A study of P2X$_1$ receptor function in murine megakaryocytes and human platelets reveals synergy with P2Y receptors

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1 We have examined the role of ATP-dependent P2X$_1$ receptors in megakaryocytes (MKs) and platelets using receptor-deficient mice and selective agonists.

2 α,β-meATP- and ATP- evoked ionotropic inward currents were absent in whole-cell recordings from MKs of P2X$_1^{-/-}$ mice, demonstrating that the P2X receptor phenotype in MKs, and by inference, platelets, is due to expression of homomeric P2X$_1$ receptors.

3 P2X$_1$ receptor deficiency had no effect on MK (CD 41) numbers or size distribution, showing that it is not essential for normal MK development.

4 P2Y receptor-stimulated $[Ca^{2+}]_i$ responses were unaffected in MKs from P2X$_1^{-/-}$ mice, however the inward cation current associated with Ca^{2+} release was reduced by $\sim 50\%$, suggesting an interaction between the membrane conductances activated by P2X$_1$ and P2Y receptors.

5 Interaction between P2X$_1$ and P2Y receptors in human platelets was also examined using $[Ca^{2+}]_i$ recordings from cell suspensions. α,β-meATP (10 μM) evoked a rapid transient P2X$_1$ receptor-mediated increase in $[Ca^{2+}]_i$, whereas ADP-(10 μM) evoked P2Y receptor responses were slower, peaked at a higher level and remained elevated for longer periods. Co-application of α,β-meATP and ADP resulted in marked acceleration and amplification of the peak $[Ca^{2+}]_i$ response.

6 We conclude that ionotropic P2X$_1$ receptors may play a priming role in the subsequent activation of metabotropic P2Y receptors during platelet stimulation.
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Introduction

Megakaryocytes (MKs) are platelet progenitor cells, whose principal role is to maintain the normal blood platelet count (Kaushansky, 1999). Platelets have little or no capacity to manufacture proteins and consequently MKs must express most, if not all, platelet proteins. Therefore, ion channels and receptors expressed in MKs may exist solely for later use in hemostatic functions, or alternatively, could play a role in megakaryocyte development. Purine nucleotides, acting through P2 receptors, are known to play important roles in fundamental platelet responses (see Kunapuli, 1998 for review). It is not known, however, at which stage of MK development P2 purinoceptors are expressed and whether they also have a role in megakaryocytopoiesis.

P2 receptors can be divided into ligand-gated ionotropic P2X receptors and metabotropic G-protein-coupled P2Y receptors (Burnstock, 1997). Seven distinct isoforms of the P2X receptor (P2X$_{1-7}$), with a range of physiological and pharmacological properties, have been isolated at the molecular level (Burnstock, 1997). They have a novel molecular architecture with two transmembrane domains, intracellular amino and carboxy termini, and a large extracellular ligand binding loop (Surprenant et al., 1995) and form homo-multimeric channels with at least three subunits (Nicke et al., 1998). P2X receptor subunits can also

co-assemble to form heteromeric channels, often with composite phenotypes, e.g. P2X$_{1/5}$, P2X$_{2/3}$, P2X$_{2/6}$ and P2X$_{4/6}$ receptors (King et al., 2000; Le et al., 1998; 1999; Lewis et al., 1995; Torres et al., 1998). P2X$_1$ receptors have been cloned from human platelets (Clifford et al., 1998; Scase et al., 1998; Sun et al., 1998; Vial et al., 1997) and are expressed by smooth muscle, neurons (Collo et al., 1996; Vulchanova et al., 1996) and a range of hematopoietic cells including megakaryoblastic cell lines (Vial et al., 1997), monocyte/macrophage lineage-HL60 and granulocyte-rat basophilic leukaemia cells (Buell et al., 1996).

The properties of MK and platelet P2X receptors, α,β-methylene ATP- (α,β-meATP) evoked rapidly desensitizing responses, correspond closely to those of recombinant P2X$_1$ receptors (Mackenzie et al., 1996; Mahaut-Smith et al., 2000). Recently a P2X$_1$ receptor deletion mutant (P2X$_{1del}$) that is ADP-sensitive and α,β-meATP-insensitive has been isolated from MK cell lines and platelets (Greco et al., 2001), however the functional role of this mutant receptor was not determined. In our studies on human platelets, α,β-meATP always evoked a rapid transient increase in $[Ca^{2+}]_i$ and purified ADP only evoked a slower P2Y mediated calcium increase (platelets from 11 donors; Mahaut-Smith et al., 2000). Thus we have no evidence for an ADP sensitive P2X$_1$ receptor-mediated response in human platelets, suggesting that if the P2X$_{1del}$ mutant is functionally expressed in human platelets it forms a heteromeric receptor dominated by the

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properties of full-length P2X$_1$ receptors. However, it remains unclear whether other cloned or yet to be identified P2X receptor isoforms are also expressed by MKs and contribute to the P2X receptor phenotype. Furthermore, the role of P2X$_1$ receptors in platelet function is unclear. P2X receptors are Ca^{2+} permeable (Benham & Tsien, 1987; Evans *et al.*, 1996) and their activation results in Ca^{2+} influx (Mackenzie *et al.*, 1996; Vial *et al.*, 1997). Two groups failed to detect a functional response linked to P2X receptor activation (Jin & Kunapuli, 1998; Savi *et al.*, 1998), however, it has recently been shown that the transient Ca^{2+} influx following selective P2X$_1$ receptor activation can stimulate a shape change response in human platelets *in vitro* if receptor desensitization is limited (Rolf *et al.*, 2001). A dominant negative P2X$_1$ receptor mutation has been reported in platelets from a patient with a severe bleeding disorder (Oury *et al.*, 2000), however the relative contribution of this mutation to hemostatic function is not known.

It has been suggested that P2X$_1$ receptors contribute to the apoptosis of thymocytes but not peripheral T cells (Chvatchko *et al.*, 1996). In addition, P2X$_1$ receptor expression was markedly upregulated when promyelocytes (HL60 cells) were treated with dibutyryl cyclic AMP to induce a neutrophil-like phenotype (Buell *et al.*, 1996) In contrast, P2X$_1$ receptor expression may be repressed during phagocyte differentiation (Clifford *et al.*, 1998). Thus, P2X$_1$ receptors may play an important role in the development of various hematopoietic cell lineages including MKs.

