Interplay between P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2X<sub>1</sub> receptors in the activation of megakaryocyte cation influx currents by ADP; evidence that the primary megakaryocyte represents a fully functional model of platelet P2 receptor signaling*

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Abstract

The difficulty of electrophysiological recordings from the platelet has restricted investigations into the role of ion channels in thrombosis and haemostasis. We now demonstrate that the well-established synergy between P2Y₁ and P2Y₁₂ receptors during ADP-dependent activation of the platelet α₁β₃ integrin also exists in murine marrow megakaryocytes, further supporting the progenitor cell as a bonafide model of platelet P2 receptor signalling. In patch clamp recordings, ADP (30 µM) stimulated a transient inward current at –70 mV, which was carried by Na⁺ and Ca²⁺ and amplified by phenylarsine oxide, a potentiator of certain TRP channels via phosphatidylinositol 4,5-bisphosphate depletion. This initial current decayed to a sustained phase, upon which were superimposed repetitive transient inward cation currents with predominantly P2X₁-like kinetics. Abolishing P2X₁ receptor activity prevented most of the repetitive currents, consistent with their activation by secreted ATP. Recordings in P2Y₁ receptor-deficient megakaryocytes demonstrated an essential requirement of this receptor for activation of all ADP-evoked inward currents. However, P2Y₁₂ receptors, via activation of PI-3 kinase, played a synergistic role in both P2Y₁ and P2X₁ receptor-dependent currents. Thus, direct stimulation of P2Y₁ and P2Y₁₂ receptors, together with autocrine P2X₁ activation, are responsible for activation of non-selective cation currents by the platelet agonist ADP.
Introduction

Platelets possess three P2 receptors, P2X1, P2Y1 and P2Y12, which all play important roles in haemostasis and thrombosis. P2X1 receptors are cation channels that allow significant Na⁺ and Ca²⁺ entry and are activated by ATP, but not ADP. P2Y1 and P2Y12 receptors are G-protein-coupled receptors at which ADP is the physiological ligand during platelet activation. P2Y1 receptors are principally coupled through Ga₄ proteins leading to activation of phospholipase-Cβ and thus IP3-dependent Ca²⁺ mobilisation. The signalling pathway of Gaᵢ-coupled P2Y12 receptors is less clear, with roles proposed for PI-3 kinase, Rap1B, K⁺ channels and Akt. P2Y12 receptors also cause inhibition of adenylate cyclase, although the importance of this pathway is unclear as it is not essential for activation of functional responses by ADP in vitro. One possibility is that in the circulation this P2Y12 receptor signal serves to counteract the stimulation of cAMP formation by endothelial-derived prostacyclin and thus relieve protein kinase A-dependent inhibition of IP3-mediated Ca²⁺ release.

An important feature of P2 receptor-dependent platelet activation is the synergy that exists between their signalling pathways leading to amplification of functional responses. For example, co-activation of P2Y1 and P2Y12 receptors is required for full ADP-dependent exposure of fibrinogen binding sites on the αIIbβ3 integrin, and thus aggregation. Furthermore, P2X1 receptors can amplify ADP-evoked Ca²⁺ increases, and thereby amplify platelet activation during co-release of ATP and ADP from dense granules. The underlying mechanisms whereby P2 receptor signalling pathways interact in the platelet are important issues that remain poorly resolved.
Ion channels play fundamental roles in all cell types, however their study has proven difficult in the tiny, fragile platelet. Since the platelet has an extremely limited capacity to synthesize its own proteins, the mature megakaryocyte (MK) is likely to express all ion channels of its daughter cell. Indeed, patch clamp studies of MKs were crucial in the identification of P2X1 as the sole ionotropic receptor underlying ATP-evoked Ca\textsuperscript{2+} influx in the platelet \textsuperscript{1,25}. P2Y receptor activation in the MK also stimulates cation-permeable ion channels that are candidates for store-dependent and store-independent Ca\textsuperscript{2+} influx in the platelet/MK lineage \textsuperscript{1,26-29}. However, the extent to which MKs express both P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors is unclear, as is the ability of these receptors to couple to Ca\textsuperscript{2+}-permeable conductances and downstream functional responses in the progenitor cell. The present study has investigated these issues in freshly isolated MKs from murine marrow with the aid of receptor-deficient models and pharmacological reagents. We demonstrate that the marrow-derived MK is a high fidelity model of platelet P2Y receptor interactions and that all three platelet P2 receptors are involved in the activation of cation influx currents during ADP stimulation. Furthermore, we provide the first direct recordings under physiological Ca\textsuperscript{2+} buffering conditions of a Ca\textsuperscript{2+} current linked directly to G-protein receptor activation in the MK/platelet lineage. The MK therefore provides a means to identify the underlying channel which will contribute to agonist-evoked Ca\textsuperscript{2+} influx in the platelet.
Methods

Megakaryocyte Isolation. Megakaryocytes from the tibial and femoral marrow of adult C57BL/6 mice were prepared for patch clamp recordings as described in detail elsewhere \(^{25,27}\). P2X\(_1^{-/-}\) and P2Y\(_1^{-/-}\) mice were bred as previously described \(^{10,30}\).

