADP has no agonist activity at native or recombinant P2X1 receptors [64, 94].

Another important feature of platelet P2X1 receptors is their resistance to inhibition by cyclic nucleotides [91, 111, 159–161]. Thus, the major endothelial-derived platelet inhibitors, prostacyclin and nitric oxide, which inhibit IP_3-dependent Ca^{2+} mobilisation in platelets, will have little or no effect on Ca^{2+} influx through P2X1 receptors. Therefore, P2X1 could be active under conditions where other Ca^{2+} mobilisation pathways are normally totally suppressed, and thus represent a particularly important early signal in the cascade of reactions leading to thrombus development.

Conclusions

A wealth of studies have shown that P2X1 receptors can enhance platelet activation by a variety of agonists and contribute to thrombus development in a number of murine models. These rapidly gated non-selective cation channels are the only platelet receptor to be activated by ATP released from blood cells or from damaged vascular cells. Furthermore, unlike IP_3 receptor-dependent Ca^{2+}-mobilisation, they are not inhibited by increased cytosolic cyclic nucleotides and thus are resistant to the main mechanism whereby platelet activation is controlled in the intact circulation. Consequently, pharmacological block of P2X1 receptors may provide novel therapeutic outcomes, particularly when one considers that platelets have proposed roles in the aetiology of cancer, atherogenesis and asthma, in addition to their recognised major precipitating role in arterial thrombosis.

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