At least two types of G-protein coupled P2Y receptors are also expressed by platelets (Kunapuli, 1998). P2Y$_1$ receptors activate phospholipase-C, leading to Ca^{2+} mobilization, *via* Gα$_q$ proteins, and also stimulate Rho/Rho kinase *via* Gα$_{12/13}$ (Leon *et al.*, 1997; Paul *et al.*, 1999). These two intracellular signalling pathways can account for the ADP-evoked platelet shape change response, with the more important contribution arising from the increase in [Ca^{2+}]$_i$ (Paul *et al.*, 1999). In addition, platelets express a P2Y receptor, the P2Y$_{AC}$ receptor (also referred to as the P2$_{cyc}$ and P2T$_{AC}$), that couples to the inhibition of adenylyl cyclase and activation of PI 3-kinase (Kunapuli, 1998; Trumel *et al.*, 1999). An ADP receptor has been cloned recently from platelets that corresponds to this receptor and has been designated P2Y$_{12}$ (Hollopeter *et al.*, 2001). Co-activation of P2Y$_1$ and P2T$_{AC}$ (P2Y$_{12}$) receptors is required for normal ADP-evoked aggregation (Hechler *et al.*, 1998; Kunapuli, 1998; Savi *et al.*, 1998), although some degree of aggregation can occur *via* either P2Y$_1$ or P2T$_{AC}$ (P2Y$_{12}$) receptors (Fabre *et al.*, 1999; Jarvis *et al.*, 2000; Leon *et al.*, 1999). Megakaryocytes most likely express both P2Y receptors found in the platelet, although their identity in the progenitor cell has not been reported in molecular studies. P2Y receptor activation in rat and mouse megakaryocytes evokes Ca^{2+} mobilization and a monovalent cation conductance (Somasundaram & Mahaut-Smith, 1994; Uneyama *et al.*, 1993). Given the variety of P2 receptors expressed on MKs and platelets, it is possible that P2X and P2Y receptors interact in the control of these cells.

Physiological P2X receptor agonists include ATP and diadenosine polyphosphates (Ap$_4$A, Ap$_5$A, and Ap$_6$A) (Evans *et al.*, 1995; Wildman *et al.*, 1999). Megakaryocyte and platelet P2Y receptors are principally activated by ADP (Kunapuli, 1998; Somasundaram & Mahaut-Smith, 1994; Uneyama *et al.*, 1993), although ATP is a weak agonist at the

MK P2Y receptors coupled to Ca^{2+} mobilization. On the other hand, ATP can act as an antagonist of ADP-dependent activation of P2Y$_1$ and P2T$_{AC}$ (P2Y$_{12}$) receptors in the platelet (Hechler *et al.*, 1998; Hourani, 2000). This apparent discrepancy can be accounted for by a low efficacy of ATP at P2Y$_1$ receptors and a low receptor density in the platelet compared to the MK (Hechler *et al.*, 1998; Palmer *et al.*, 1998). Although it had previously been thought that ADP was an agonist at both P2X and P2Y receptors (Mackenzie *et al.*, 1996; Mahaut-Smith *et al.*, 1992; Sage *et al.*, 2000) it has recently been shown that the action of ADP at P2X receptors results from low levels of ATP contamination of commercially available ADP (Mahaut-Smith *et al.*, 2000). During hemostasis, large amounts of ATP will be released from damaged cells at the site of vascular injury and ADP, ATP and diadenosine polyphosphates will be released from the dense granules of activated platelets. Thus, under physiological conditions, it is likely that platelet P2X and P2Y receptors will be activated simultaneously, although by separate agonists, and thus interactions between these two receptor subtypes could have important functional consequences for hemostasis and thrombosis.

In this study we have compared the properties of MKs from normal and P2X$_1$ receptor-deficient mice (Mulryan *et al.*, 2000) to determine (1) whether P2X$_1$ receptor expression is essential for normal MK development and (2) the role of P2X$_1$ receptor subunits in activation of mouse MKs by extracellular purines. We have also used selective agonists for ionotropic and metabotropic platelet purinoceptors to examine the interaction of P2X and P2Y receptors during receptor-operated Ca^{2+} mobilization in human platelets.

Methods

Marrow preparation and antibody staining

Adult male P2X$_1$ receptor deficient ($-/-$), heterozygote ($+/-$) or wild type ($+/+$) mice (Mulryan *et al.*, 2000) were killed by cervical dislocation and exsanguinated. For immunohistochemical studies the spleen and bone marrow (from femur and tibia) were dissected and rapidly frozen in Tissue-Tek® (Sakuna, The Netherlands). 12 μm sections were cut, thaw-mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma-Aldrich, U.K.) and fixed in 2% paraformaldehyde (Sigma-Aldrich, U.K.), 80 mM Na$_2$HPO$_4$, 20 mM NaH$_2$PO$_4$ for 10 min and washed in PBS (phosphate buffered saline). The sections were incubated in 0.5% Triton X-100 (Sigma-Aldrich, U.K.), and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, U.S.A.) in PBS for 30 min at room temperature, washed in PBS and incubated with rabbit anti-rat P2X$_1$ polyclonal antibody (Alomone Labs, Israel) at a dilution of 1:200 with 10% normal donkey serum in PBS at 4°C overnight. The sections were subsequently washed in PBS, incubated with Texas Red® dye-conjugated donkey anti-rabbit IgG (dilution of 1:100) (Jackson ImmunoResearch Laboratories, U.S.A.) and for double labelling studies FITC-conjugated rat anti-mouse CD41 monoclonal antibody (dilution of 1:500) (Pharmingen International, U.S.A.) was added in the presence of 10% normal donkey serum in PBS for 2 h at room temperature, washed in PBS and then mounted in Citifluor (Citifluor,