Reagents and Salines. The saline used for marrow isolation, storage and for confocal experiments contained (in mM): 145 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes, 10 Glucose, pH 7.35 (NaOH). 0.32 U/ml type VII apyrase (Sigma-Aldrich, Poole, UK) was also included in the preparation of cells for electrophysiological experiments. Electrophysiological salines were designed to eliminate K\(^+\) currents \(^{25,26,28}\); the standard bath saline contained (mM): 150 NaCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes, 10 Glucose, pH 7.35 (NaOH) and the standard pipette saline contained (mM): 150 CsCl, 2 MgCl\(_2\), 0.05 Na\(_2\)GTP, 0.05 K\(_5\)Fura-2, 10 Hepes, 0.1 EGTA, pH 7.2 (CsOH). For studies of Ca\(^{2+}\) permeability, Na\(^+\) was replaced by the large, impermeant cation NMDG\(^+\) and the saline pH adjusted with HCl. The absence of voltage-gated outward K\(^+\) currents following a depolarizing step to from –70 to 0 mV was used to confirm that adequate dialysis had occurred to eliminate K\(^+\)-selective currents \(^{31,32}\).

We have previously demonstrated that Cl\(^-\) conductances do not contribute to the nucleotide-evoked currents under these conditions \(^{26,28,29}\). K\(_5\)fura-2 and Oregon green-labelled fibrinogen were from Molecular Probes (Leiden, Netherlands). The latter was centrifuged at 13,200 rpm for 5 min prior to use. Unless otherwise stated, all other reagents were from Sigma Aldrich (Poole, UK). ADP was treated with hexokinase to remove contaminating ATP, as previously reported \(^{7}\) and a luciferin: luciferase assay used to test that treated samples contained negligible levels of ATP (C.Cendana & M.P.Mahaut-Smith, unpublished). Cells were incubated with LY294002 (Tocris Cookson Ltd, Bristol, UK) for 15 minutes prior to experiments. All other pre-
treatments were applied 2 minutes prior to ADP application. Vanadate was prepared either by dissolving sodium orthovanadate directly in the standard bath saline or as described previously \(^{33}\); no difference was observed between these two preparations. AR-C69931MX was a kind donation from AstraZeneca (Moindal, Sweden).

**Electrophysiology.** Conventional whole-cell patch clamp recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) in voltage clamp mode as described in detail elsewhere \(^{25,27}\). Membrane currents were filtered at 1 kHz and acquired to computer at a rate of 5 kHz using a Digidata 1200 series A/D converter and Axotape software (Axon Instruments, Foster City, CA). All experiments were carried out at room temperature.

\([\text{Ca}^{2+}]_i\), recordings. \([\text{Ca}^{2+}]_i\) was measured by single cell fluorescence photometry as described in detail elsewhere \(^{34,35}\). Briefly, fura-2 was excited at 340 and 380 nm wavelength and emitted light detected at 480-600 nm. Data were sampled at 60 Hz and averaged to give a final sample rate of 15 Hz. Background fluorescence was subtracted and \([\text{Ca}^{2+}]_i\) calculated from the 340/380 nm fluorescence ratios as described previously using a viscosity correction factor of 0.85 \(^{34-36}\).

**Fluorescence imaging of fibrinogen receptor activation.** Confocal fluorescence imaging was conducted, at room temperature, using a Zeiss LSM 510 (Carl Zeiss Ltd, Welwyn Garden City, UK) with Plan-Neofluar 40x 1.3 oil and Plan-Apochromat 63x 1.4 oil DIC objectives, excitation and emission wavelengths of 488 and \(>505\) nm, respectively, and an optical section of \(\leq 3\mu m\). Cells were mixed with 30 \(\mu g\) ml\(^{-1}\) Oregon Green-labelled fibrinogen prior to addition to the experimental chamber. Images acquired before and after addition of ADP were analysed using either Image J 1.32 (a public domain program developed at the National Institutes of Health by Wayne Rasband and available at \(http://rsb.info.nih.gov/ij/\)) or in-house custom-
designed software (LSM Toolbox, written by Dr C.J.Schwiening, University of Cambridge). For comparison of spatial fluorescence profiles (Fig. 1B), background-subtracted fluorescence levels ($f$) were expressed relative to the average extracellular fluorescence ($f_e$). ADP promoted the binding of exogenous fibrinogen preferentially to the periphery of the megakaryocyte, which was further quantified as the ratio of the peripheral fluorescence ($f_p$), relative to $f_e$ (see Fig. 1C). In control, time-matched experiments this ratio remained constant in the absence of added agonist (G.Tolhurst & M.P.Mahaut-Smith, unpublished observations).

**Analysis.** Membrane currents were analysed in Clampfit v9.0 (Axon Instruments). Electrophysiological data and fura-2 fluorescence were also exported, for analysis and presentation, within Microcal Origin (Microcal Software Inc, Northampton, MA, USA). Where applicable, current decays were fitted with a single exponential decay ($y = y_0 + A_1 e^{-(x-x_0)/t_1}$). The activation time for both the $[Ca^{2+}]_i$ increase and membrane current were taken as the point where the trace first increased above (or decreased below) the noise level of the steady-state recording under basal conditions. Data are expressed as the mean ± s.e.m and statistical analysis was performed using a Student’s paired or unpaired $t$-test.
Results

Synergy between P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors at the level of fibrinogen receptor activation in single marrow megakaryocytes