A. Bone marrow
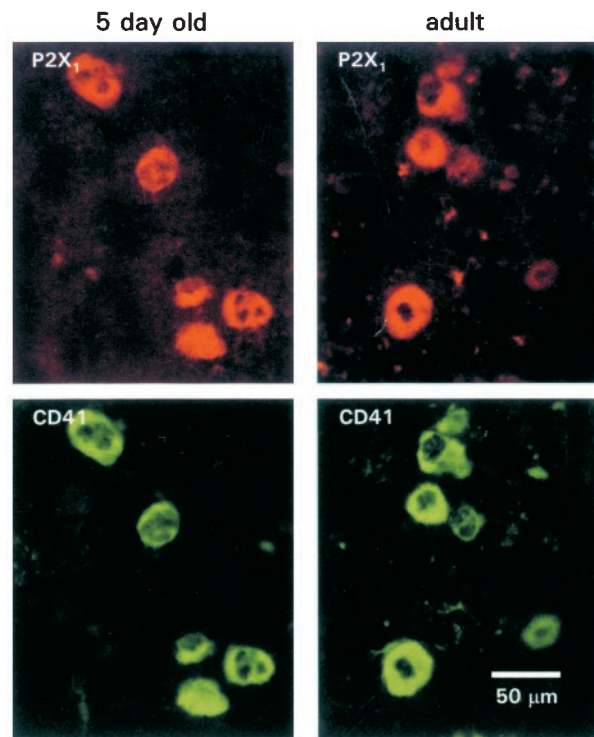
B. Spleen
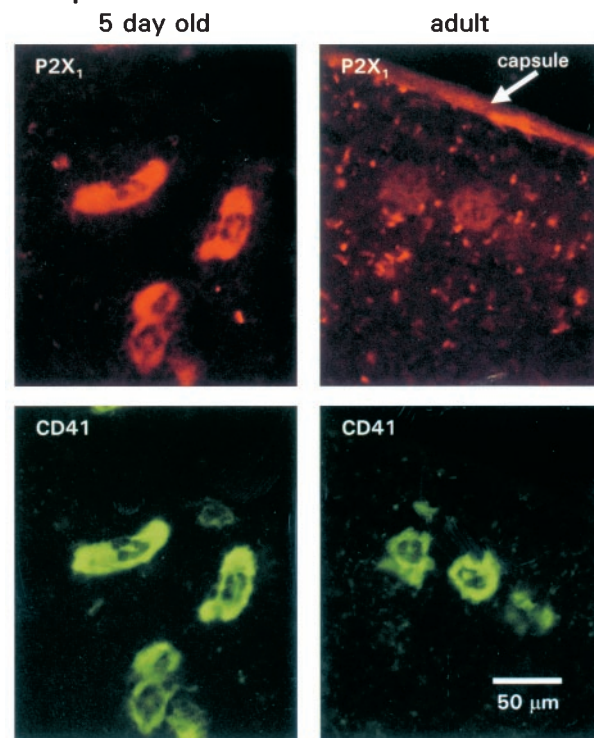
Figure 1 Immunoreactivity for P2X$_1$ receptors and the MK lineage marker CD41 in bone marrow and spleen. P2X$_1$ receptor immunoreactivity is co-localized with the MK marker CD41 in bone marrow. (A) Similar levels of immunoreactivity were detected in tissues from 5-day-old (left panel) and adult mice (right panel). P2X$_1$ receptor immunoreactivity was also co-localized with CD41 immunoreactivity in the spleen from 5-day-old mice (B, left hand panels), in contrast

U.K.). Images were captured using Scion Imaging v 4.0.2 software (a modified version of the public domain program, NIH Image) and the intensity of P2X$_1$ receptor immunofluorescence was determined.

For determination of the size distribution of MKs, bone marrow from a total of 3 (+/+) and 4 P2X$_1$ receptor deficient (−/−) mice were collected and frozen in Tissue-Tek®. For each mouse, two different sections were cut and fixed as described above, incubated in 10% normal donkey serum in PBS for 30 min at room temperature, rinsed, incubated for 2 h at room temperature in FITC-conjugated rat anti-mouse CD41 monoclonal antibody (dilution of 1 : 500), then washed and mounted with Citifluor. The sections were photographed with an epifluorescence microscope and the size distribution of CD41 immunoreactive MKs was determined using NIH Image as described previously (Chopra et al., 2000).

Electrophysiological and [Ca^{2+}]$_i$ measurements from single megakaryocytes

For these studies mouse MKs were isolated by gentle trituration from the femoral and tibial marrow and conventional whole cell patch clamp and intracellular Ca^{2+} recordings were made as described previously (Mahaut-Smith et al., 2000). To isolate the P2X receptor-mediated current, short pulses (<2 s) of α,β-meATP (Sigma-Aldrich, U.K.) were applied to cells held at −60 mV. The pipette filling solution contained (in mM) Kgluconate 140, NaCl 5, EGTA 9, HEPES 10, pH 7.3 (KOH) and the external solution contained (in mM) NaCl 150, HEPES 10, KCl 2.5, MgCl$_2$ 1, CaCl$_2$ 2.5, pH 7.3 (NaOH). For dual measurement of ATP-activated P2 receptor inward currents and changes in intracellular Ca^{2+}, the holding potential was −70 mV, the pipette filling solution contained (in mM) CsCl 150, MgCl$_2$ 2, EGTA 0.1, K$_5$fura-2 0.05, Na$_2$GTP 0.05, HEPES 10, pH 7.2 (CsOH) and the external solution contained (in mM) NaCl 150, HEPES 10, CaCl$_2$ 1, MgCl$_2$ 1, glucose 10, pH 7.35 (NaOH). All megakaryocyte electrophysiological and [Ca^{2+}]$_i$ studies were conducted at room temperature (20 – 24°C).

Platelet [Ca^{2+}]$_i$ measurements

Cuvette fluorescence measurements of [Ca^{2+}]$_i$ in fura-2-loaded human platelets were performed essentially as described previously (Mahaut-Smith et al., 2000; Rolf et al., 2001) at a temperature of either 13°C or 37°C. The saline contained (in mM), NaCl 145, KCl 5, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, glucose 10, pH 7.35 (NaOH) and 0.32 U ml^{-1} apyrase. For data collected at 13°C, background-corrected 340/380 nm ratios are used to indicate [Ca^{2+}]$_i$ due to problems associated with accurately calibrating fura-2 fluorescence at the reduced temperature (Mahaut-Smith et al., 2000). ATP-free ADP was prepared by incubation of a stock of 10 mM ADP (Sigma-Aldrich, U.K.) in a high glucose saline (composition, in mM: NaCl 150, CaCl$_2$ 2.5, HEPES 10, KCl 2.5, MgCl$_2$ 1, glucose 22, pH 8 (NaOH), with 3 units/ml hexokinase for 1 h at 37°C (Mahaut-Smith et al., 2000).

P2X$_1$ receptor immunoreactivity was barely detectable in CD41 immunoreactive cells from adult spleen.
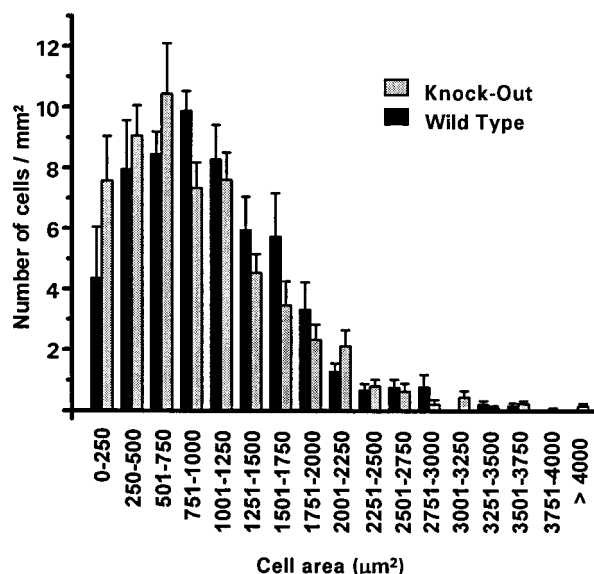
Figure 2 Size distribution of MKs from normal and P2X₁ receptor deficient mice. Analysis of the area of CD41 immunoreactive cells in bone marrow. Two sections per mouse were analysed ($n = 3$ and 4 mice for $+/+$ and $-/-$ mice).

Figure 3 P2X receptor-mediated inward currents are absent in P2X₁ receptor deficient mice MKs. α,β-meATP (10 μM) evoked rapid transient inward currents in $+/+$ MKs these were absent in MKs from P2X₁ receptor deficient $-/-$ mice. Bar indicates period of drug application.
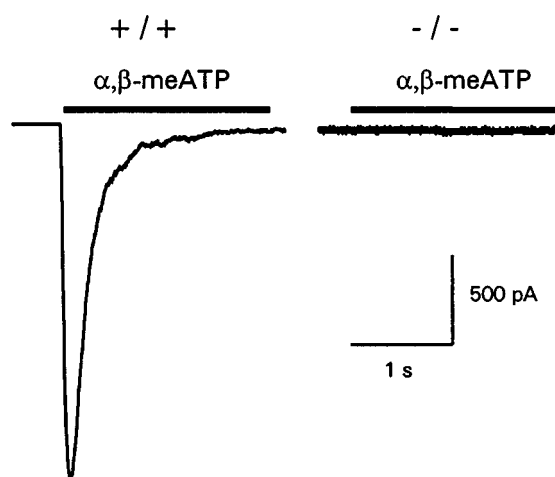
Data analysis

Data are presented throughout as mean $\pm$ s.e.mean, $n =$ number of observations. Differences between means were determined by the appropriate Student's t-test and were considered significant when $P < 0.05$. Differences between the size distribution of MKs were tested using two-way analysis of variance.

Results

P2X₁ receptor expression in bone marrow and spleen

P2X₁ receptor immunoreactivity was detected in sections of bone marrow (Figure 1) associated with large diameter hematopoietic cells. In addition, P2X₁ receptor immunoreactivity was seen in arteries coursing through the marrow (not shown). This pattern of expression is consistent with P2X₁ receptor expression in MKs and smooth muscle (Vial et al., 1997; Vulchanova et al., 1996). To confirm that the P2X₁ receptor is expressed by MKs we looked for co-localization of immunoreactivity with the MK and platelet-specific marker glycoprotein-IIb (α_{IIb} integrin or CD41) (Uzan et al., 1991). In dual labelling studies P2X₁ receptor and CD41 immunoreactivity were co-localized in sections of bone marrow from wild type ($+/+$) mice (Figure 1) (excluding P2X₁ receptor expression in vascular smooth muscle). This confirms that P2X₁ receptors are expressed in MKs.

In rodents, the major sites of megakaryocyte development and platelet production are the bone marrow and spleen (Davis et al., 1997). In 1-5-day-old animals P2X₁ receptor immunoreactivity (co-localized with CD41 immunoreactivity) was detected at similar levels of intensity in both the spleen and bone marrow (61.7 ± 3.6 and 61.5 ± 5.5 units of intensity respectively, $n = 34$, 28 MKs) (Figure 1).

Interestingly, in contrast to bone marrow where P2X₁ receptor immunoreactivity was at similar levels in adults to that in 1-5-day-old animals (61.5 ± 5.5 and 67.2 ± 5.7 intensity units, $n = 28$, 29 cells respectively), P2X₁ receptor immunoreactivity was reduced significantly ($P < 0.001$) in CD41 immunoreactive MKs in adult spleen (61.7 ± 3.6 and 42.4 ± 2.0 intensity units, $n = 34$, 76 MKs from 1-5 day and adult respectively) (Figure 1). P2X₁ receptor immunoreactivity was detected in the artery walls, the capsule layer of the spleen and in tuberculae of adult marrow. These results suggest the down regulation of P2X₁ receptor expression in splenic MKs.

MK development in P2X₁ receptor deficient mice

P2X₁ receptor immunoreactivity was not detected in bone marrow from P2X₁ receptor deficient ($-/-$) mice. These results confirm that the P2X₁ receptor is not produced in these mice (as shown previously (Mulryan et al., 2000)). MKs are polyploid cells with different levels of ploidy reflected by the size of the cell (Levine et al., 1982). In order to test whether P2X₁ receptor expression is essential for normal MK development we have determined the numbers and size distribution of megakaryocytes for $+/+$ and $-/-$ mice using the MK marker CD41. The P2X₁ receptor deficiency had no effect on the number or size distribution of MKs in bone marrow from adult mice (ANOVA $p = 0.8$) (Figure 2). This finding is also supported by the electrophysiological studies in which the capacitance of the MKs (a measure of plasma membrane area) was not significantly different for $+/+$ and $-/-$ mice (298 ± 32 pF and 261 ± 26 pF, respectively $n = 12-22$). These results indicate that the P2X₁ receptor is not essential for MK development.