To investigate the ability of P2Y receptors to couple to functional platelet responses in marrow-derived MKs, we examined ADP-dependent α<sub>IIb</sub>β<sub>3</sub> integrin activation using confocal fluorescence measurements of fibrinogen binding. Prior to the addition of agonist, negligible levels of fibrinogen were bound to the MK (see fluorescence image in Fig. 1Aii and line profile plots in Fig. 1Bi). 30 µM ADP induced a marked increase in fluorescence predominantly at the periphery of the MK (Fig. 1Aiii, and Bii, control; see also supplementary movie). The peripheral/external fluorescence ratio (fp/fe) increased from 0.85 ± 0.05 to a peak of 5.69 ± 0.22 (n=10) (Fig. 1C). This response was virtually eliminated by the P2Y<sub>12</sub> receptor inhibitor AR-C69931MX (1 µM) or in P2Y<sub>1</sub>-/- MKs (Fig. 1B,C). Compared to control cells, the peak fp/fe increase was only 3 % in AR-C69931MX (an increase from 0.95 ± 0.05 to 1.08 ± 1.3, n=10; P=<0.001) and 2% in P2Y<sub>1</sub>-/- MKs (an increase from 0.88 ± 0.02 to 0.96 ± 0.03, n=10; P=<0.001). Thus, co-activation of P2Y<sub>1</sub> and P2Y<sub>12</sub> is essential for ADP-evoked fibrinogen receptor activation in the primary mouse MK, as previously shown in human and murine platelets<sup>20-22,37,38</sup>. Together with previous reports that megakaryocytes, like platelets, possess only P2X<sub>1</sub> receptors within this family of ATP-gated ion channels<sup>25</sup>, these data support use of the MK as a fully functional model for investigations of platelet P2 receptor signalling.
**ADP elicits multiple phases of cation influx currents which are all dependent upon activation of P2Y<sub>1</sub> receptors**

Under conditions that eliminate K<sup>+</sup>-selective currents and thereby allow measurements of cation influx currents, 30 µM ADP activated an early transient inward current which coincided with the initial increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]) (Fig. 2A). This current corresponds to the P2Y receptor-evoked transient inward current that we have previously reported in MKs from wild type rodents and P2X<sub>1</sub> knock-out mice<sup>25</sup>, and is largely carried by Na<sup>+</sup> in physiological salines<sup>25,28,29</sup>. During maintained exposure to 30 µM ADP, the initial current declined to a sustained phase and followed a timecourse which mirrored the plateau phase of the [Ca<sup>2+</sup>] increase. In addition, many cells displayed repetitive transient inward currents superimposed upon the sustained phase of the response (see Fig. 2B). Although similar transient currents were also observed spontaneously, their frequency markedly increased during application of ADP (see Fig. 2D). 30µM ADP failed to activate detectable [Ca<sup>2+</sup>] increases and membrane currents in MKs from P2Y<sub>1</sub><sup>−/+</sup> mice (n=7, Fig. 2C). Spontaneous repetitive-type transient currents were occasionally detected in P2Y<sub>1</sub><sup>−/+</sup> MKs, although the frequency of these events was also unaffected by application of ADP (P=>0.05, Fig. 2D). Thus, P2Y<sub>1</sub> receptors are essential for all membrane currents activated in the MK by this high concentration of ADP. In the absence of ADP, the repetitive transient currents were less frequent in P2Y<sub>1</sub><sup>−/+</sup> versus wild type MKs (n=7, P=>0.05, Fig.2D), thus this receptor is also involved in the spontaneous activation of inward cation currents.
The repetitive transient currents are predominantly due to autocrine activation of P2X1 receptors

The repetitive inward currents during the plateau phase of the ADP-stimulated \([\text{Ca}^{2+}]_i\) increase exhibited a range of kinetics, although the majority (84%) had a rise time (time to peak) of ≤ 100 msec and a decay constant of < 200 ms (Fig. 3A). These rapid kinetics are characteristic of P2X1 receptors in the platelet and other cell types \(^6,39,40\). Consistent with this interpretation, the majority of the transient events were absent in MKs from P2X1\(^{-/-}\) mice (n=11, Fig. 3B). A similar effect was observed after desensitisation of P2X1 receptors with 10 \(\mu\text{M}\) \(\alpha,\beta\)-meATP and or following their inhibition with 1 \(\mu\text{M}\) NF449 \(^6,41\) (n=7, \(P<0.001\); see Fig 3B). As expected, the remaining repetitive transient currents under these conditions had slower kinetics; they decayed either with a single exponential (range 395 – 1314 msec) or with a more complex timecourse (not shown) and followed a rise time of 340 - 2060 msec (see examples in Fig. 3C for P2X1\(^{-/-}\) MKs). As we have previously shown, these slower ADP-evoked repetitive transient currents were associated with a small transient increase in \([\text{Ca}^{2+}]_i\) in the presence or absence of extracellular \(\text{Ca}^{2+}\) \(^26\), suggesting that they may be dependent upon the cytosolic free \([\text{Ca}^{2+}]_i\).

A similar mixture of rapid, P2X1 and slower non-P2X1 inward currents were also detected in the absence of exogenous agonist, but at a lower frequency (Fig. 3D,E). As shown for ADP stimulation, conditions that remove P2X1 receptor activation (P2X1\(^{-/-}\) or pre-exposure to either 1 \(\mu\text{M}\) NF449 or 10 \(\mu\text{M}\) \(\alpha,\beta\)-meATP) abolished the majority of the spontaneous membrane currents (Fig. 3E). The remaining spontaneous slower inward currents often co-incided with a small spontaneous increase in \([\text{Ca}^{2+}]_i\), and were absent in P2Y1\(^{-/-}\) MKs (not shown, but see earlier). Together, these data
suggest that the repetitive inward transient currents under both spontaneous and ADP-stimulated conditions are predominantly mediated by P2X$_1$ receptors, with a contribution from another pathway dependent upon P2Y$_1$ receptor activation. Since ADP is not an agonist at P2X$_1$ receptors $^7,42$, and the MKs were moved away from other cells in these experiments, activation of the ionotropic receptor must be mediated via an autocrine mechanism, following secretion of ATP or diadenosine polyphosphates, as suggested previously by our group $^1,43$ and by Kawa $^{44}$.