### P2X₁ receptors are essential for the production of functional MK P2X receptors

The P2X₁, P2X₁/₅, P2X₃ and P2X₂/₃ receptor agonist α,β-meATP (10 μM, a maximal concentration at these receptors)
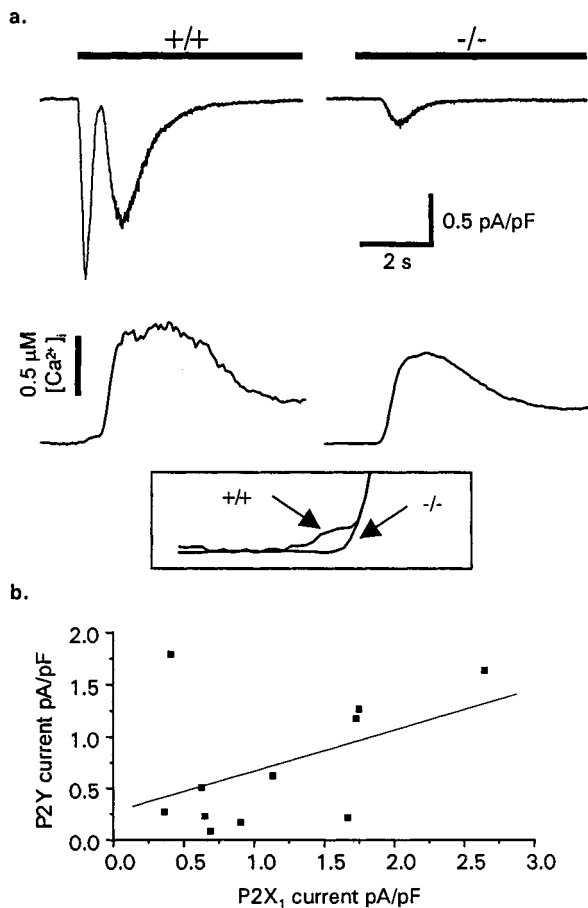
Figure 4 ATP-induced $[Ca^{2+}]_i$ and inward current responses in murine megakaryocytes: effects of P2X$_1$ receptor deficiency. (a) Whole-cell membrane currents (upper traces) and $[Ca^{2+}]_i$ responses (lower traces) activated by ATP (30 μM, bar) in wild type (+/+) and P2X$_1$ receptor-deficient (−/−) mice. Inset box shows the expanded region of the calcium trace showing the initial P2X$_1$ receptor mediated increase in intracellular calcium in +/+ compared to P2X$_1$ −/− MKs. Holding potential: −70 mV. Membrane currents have been normalized for whole-cell capacitance. (b) Correlation of the amplitude of P2X$_1$ receptor and secondary currents recorded from mouse megakaryocytes.

evoked rapid transient inward currents in +/+ and +/− MKs (707±116 and 680±200 pA respectively, $n=15-16$) but had no effect on MKs ($n=12$) from P2X$_1$ receptor deficient mice (Figure 3). P2X$_1$ receptors are non-selective cation channels; monovalent cations predominate ionic flow and calcium accounts for 5−10% of current flow under physiologcal conditions (Benham, 1989; Evans *et al.*, 1996). ATP (30 μM), an effective agonist at all P2X receptors, also evoked rapid transient inward currents from +/+ but not −/− mice when P2Y receptor-mediated $[Ca^{2+}]_i$ increases were prevented using the chelator EGTA (9 mM) in the recording pipette solution (not shown, but see responses to ATP with low Ca^{2+} buffering conditions, Figure 4). These results indicate that there is no residual P2X receptor mediated current in the MK in P2X$_1$ −/− mice and demonstrates that the P2X$_1$ receptor is essential for the expression of functional P2X receptor channels in this cell type.

At physiological levels of intracellular Ca^{2+} buffering, ATP application evokes a complex response consisting of multiple cationic currents and an increase in intracellular Ca^{2+} mediated by the activation of P2X and P2Y receptors (Uneyama *et al.*, 1993; Somasundaram & Mahaut-Smith, 1994). The interaction of P2Y$_1$ and P2Y$_{AC}$ receptors is important in platelet activation (Jarvis *et al.*, 2000), however the level of cross-talk between P2X receptors and P2Y receptors is unclear. The lack of functional P2X receptor-mediated responses in the P2X$_1$ receptor deficient mouse allows assessment of P2X and P2Y receptor interaction in MKs. These experiments were conducted using Cs$^+$-containing internal salines to fully resolve inward cationic currents including the P2X$_1$ receptor current. In normal (+/+) mouse MKs, ATP (30 μM, a maximal concentration at P2X$_1$ receptors (Valera *et al.*, 1994) and P2Y receptors in MKs (Uneyama *et al.*, 1993)), evoked a rapid initial transient P2X current (peak amplitude 1.31±0.32 pA/pF, $n=11$) and
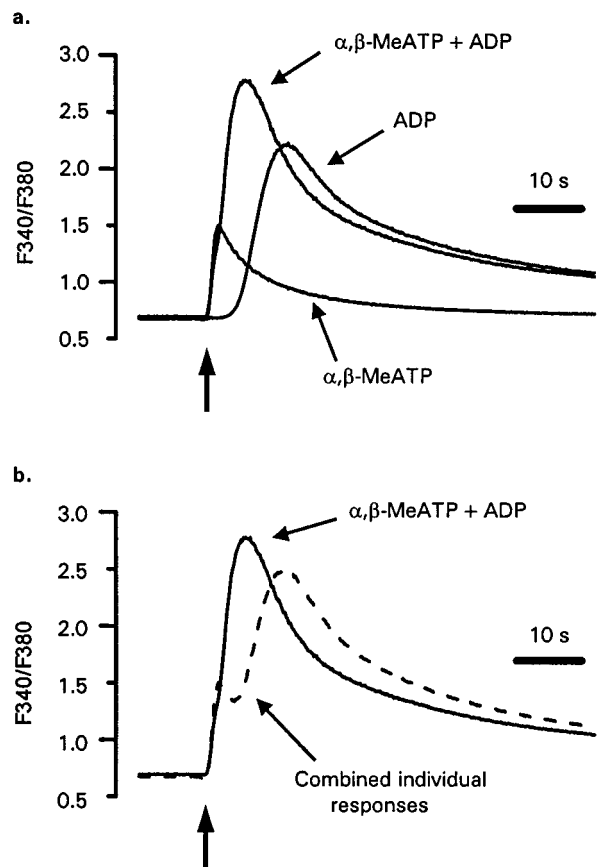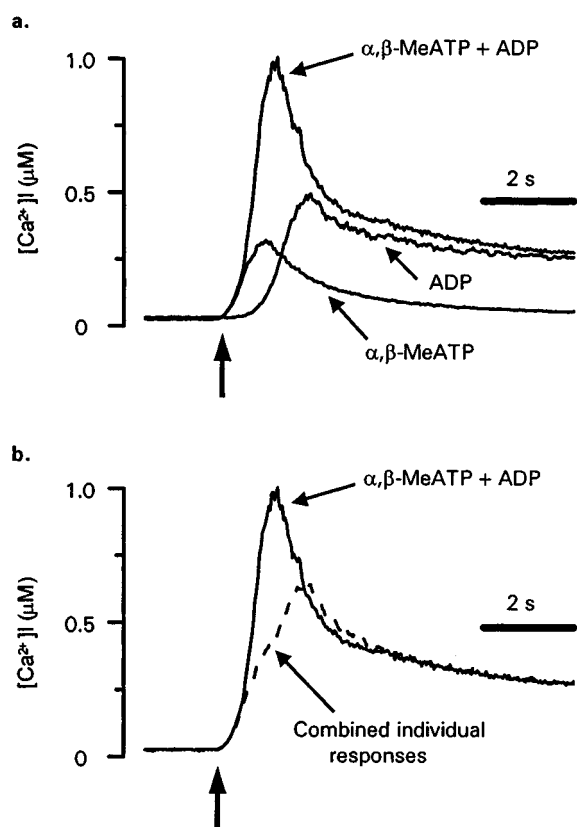