**P2X$_1$ receptor-dependent priming of the ADP-evoked current**

Genetic deletion of P2X$_1$ receptors had no significant effect on the amplitude or kinetics of the [Ca$^{2+}$]$_i$ increase or the amplitude of the initial membrane current evoked by 30 $\mu$M ADP ($P=>0.05$; $n=8$; see examples in Fig. 4A). However, one noticeable effect of a loss of P2X$_1$ receptors was a small, but significant shift in the onset of the initial inward current relative to the [Ca$^{2+}$]$_i$ increase (Fig. 4A,B). In control cells, the inward current commenced, on average, prior to the first detectable [Ca$^{2+}$]$_i$ increase ($-36.67 \pm 88.99$ msec, $n=9$). In P2X$_1^{-/-}$ MKs, the current shifted to $224 \pm 64$ msec relative to the first detectable [Ca$^{2+}$]$_i$ increase, $P=<0.05$ (n=8; see Fig. 4C). This increased latency was also observed following pre-incubation with 1 $\mu$M NF449 ($894 \pm 297$ msec, $n=5$, $P=<0.05$) or 10 $\mu$M $\alpha,\beta$-meATP ($280 \pm 84$ msec, $n=5$, $P=<0.05$; Fig. 4C). We have previously reported that P2X$_1$ receptors potentiate the peak amplitude of the P2Y-dependent cation current during co-stimulation of the ionotropic and G-protein-coupled purinoceptors $^{25}$. The present study therefore adds a further synergistic role for the ionotropic receptor in that it accelerates the onset of the P2Y-evoked current relative to the [Ca$^{2+}$]$_i$ increase. One explanation for this effect is
that the spontaneous activation of P2X1 receptors leads to a basal elevation of [Ca\(^{2+}\)]\(_i\) levels immediately under the plasma membrane, and thereby accelerates the onset of the P2Y receptor-evoked current, either by acting directly on the channel or on an earlier Ca\(^{2+}\)-dependent component of the cascade such as phospholipase-C\(^{25,26,45}\). This “priming” of a Ca\(^{2+}\)-permeable conductance may represent one mechanism whereby P2X1 receptors can accelerate P2Y-mediated Ca\(^{2+}\) signals and thereby potentiate platelet activation\(^{1,3,25}\).

**A synergistic role for P2Y\(_{12}\) receptors in the P2Y\(_{1}\) and P2X\(_1\)-dependent membrane currents**

In contrast to the complete loss of responses in P2Y\(_{1}\)^{−/−} MKs, ADP was still able to induce [Ca\(^{2+}\)]\(_i\) increases and inward currents following inhibition of P2Y\(_{12}\) receptors by a supramaximal concentration of AR-C69931MX (1 µM)\(^{46}\). No significant effect of this compound was observed on the amplitude or time to peak of the [Ca\(^{2+}\)]\(_i\) increase (Fig. 5C; \(P=>0.05\); n=8), although due to the marked single cell heterogeneity of the Ca\(^{2+}\) responses, we cannot rule out small effects on the ADP-evoked Ca\(^{2+}\) mobilisation. However, AR-C69931MX significantly reduced the amplitude of the initial ADP-evoked current from \(-0.72 ± 0.19\) pA/pF, n=9 to \(-0.17 ± 0.04\) pA/pF, \(P=<0.05\) (Fig. 5A,B). P2Y\(_{12}\) receptor inhibition also delayed the onset of the current relative to the first detectable [Ca\(^{2+}\)]\(_i\) increase (from \(-36.67 ± 88.99\) msec, n=9 to \(374.37 ± 77.34\) msec, n=8, \(P=<0.05\)), although this may simply reflect an increased time to detection above background noise levels for the smaller amplitude current. The ability of the P2Y\(_{12}\) receptor to potentiate this ADP-evoked conductance was largely due to signalling via PI 3-kinase since LY294002 (30 µM,
n=5), a blocker of this enzyme, decreased the current amplitude to a similar extent to AR-C69931MX (Fig. 5A,B). The average ADP-evoked Ca\(^{2+}\) increase was slightly suppressed compared to control (Fig. 5C), however as observed for AR-C69931MX, this was not statistically significant (\(P=>0.05\)).

AR-C69931MX (1 \(\mu\)M), and LY294002 (30 \(\mu\)M), also caused a marked, significant reduction in the repetitive transient currents evoked by a sustained ADP application, from 8.85 ± 2.04 events per min, n=15, to 1.87 ± 0.48, n=6 (\(P=<0.005\)) and 0.46 ± 0.30 events per min, n=7 (\(P=<0.005\)), respectively for AR-C69931MX and LY294002 (see Fig. 5D). Inhibition of P2Y\(_{12}\) and PI 3-kinase using these compounds also decreased the basal activity of the transient currents, though the reduction was only significant in the presence of LY294002 when compared to control, 0.32 ± 0.0.21 (n=5) from 2.63 ± 0.73 events per min, n=15, (\(P=<0.01\)), respectively. As described above, these transient currents represent autocrine activation of predominantly P2X\(_1\), following co-secretion of ATP and ADP, under both basal and ADP-stimulated conditions. Taken together, these data show that in addition to a synergistic effect on the main P2Y\(_1\) receptor-evoked conductance, P2Y\(_{12}\) receptors also contribute, via PI-3 kinase, to autocrine activation of P2X\(_1\) receptors during ADP stimulation.

The main P2Y receptor-evoked conductance is Ca\(^{2+}\)-permeable and markedly amplified by phenylarsine oxide, an agent known to amplify TRP channels via PIP\(_2\) depletion.
Previous work has shown that \( \text{Na}^+ \) conducts most of the initial P2Y receptor-evoked inward current in physiological external salines\(^{28,29}\). Detection of a \( \text{Ca}^{2+} \) permeability through this conductance has proven difficult, in part due to the reduction in both cell viability and responses to ADP as external \( \text{Ca}^{2+} \) levels are increased\(^{28}\). More recent work has demonstrated the presence of three members of the transient receptor potential family of ion channels, TRPC1, 4 and 6 in platelets and/or MKs\(^{47-49}\), which are all non-selective cation channels and activated by G-protein-coupled receptors coupled to phospholipase-C\(^{50,51}\). Therefore, we further examined the extent to which \( \text{Ca}^{2+} \) can permeate the P2Y receptor-dependent channel. External \( \text{Na}^+ \) was replaced with the large impermeant cation NMDG\(^+\), Mg\(^{2+}\) ions were omitted, and \( \text{Ca}^{2+} \) was increased to as high a concentration as the cell would withstand (2.5mM) yet still generate a robust P2Y receptor-evoked \([\text{Ca}^{2+}]_i\) increase. These conditions greatly suppressed the current amplitude, as expected for a \( \text{Na}^+ \)-permeable conductance, however we were able to detect a small initial transient current with a timecourse similar to that observed in \( \text{Na}^+ \)-containing saline and mirroring the initial \( \text{Ca}^{2+} \) spike (Fig. 6A, representative of 6 cells). This residual current was carried by \( \text{Ca}^{2+} \) and not carried by NMDG\(^+\) as no detectable ADP-evoked inward current was observed in divalent cation-free, NMDGCl saline (n=9). Furthermore, the current was also not due to P2X\(_1\) receptors, as it was observed in the presence of the P2X\(_1\) blocker NF449 (1 \( \mu \text{M}, \) n=6).

Phenylarsine oxide (PAO, 15 \( \mu \text{M} \)), an agent shown to potentiate a phospholipase-C-dependent TRP channel via PIP\(_2\) depletion\(^{52}\), greatly amplified the ADP-evoked inward current in the MK (Fig. 6B). This action of PAO was not due to inhibition of tyrosine phosphatases\(^{53}\) as vanadate (100 \( \mu \text{M} \)), another blocker of such enzymes, had
no significant effect on the ADP-evoked current (n=5, P=>0.05; Fig. 6C). PAO also amplified the ADP-evoked current in Na\(^+\) and Mg\(^{2+}\)-free, 2.5mM Ca\(^{2+}\) medium (n = 4, P=<0.05, Fig. 6C), further indicating an underlying Ca\(^{2+}\) permeability.

**Discussion**

Activation of platelets by extracellular ADP plays a key role in thrombosis and haemostasis. It is well established that co-stimulation of P2Y\(_1\) and P2Y\(_{12}\) receptors is required for ADP-dependent exposure of fibrinogen binding sites on the platelet integrin \(\alpha_{\text{IIb}}\beta_{3}\), leading to the main functional response of aggregation\(^2,4\). However, the mechanism whereby these two receptors interact remains poorly resolved. We now show that the functional synergy between P2Y\(_1\) and P2Y\(_{12}\) receptors also exists in the primary MK, further supporting direct use of these large cells for studies of platelet signalling. In particular, the MK provides an important tool for investigations of ionic conductances during platelet activation. In the present study we have focused on understanding the relative role of different P2 receptors in the activation of pathways that conduct cations into the cell during stimulation by ADP. The complete absence of ADP-evoked cation influx currents in the P2Y\(_{1}\)^{-/-} MKs demonstrates an essential role for this receptor, however the present work also provides evidence for synergistic roles for both P2Y\(_{12}\) and P2X\(_1\) receptors. Another important observation within this study is that the P2Y receptor-evoked conductance previously shown to be mainly Na\(^+\) influx, is also permeable to Ca\(^{2+}\), a key second messenger in the platelet. Therefore, this conductance provides a means whereby P2Y\(_{12}\), P2Y\(_1\) and P2X\(_1\) receptors can positively interact in the stimulation of Ca\(^{2+}\) influx.
The mechanism whereby GPCRs can activate Ca\(^{2+}\)-permeable channels remains highly controversial in a variety of non-excitablc cell types, including the platelet. One extensively studied system is the activation of TRP and TRP-like channels by rhodopsin in the Drosophila photoreceptor\(^{54}\). Considerable note has been taken of this pathway due to the relative ease of manufacturing specific mutants and the fact that TRP channel homologues are the principal candidates for non voltage-gated Ca\(^{2+}\) influx pathways in mammalian cells. A crucial requirement has been demonstrated for phospholipase-C activity in the gating of Drosophila TRP channels by rhodopsin, however the specific mechanism of channel activation is still unclear\(^{55}\). In the Drosophila photoreceptor as well as in mammalian cells, roles have been proposed for second messengers generated by PIP\(_2\) hydrolysis, particularly diacylglycerol and products of its own metabolism\(^{56,57}\), although it is becoming increasingly apparent that multiple signalling modalities are involved\(^{50,58}\). Indeed, we have previously provided evidence that an increase in cytosolic IP\(_3\) (or a metabolite of IP\(_3\)) can activate a whole-cell cationic conductance and that cytosolic Ca\(^{2+}\) has at least a modulatory role in the ADP-evoked cation channel\(^{26,28,29}\). Of particular note, it has recently been postulated that a decrease in PIP\(_2\) may act as signal to open TRP channels in the Drosophila photoreceptor\(^{55}\). This is not surprising given the considerable number of other membrane proteins known to be modulated by the level of PIP\(_2\) in the membrane\(^{59}\). In mammalian cells the activation of TRPV1 channels by receptors coupled to phospholipase-C is greatly potentiated by depletion of PIP\(_2\) levels with PAO\(^{52}\). We also show that this compound potentiates the initial ADP-evoked cation current (Fig. 6) and therefore PIP\(_2\) depletion may also play a major role in the P2Y-evoked cation current in the MK/platelet. Since a major effect of P2Y\(_{12}\) receptors is the conversion of PIP\(_2\) to PIP\(_3\) by PI 3-kinase, PIP\(_2\) depletion provides a
mechanism whereby this G\(\alpha\)-coupled receptor can potentiate the P2Y\(_1\)-induced conductance. Other P2Y\(_12\) events may contribute, including an increase in PIP\(_3\), diacylglycerol, modulation of the cytoskeleton, and activation of lipid kinases such as Btk. Thus, further studies are required to clarify the relative roles of these different signals and the specific mode of action by PAO. Nevertheless, a role for PIP\(_2\) depletion seems plausible, especially given the onset of the current prior to the rise in cytosolic Ca\(^{2+}\) under control conditions (see Fig. 4).