Figure 5 Low temperature studies of P2X and P2Y receptor interactions during platelet Ca^{2+} mobilization. (a) $[Ca^{2+}]_i$ responses of human platelets in stirred suspension at 13°C to α,β-MeATP (10 μM), ADP (10 μM) or simultaneous addition of both agonists (10 μM). Background-corrected 340/380 nm ratios are used to indicate $[Ca^{2+}]_i$ at the low temperature due to problems associated with accurately calibrating fura-2 fluorescence. (b) Comparison of the experimental trace following co-addition of α,β-MeATP and ADP with the result expected from summation of the responses to individual agonists (dashed line). The dashed line was derived by addition of the individual responses in panel a and subtraction of a F340/F380 nm ratio equal to the resting level prior to agonist stimulation. The vertical arrows indicate the point of agonist addition.

Table 1 Comparison of the kinetics and amplitude of platelet $[Ca^{2+}]_i$ responses following selective or simultaneous stimulation of P2X and P2Y receptors at 13°C

Agonist	Peak (F340/F380)	60s integral (F340/F380.60s)	Time to peak (s)	Time to 50% peak (s)
α,β-meATP	1.39 ± 0.08	11.46 ± 1.47	2.00 ± 0.09	1.12 ± 0.04
ADP	2.18 ± 0.16	41.41 ± 2.69	10.6 ± 0.66	6.28 ± 0.47
Co-stimulation:α,β-meATP+ADP	2.84 ± 0.57	48.48 ± 8.46	6.55 ± 0.23	2.93 ± 0.28
Theoretically combined individual: α,β-meATP+ADP	2.46 ± 0.10	52.86 ± 2.96	9.89 ± 0.72	5.31 ± 0.60
t-Test *p* value (co-stimulation *vs.* combined individual)	0.50	0.59	0.002	0.003

Parameters were measured for the experimental responses to α,β-meATP, ADP, or co-stimulation with both agonists, and also for the theoretically combined individual responses to both agonists. α,β-meATP and ADP were added at 10 μM in all experiments. The means and standard errors of the mean are given for seven experiments in each case. The theoretically combined individual responses were calculated as described in the legend to Figure 5.

Figure 6 P2X and P2Y receptor interactions during platelet Ca^{2+} mobilization at 37°C. (a) $[Ca^{2+}]_i$ responses of human platelets in stirred suspension at 37°C to α,β-MeATP (10 μM), ADP (10 μM) or simultaneous addition of both agonists (10 μM). (b) Comparison of the experimental trace following co-addition of α,β-MeATP and ADP with the result expected from summation of the responses to individual agonists (dashed line). The dashed line was derived by addition of the individual responses in panel a and subtraction of an $[Ca^{2+}]_i$ equal to the resting level prior to agonist stimulation. The vertical arrows indicate the point of agonist addition.

a slower P2Y receptor-coupled inward current (peak amplitude 0.62 ± 0.17 pA/pF, $n=11$) (Figure 4a) as described in rat megakaryocytes (Somasundaram & Mahaut-Smith, 1994). The P2X receptor and slower P2Y receptor coupled currents from individual cells are shown in Figure 4b, the best fit correlation line is shown, however this is not significant ($r=0.45$, $P=0.16$). ATP application evoked a

biphasic increase in intracellular Ca^{2+}; an initial small but rapid rise (18 ± 5 nM, $n=12$) associated with P2X receptor activation and a larger sustained P2Y receptor-mediated increase (peak increase 514 ± 81 nM, $n=12$) (Figure 4).

In P2X₁ receptor deficient mice, ATP failed to produce the fast transient inward P2X receptor current and intial increase in $[Ca^{2+}]_i$ observed in normal MKs. In addition the amplitude of the slower secondary current was reduced significantly to 0.24 ± 0.06 pA/pF ($n=15$; $P<0.05$). The amplitude of the ATP-evoked Ca^{2+} rise was not significantly affected by the P2X₁ receptor deficiency (412 ± 76 nM, $n=13$). Resting $[Ca^{2+}]_i$ levels were slightly higher in P2X₁ $-/-$ megakaryocytes (169 ± 25 nM, $n=15$) compared to $+/+$ mice (96 ± 22 nM; $n=15$), however this difference was not significant. The above data suggest a role for interaction of P2X and P2Y receptors in the megakaryocytes by potentiation of the delayed inward current that is associated with P2Y₁ receptor activation.

Interaction of P2X₁ and P2Y receptors in the activation of human platelet $[Ca^{2+}]_i$ responses

P2X₁ receptors stimulate a much larger increase in $[Ca^{2+}]_i$ in platelets compared to megakaryocytes (Rolf *et al.*, 2001; Somasundaram & Mahaut-Smith, 1994), therefore we also investigated interactions between P2X₁ and P2Y₁ receptor Ca^{2+} signalling in human platelets using α,β-meATP and purified ADP to selectively activate ionotropic and metabotropic purinoceptors, respectively (Ennion *et al.*, 2000). Initially, experiments were conducted at a reduced ambient temperature (13°C) in order to provide a clearer indication of the kinetics and interactions of P2X₁ and P2Y₁ receptor-evoked $[Ca^{2+}]_i$ responses (see Mahaut-Smith *et al.*, 2000). α,β-meATP (10 μM) evoked an immediate, transient increase in $[Ca^{2+}]_i$ (Figure 5a). In contrast, the $[Ca^{2+}]_i$ response to ADP (10 μM) had a characteristic lag, peaked at a higher level and remained elevated for longer periods. Average values for $[Ca^{2+}]_i$ changes; the peak increase, integral over the first 60 s and two estimates of speed of the response, time to peak and time to 50% of the peak, are shown in Table 1. Co-application of α,β-meATP and ADP (both 10 μM) resulted in a marked acceleration of the peak response compared with ADP stimulation alone (Figure 5a; Table 1). Figure 5b compares the mathematically-derived combined $[Ca^{2+}]_i$ response (dashed line) with the experimental effect of combined α,β-meATP and ADP stimulation. This demonstrates that the change in time-course of the peak response

Table 2 Comparison of the kinetics and amplitude of platelet [Ca^{2+}]$_i$ responses following selective or simultaneous stimulation of P2X and P2Y receptors at 37°C

Agonist	Peak (nM)	60s integral (nM.60s)	Time to peak (s)	Time to 50% Peak (s)
α,β-meATP	264 ± 18	1254 ± 117	1.09 ± 0.02	0.62 ± 0.02
ADP	508 ± 45	6931 ± 578	1.84 ± 0.07	1.29 ± 0.05
Co-stimulation: α,β-meATP + ADP	858 ± 46	7792 ± 578	1.35 ± 0.02	0.89 ± 0.01
Theoretically combined individual: α,β-MeATP + ADP	654 ± 43	8185 ± 584	1.75 ± 0.06	0.95 ± 0.03
t-Test *p* value(co-stimulation vs combined individual)	< <0.001	0.12	< <0.001	0.07

Parameters were measured for the experimental responses to α,β-meATP, ADP, or co-stimulation with both agonists, and also for the theoretically combined individual responses to both agonists. α,β-meATP and ADP were added at 10 μM in all experiments. The means and standard errors of the mean are given for 10 experiments in each case. The theoretically combined individual responses were calculated as described in the legend to Figure 6. $n = 10$, temperature = 37°C.

was not simply due to summation of the individual responses to the two agonists. The time to peak and time to 50% of the peak were both significantly reduced by co-stimulation (Table 1). However, there was no significant change in the peak Ca^{2+} response or the total Ca^{2+} increase, where the latter was measured as the integral of the Ca^{2+} response over the first 60 s (Table 1). At 37°C, the responses to ADP were considerably faster compared with 13°C, yet co-stimulation with α,β-MeATP still accelerated the overall response (Figure 6 and Table 2). The time to peak was significantly shorter following co-stimulation with α,β-MeATP and ADP compared to the combined individual responses. The time to 50% was slightly reduced for the co-stimulated compared to theoretically combined responses, however this difference was not significant. It is likely that the lack of significance results from the difficulty of accurately measuring responses with very short latencies. The integral of the Ca^{2+} response over the first 60 s remained unaltered by co-stimulation. Importantly, however, the peak [Ca^{2+}]$_i$ increase was significantly increased by co-stimulation at 37°C. Taken together, these data suggest that platelet P2X$_1$ and P2Y receptors display marked synergy by accelerating and amplifying the overall peak increase in [Ca^{2+}]$_i$.

Discussion

This study shows that the P2X$_1$ receptor is expressed in MKs and is essential for the production of functional P2X receptors. The data also indicate that MK size or number is not affected by the P2X$_1$ receptor deficiency demonstrating that these receptors do not play a central role in megakaryocytopoiesis. However, in electrophysiological and [Ca^{2+}]$_i$ studies we have detected synergistic interactions between P2X and P2Y receptors in MKs and platelets and this may resolve a functional role for P2X$_1$ receptors in these cells.

In bone marrow and spleen the co-expression of P2X$_1$ and CD41 immunoreactivity in hematopoietic cells indicates significant expression of P2X$_1$ receptors in MKs. This confirms previous studies showing that P2X$_1$ receptors are expressed by megakaryoblastic cell lines and platelets (Mackenzie et al., 1996; Vial et al., 1997) and indicates that if P2X$_1$ receptors are expressed by other hematopoietic cells in the marrow or spleen that they are below detection levels. In a variety of blood cell types changes in expression of P2X$_1$ receptors have been associated with differentiation or

apoptosis (Buell et al., 1996; Chvatchko et al., 1996; Clifford et al., 1998). The P2X$_1$ receptor is expressed by MKs from 1 day old (the earliest time-point tested) as suggested by Clifford et al. (1998). The normal distribution of MKs in P2X$_1$ receptor deficient mice demonstrates that the P2X$_1$ receptor is not essential for MK development. In MKs derived from the long bones the level of P2X$_1$ receptor immunoreactivity is maintained in the adult. In contrast the level of P2X$_1$ receptor immunoreactivity declines with age in splenic MKs suggesting that in the adult the properties of long-bone and spleen derived MKs are different. The reason for this difference is unclear. We were unable to obtain high resistance giga-seals on adult splenic MKs due to what appeared to be a reduced membrane deformability compared to marrow megakaryocytes and thus could not further assess P2X$_1$ current density in splenic MKs (R.J. Evans and M.P. Mahaut-Smith, unpublished observations). However, this property supports the conclusion that differences do exist between MKs from the two tissues.

The rapidly desensitizing α,β-meATP sensitive (and L-β,γ-methylene ATP sensitive, data not shown) P2X receptor mediated currents in the MKs bear the hallmark of the properties of recombinant P2X$_1$ receptors (Valera et al., 1994) and are essentially the same as those recorded from platelets (Mackenzie et al., 1996). The lack of rapid P2X receptor mediated responses to α,β-meATP or ATP in MKs from P2X$_1$ receptor deficient mice demonstrates that the P2X$_1$ receptor is essential for the production of functional P2X receptors in MKs. The fact that there is no residual response indicates that MKs do not express other P2X receptor isoforms and confirms previous studies using a degenerate PCR screening approach which failed to detect RNA transcripts for P2X receptor isoforms other than the P2X$_1$ subtype (Sun et al., 1998). It is therefore likely that MKs express homomeric P2X$_1$ receptors, as shown in smooth muscle cells of the vas deferens and bladder (Mulryan et al., 2000; Vial & Evans, 2000).