The molecular identity of the Ca\(^{2+}\)-permeable ion channel underlying the main ADP-evoked conductance in the MK/platelet also remains unknown, however its ability to be amplified by PAO should provide a useful tool in future studies. Evidence to date suggests that Ca\(^{2+}\) influx triggered by P2Y and other G\(\alpha\)-coupled receptors can occur via second messenger-dependent and store depletion-dependent pathways (termed store-independent and store-dependent Ca\(^{2+}\) influx)\(^{62}\). Previous work has proposed roles for TRPC1 and TRPC6 in Ca\(^{2+}\) influx in the platelet and megakaryocyte\(^{47-49}\), although the relative contribution by these and other TRP channels remains controversial\(^{63}\). The main electrophysiological candidate for the ionic conductance activated by store depletion in the platelet/MK as in many other non-excitable cells is the highly Ca\(^{2+}\)-selective CRAC conductance (CRAC = Ca\(^{2+}\)-release-activated Ca\(^{2+}\))\(^{27,28,64}\). However, this current can only be recorded in the megakaryocyte when the intracellular Ca\(^{2+}\) levels are highly buffered with a chelator such as EGTA or BAPTA\(^{28}\), and it is unclear whether this current is active under normal conditions.

Furthermore, although direct depletion of intracellular Ca\(^{2+}\) stores by agents such as thapsigargin lead to Ca\(^{2+}\) influx, and thus is a pathway assumed to occur in response to agonists, recent work suggests that activation of IP\(_3\) receptors rather than store
depletion may be the crucial transducing event required for activation of this influx component. Thus, considerably more work is required to identify the agonist-evoked Ca\(^{2+}\)-permeable ion channels in the platelet. The present study provides the first direct electrophysiological evidence for a Ca\(^{2+}\) current coupled to a G-protein-coupled receptor in the platelet/MK under physiological Ca\(^{2+}\) buffering conditions. Due to the small Ca\(^{2+}\) permeability, the Ca\(^{2+}\) current was only clearly visible when the conductance was at its largest, that is during the large initial [Ca\(^{2+}\)]\(_i\) increase. In physiological salines, where Na\(^+\) is the main current carrier, this conductance also continued as a smaller sustained current in many recordings throughout the plateau phase of the [Ca\(^{2+}\)]\(_i\) increase. We therefore interpret this as evidence for a pathway that provides a biphasic Ca\(^{2+}\) and Na\(^+\) influx during stimulation of P2Y receptors, with initial and sustained phases.

Although we observe marked effects of P2Y\(_{12}\) and PI 3-kinase blockade on the ADP-evoked membrane currents, we did not observe significant effects on the intracellular Ca\(^{2+}\) increase evoked by ADP in the MK. This is in contrast to the recent study by Hardy et al., who report a reduction in ADP-evoked Ca\(^{2+}\) increases following inhibition of P2Y\(_{12}\) receptors or PI 3-kinase in human platelets. In part this may reflect the heterogeneity of the MK Ca\(^{2+}\) responses and thus the inability to detect small changes in responses averaged from single cell experiments compared to measurements in platelet populations. However, it may also result from a greater contribution of Ca\(^{2+}\) release relative to influx in the MK due to the larger cellular volume compared to the platelet. In the platelet, it is likely therefore that P2Y\(_{12}\)/PI 3-kinase-dependent potentiation of a non-selective cation conductance could
significantly contribute to the synergy reported between P2Y$_1$ and P2Y$_{12}$ during ADP-stimulated Ca$^{2+}$ mobilisation$^{54}$ and is worthy of further investigation.

In comparison to the well established roles for P2Y$_1$ and P2Y$_{12}$ receptors in platelet activation, the importance of the P2X$_1$ receptor has been less clear. Recent work has shown that P2X$_1$ receptors contribute to the risk of thrombosis, particularly in small arteries$^{1,3,67}$, however the mechanisms whereby this receptor can influence platelet activation is unclear. The P2X$_1$-dependent priming of the P2Y$_1$ membrane current (see Fig. 4) may represent a means of accelerating P2Y$_1$ receptor responses as we have observed in platelet suspensions$^{25}$. Furthermore, co-stimulation of P2Y$_1$ and P2Y$_{12}$ induces repetitive autocrine activation of P2X$_1$ receptors, with a role for PI 3-kinase. Since ADP is not directly an agonist at the P2X$_1$ receptor$^{7}$ and our ADP samples were treated to remove ATP contamination (confirmed by luciferin:luciferase measurements), the P2X$_1$ responses that we observe must be due to ATP secretion. It was interesting that multiple P2X$_1$ responses could occur in the same cell and must reflect focal activation by released quanta of ATP. This type of local activation has also been observed in PC12 cells$^{68}$ and human platelets$^{43}$ and provides a means whereby P2X$_1$ stimulation can be sustained. Autocrine activation may allow the P2X$_1$ receptor to signal in a prolonged and more efficient manner compared to the one-off activation observed following exogenous $\alpha,\beta$-meATP$^{1,3,25,26,45}$.

In conclusion, we have established that the marrow-derived MK displays fully functional interactions between platelet P2Y receptors, thereby supporting use of the progenitor cell for studies of platelet signalling pathways, particularly ion channels. We show that both P2Y$_1$ and P2Y$_{12}$ are required for complete activation of a non-
selective cation channel that results in Ca^{2+} as well as Na^+ entry. Furthermore, P2X_1 receptors, following secretion of ATP, can contribute repetitively to the ADP-evoked currents and act to accelerate the P2Y receptor currents. This complex interplay, as described in the model (Fig. 7), may allow all three receptors to co-operate in the generation of Ca^{2+} influx during platelet activation.
**Figure Legends**

**Figure 1.** Single cell measurements of fibrinogen binding demonstrate functional synergy between P2Y₁ and P2Y₁₂ receptors in primary murine megakaryocytes. Mouse marrow cells (Ai) were bathed in Oregon green-labelled fibrinogen and fluorescence images acquired within a 3 µm z-section at the mid-plane of a megakaryocyte by confocal microscopy before (Aii) and after addition of 30 µM ADP (Aiii). The images were analysed as described in detail in the methods section; $f$ is the fluorescence level, $f_e$ is the average extracellular fluorescence and $f_p$ is the average fluorescence measured around the cell periphery. B. $f/f_e$ across the centre of a control, P2Y₁⁻/⁻ and AR-C69931MX-treated Mks prior to addition of ADP (i) and 2 minutes after 30 µM ADP (ii). C. Average $f_p/f_e$ ratios before (open bars) and after (shaded bars) 30 µM ADP for control, P2Y₁⁻/⁻ and AR-C69931MX-treated MKs (n=10 for each). (** Indicates statistical significance of $P=<0.001$ when compared to control.)

**Figure 2.** ADP evokes multiple phases of inward cationic current via a mechanism that requires P2Y₁ receptors. Simultaneous whole-cell patch clamp and $[\text{Ca}^{2+}]_i$ recordings at −70 mV under conditions that eliminate K⁺-selective currents. A. Typical biphasic inward current and $[\text{Ca}^{2+}]_i$ increase activated by 30 µM ADP. The dashed line shows the background current level. B. Another cell displaying typical repetitive inward currents observed in many recordings during exposure to ADP. These repetitive events had rapid kinetics, as shown in the expanded section. C. Inward currents and $[\text{Ca}^{2+}]_i$ increases were not observed in response to 30 µM in P2Y₁⁻/⁻ MKs. D. Frequency of occurrence (events per minute) of the repetitive transient inward currents for wild type (WT) and P2Y₁⁻/⁻ MKs before (open boxes)
and during exposure to 30 μM ADP (shaded boxes). (* Indicates statistical significant of $P=\leq 0.05$, compared to WT.)

**Figure 3. Transient repetitive currents in the presence and absence of exogenous ADP are mainly due to P2X$_1$ receptors.** A. Scatter plot of activation and inactivation kinetics for the repetitively activated inward currents observed during application of 30 μM ADP. Each point shows the time to peak current and inactivation time constant for a single transient event where this could be fitted by a single exponential decay. Data from 15 cells. B. Frequency of occurrence (events per minute) of all transient currents during exposure to 30 μM ADP for control, P2X$_1^{-/-}$ MKs, after pre-exposure to 10 μM $\alpha,\beta$-meATP and in the presence of 1 μM NF449. C. Example of the slower transient events and concurrent $[\text{Ca}^{2+}]_i$ transients evoked by ADP in the absence of P2X$_1$ receptors. D. Example of the spontaneous $[\text{Ca}^{2+}]_i$ increases and transient inward currents displaying a mixture of rapid and slow current events. E. Frequency of all spontaneous current transients in control cells, P2X$_1^{-/-}$ MKs (n=11), after exposure to 10 μM $\alpha,\beta$-meATP (n=7) and in the presence of 1 μM NF449 (n=7). (Asterisks indicate statistical significance compared to control, * $P=\leq 0.05$; ** $P=\leq 0.01$; *** $P=\leq 0.001$.)

**Figure 4. Priming of the initial ADP-activated inward current by P2X$_1$ receptors.** Membrane currents and intracellular $\text{Ca}^{2+}$ responses were recorded simultaneously at −70 mV in response to 30 μM ADP. A. Representative recordings of the initial increase in intracellular $[\text{Ca}^{2+}]_i$ (top panel) and whole cell current (lower panel) in wild type and P2X$_1^{-/-}$ MKs. B. Expanded section of the traces showing current activation
prior to the [Ca\textsuperscript{2+}]\textsubscript{i} increase in a wild type MK, but at or later than the [Ca\textsuperscript{2+}]\textsubscript{i} response in a P2X\textsubscript{1}\textsuperscript{-/-} MK. C. Average onset of the ADP-stimulated current relative to the first detectable [Ca\textsuperscript{2+}]\textsubscript{i} increase in control (n=9), P2X\textsubscript{1}\textsuperscript{-/-} MKs (n=8), in the presence of 1 \mu M NF449 (n=5) and after pre-exposure to 10 \mu M \alpha,\beta\text{meATP} (n=5). (* Indicates statistical significance of \(P=\leq 0.05\) when compared to control.)