A role for P2X$_1$ receptors in platelets has been disputed. Two groups have reported a lack of P2X receptor-evoked shape change or aggregation (Jin & Kunapuli, 1998; Savi et al., 1998). This probably results from desensitization of the receptors during the isolation procedures. For example it has been shown that HL60 cells have to be treated with the P2 receptor antagonist suramin and/or apyrase (to breakdown endogenous ATP) to protect the cells from endogenously released ATP in culture to reveal the P2X$_1$-like phenotype (Buell et al., 1996). In human platelets, using conditions

designed to minimize desensitization, Rolf *et al.* (2001) have demonstrated a P2X receptor-mediated shape change. Therefore release of ATP and diadenosine polyphosphates during phlebotomy and/or from spontaneously activated platelets most likely accounts for the apparent difficulty in detecting a functional platelet response linked to the ionotropic purinoceptor (Rolf *et al.*, 2001). Apyrase is required *in vitro* to observe a platelet $P2X_1$ response, which may reflect the situation *in vivo* where ectonucleotidases metabolise ATP and thus limit P2X receptor desensitisation. A functional role of $P2X_1$ receptors on platelets has been suggested by the identification of a patient with a severe bleeding disorder that has a point deletion mutation in the second transmembrane domain resulting in the production of a dominant negative mutant (Oury *et al.*, 2000). Recent studies have shown that the $P2X_1$ receptor deficiency in mice leads to a significant ~70% increase in bleeding time (Hechler *et al.*, 2001) similar to that for the $P2Y_1$ receptor deficient mouse (Leon *et al.*, 1999). These results indicate that the $P2X_1$ receptor does play a role in the regulation of hemostasis.

P2X and P2Y receptors will normally be co-activated under physiological conditions, thus crosstalk between their signalling pathways may have potentially important consequences. $P2X_1$ receptor activation will result in Na^+ and Ca^{2+} influx and membrane depolarization (Evans *et al.*, 1996; Mackenzie *et al.*, 1996; Mulryan *et al.*, 2000). Activation of metabotropic $P2Y_1$ and $P2T_{AC}$ ($P2Y_{12}$) receptors will lead to a combination of signals including IP_3-mediated release of Ca^{2+} from intracellular stores, the stimulation of a slow secondary inward current, inhibition of adenylyl cyclase activity and activation of PI 3-kinase (Somasundaram & Mahaut-Smith, 1994; Kunapuli, 1998; Trumel *et al.*, 1999). Phospholipase-C and IP_3 receptor activity are known to be potentiated by an increase in $[Ca^{2+}]_i$ and therefore $P2X_1$ receptors could play a priming role in subsequent P2Y receptor responses (Bezprozvanny *et al.*, 1991; Eberhard & Holz, 1988). To investigate interactions between $P2X_1$ and P2Y receptor signalling we looked at agonist-induced membrane currents and $[Ca^{2+}]_i$ increases in MKs and platelets. In MKs the amplitude of the secondary current was significantly reduced in MKs from $P2X_1^{-/-}$ mice. These results suggest a functional interaction of signalling through $P2X_1$ and P2Y receptors.

An increase in cytosolic levels of both IP_3 and Ca^{2+} has been proposed to activate the P2Y receptor conductance (Hussain & Mahaut-Smith, 1998) therefore Ca^{2+} influx through $P2X_1$ receptors (Benham, 1989; Evans *et al.*, 1996) may explain the potentiation of the P2Y receptor current. Although the global Ca^{2+} increase mediated *via* $P2X_1$ receptors in MKs was extremely small (Figure 4a), a larger Ca^{2+} increase will occur immediately under the plasma membrane compared to further into the cell, and thus could act locally to enhance the subsequent secondary current. The functional relevance of P2 receptor-evoked $[Ca^{2+}]_i$ increases and associated membrane conductances in the MK are unknown. The P2Y receptor current carries Na^+ into the cell under normal conditions, however the ability to conduct Ca^{2+} is unclear. The fact that megakaryocytopoiesis was

unaltered in mice lacking $P2X_1$ receptors suggests that the reduction in the delayed P2Y receptor activated current does not have a major influence on MK development. However the data from the MKs clearly indicate that $P2X_1$ receptors can potentiate P2Y receptor signalling and this may be functionally more important when when $P2X_1$ receptor-evoked $[Ca^{2+}]_i$ responses are larger.

The rapid influx of Ca^{2+} through $P2X_1$ receptors causes a larger increase in $[Ca^{2+}]_i$ in platelets compared to megakaryocytes, presumably due to the larger surface area to volume ratio of the former (Mackenzie *et al.*, 1996; Somasundaram & Mahaut-Smith, 1994). In studies of human platelet $[Ca^{2+}]_i$ signalling, a significant interaction was observed between $P2X_1$ and P2Y receptors (Figures 5,6 and Tables 1,2). Co-activation of $P2X_1$ and P2Y receptors caused both an acceleration and an enhancement of the $[Ca^{2+}]_i$ increase compared with the result predicted from summation of the individual responses. Potentiation of $P2Y_1$ receptor-stimulated phospholipase-C or IP_3 receptor activity by $P2X_1$ receptor-derived intracellular Ca^{2+} can explain the synergistic action between the two receptors (Bezprozvanny *et al.*, 1991; Eberhard & Holz, 1988). Intracellular Na^+ also increases in platelets following stimulation of P2X receptors (Sage *et al.*, 1997), although there is less direct evidence for a Na-dependence to metabotropic receptor-dependent Ca^{2+} signalling. However, in the platelet suspension experiments $P2X_1$ receptors will depolarize the cell which has been shown to stimulate Ca^{2+} release during P2Y receptor activation in the megakaryocyte (Mahaut-Smith *et al.*, 1999). The ability of P2X receptors to potentiate metabotropic receptor events and thus accelerate platelet activation may play a part in determining the overall speed of the hemostatic response. Alternatively, this synergistic action of $P2X_1$ receptors may make platelets more prone to unwanted activation during thrombosis.

In conclusion, our results in MKs demonstrate that $P2X_1$ receptors mediate the initial transient inward current and Ca^{2+} influx triggered by ATP, but that this receptor is not essential for normal megakaryocytopoiesis. We also show a synergistic interaction between $P2X_1$ and P2Y receptors in both megakaryocytes and platelets which suggests that $P2X_1$ receptors could have a priming role in the activation of P2Y receptors. Since large amounts of ATP will be rapidly released from damaged cells at the site of vascular injury, $P2X_1$ receptors may therefore act to accelerate platelet activation during hemostasis or thrombosis.

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