Figure 5. Synergistic role for P2Y\textsubscript{12} receptors via PI-3 kinase in the activation of inward cation currents by ADP. Membrane currents and intracellular Ca\textsuperscript{2+} responses were recorded simultaneously at \(-70\) mV in response to 30 \mu M ADP in control MKs (n=9) and following exposure to the P2Y\textsubscript{12} antagonist AR-C69931MX (1 \mu M, n=8) or the PI 3-kinase blocker LY294002 (30 \mu M, n=5). A. Timing of the initial ADP-evoked membrane current relative to the intracellular Ca\textsuperscript{2+} increase. B. Average initial peak ADP-evoked currents. C. Normalised ADP-evoked Ca\textsuperscript{2+} peak amplitude (dark boxes) and time to peak (light boxes), expressed as a percentage of control. D. Frequency of occurrence of repetitive transient inward currents. (Asterisks indicate statistical significance compared to the control (saline or ADP, as appropriate), * \(P=\leq 0.05\); ** \(P=\leq 0.01\).)

Figure 6. The P2Y receptor-dependent conductance is permeable to Ca\textsuperscript{2+} and is amplified by phenylarsine oxide. A. Representative ADP-evoked [Ca\textsuperscript{2+}]\textsubscript{i} (top panel) increase and whole cell membrane currents at \(-70\) mV in 0Na\textsuperscript{+} (NMDG\textsuperscript{+}), 0Mg\textsuperscript{2+}, 2.5 mM Ca\textsuperscript{2+} saline with 1 \mu M NF449. B. Effect of a 2 minute pre-exposure to 15 \mu M phenylarsine oxide on the ADP-evoked membrane currents. C. Average peak ADP-evoked currents in normal saline (150 mM Na, 1 mM Ca\textsuperscript{2+}) and 2.5 mM Ca\textsuperscript{2+} saline
(0 Na\(^+\), 0Mg\(^{2+}\)) with/without pre-treatment of 15 µM PAO or 100 µM vanadate. (*
Indicates statistical significance of \(P\leq0.05\) when compared to control.)

**Figure 7. Proposed mechanisms for P2 receptor-dependent activation of non-selective cation channels in the megakaryocyte and platelet.** ADP activates a Na\(^+\)- and Ca\(^{2+}\)-permeable cation channel, likely to be a member of the transient receptor potential (TRP) family of ion channels, via combined stimulation of P2Y\(_1\) and P2Y\(_{12}\) receptors. 1. P2Y\(_1\) activation of PLC\(\beta\) hydrolyses PIP\(_2\) resulting in removal of an inhibitory action on the cation channel. 2. Simultaneously, P2Y\(_{12}\) activates PI 3-kinase via its G\(\beta\gamma\) subunits, resulting in phosphorylation of PIP\(_2\) to PIP\(_3\), further decreasing the inhibition by PIP\(_2\). 3. PIP\(_3\) may be an activator of this or another cation channel. 4. Both DAG and IP\(_3\) (or an IP\(_3\) metabolite) have been reported to activate cation channels in the platelet/MK. IP\(_3\) results in release of stored Ca\(^{2+}\), which further modulates the cation channel. 5. P2Y\(_{12}\) receptors, via PI 3-kinase, stimulate dense granule release into the OCS/DMS; P2Y\(_1\)–dependent PKC stimulation is also likely to potentiate this secretion. 6. Released ATP can repetitively activate P2X\(_1\) receptors, and further modulate the P2Y current via Ca\(^{2+}\) influx.

DAG, diacylglycerol; IP\(_3\), inositol 1,4,5 trisphosphate; MLCK, myosin light chain kinase; OCS, open canalicular system in the platelet; DMS, demarcation system in the MK; PI3K, phosphatidylinositol 3-kinase; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; PIP\(_3\), phosphatidylinositol 3,4,5 trisphosphate; PKC, protein kinase C; TRP, transient receptor potential ion channel.
Figure 1
Figure 2

A. WT

\[ [\text{Ca}^{2+}]_i \]

50 nM

30 µM ADP

Current

0.5 pA/pF

10 sec

B. WT

\[ [\text{Ca}^{2+}]_i \]

100 nM

30 µM ADP

Current

0.25 pA/pF

5 sec

C. P2Y<sub>1</sub><sup>−/−</sup>

\[ [\text{Ca}^{2+}]_i \]

50 nM

30 µM ADP

Current

0.5 pA/pF

5 sec

D.

<table>
<thead>
<tr>
<th>Frequency (spurts/min)</th>
<th>WT</th>
<th>P2Y&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>WT</th>
<th>P2Y&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td></td>
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<tr>
<td>30 µM ADP</td>
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Figure 3

A  WT: 30 μM ADP

B  30 μM ADP

C  P2X<sub>1</sub><sup>-/-</sup>: 30 μM ADP

D  Spontaneous

E  Spontaneous
Figure 4

A

[Bae],
200nM

Current
0.5 nA
1 sec

30 µM ADP

WT
P2X<sup>−/−</sup>

B

[Bae],
50nM

Current
0.2 nA
0.2 sec

30 µM ADP

WT
P2X<sup>−/−</sup>

C

Current delay (ms)

alcohol
P2X<sup>−/−</sup>
NF AαB
qRT-PCR

*
Figure 5

A. 30 μM ADP

B. Current

C. Calcium

D. Repetitive Transient Currents

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Figure 6

A 0 Na (NMDG), 0 Mg²⁺, 2.5 mM Ca²⁺, 1 µM NF449

B Phenylarsine oxide

C
Figure 7
Reference List


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Interplay between P2Y$_1$, P2Y$_{12}$ and P2X$_1$ receptors in the activation of megakaryocyte cation influx currents by ADP; evidence that the primary megakaryocyte represents a fully functional model of platelet P2 receptor signaling

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