P2X1 Receptors Amplify FcγRIIa-Induced Ca^{2+} Increases and Functional Responses in Human Platelets

Zeki Ilkan1 Stephanie Watson2 Steve P. Watson2,3 Martyn P. Mahaut-Smith1

1 Department of Molecular and Cell Biology, Henry Wellcome Building, University of Leicester, Leicester, United Kingdom
2 Institute of Cardiovascular Sciences, Institute of Biomedical Research Building, University of Birmingham, Birmingham, United Kingdom
3 Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, Midlands, UK

Abstract

Platelets express key receptors of the innate immune system such as FcγRIIa and Toll-like receptors (TLR). P2X1 cation channels amplify the platelet responses to several major platelet stimuli, particularly glycoprotein (GP)VI and TLR2/1, whereas their contribution to Src tyrosine kinase-dependent FcγRIIa receptors remains unknown. We investigated the role of P2X1 receptors during activation of FcγRIIa in human platelets, following stimulation by cross-linking of an anti-FcγRIIa monoclonal antibody (mAb) IV.3, or bacterial stimulation with Streptococcus sanguinis. Activation was assessed in washed platelet suspensions via measurement of intracellular Ca^{2+} ([Ca^{2+}]_i) increases, ATP release and aggregation. P2X1 activity was abolished by pre-addition of α,β-meATP, exclusion of apyrase or the antagonist NF449. FcγRIIa activation evoked a robust increase in [Ca^{2+}]_i (441 ± 33 nM at 30 μg/mL mAb), which was reduced to a similar extent (to 66–70% of control) by NF449, pre-exposure to α,β-meATP or apyrase omission, demonstrating a significant P2X1 receptor contribution. FcγRIIa activation-dependent P2X1 responses were partially resistant to nitric oxide (NO), but abrogated by 500 nM prostacyclin (PGI2). Aggregation responses to bacteria and FcγRIIa activation were also inhibited by P2X1 receptor desensitization (to 66 and 42% of control, respectively). However, FcγRIIa-mediated tyrosine phosphorylation and ATP release were not significantly altered by the loss of P2X1 activity. In conclusion, we show that P2X1 receptors enhance platelet FcγRIIa receptor-evoked aggregation through an increase in [Ca^{2+}]_i, downstream of the initial tyrosine phosphorylation events and early dense granule release. This represents a further route whereby ATP-gated cation channels can contribute to platelet-dependent immune responses in vivo.

Keywords

► bacteria
► immunity
► inflammation
► thrombosis
► platelet

Introduction

In addition to their essential role in the process of haemostasis, platelets contribute to immune responses through several mechanisms including the interaction of surface receptors with invading pathogens. Human platelets express the low affinity receptor for immunoglobulin G, FcγRIIa (CD32a), which recognizes the immunoglobulin G (IgG) that opsonizes invading pathogens in the circulation.1 Cross-linking of FcγRIIa receptors...
results in the activation of a signal transduction pathway through an immunoreceptor tyrosine-based activation motif (ITAM) in a manner similar to that observed following stimulation of the collagen and fibrin receptor GPVI. The vital role of FcγRIIa receptor in platelet aggregation and thrombus formation has been established by several in vitro and in vivo studies. In addition, interaction between bacteria and platelets has been shown to cause formation of dangerous circulating or localized thrombi such as in infective endocarditis (IE). Despite this, our knowledge of FcγRIIa receptor involvement in platelet function remains rudimentary.

P2X1 channels are the only adenosine triphosphate (ATP)-activated receptors in platelets and represent the fastest \( \text{Ca}^{2+} \) entry route following ATP release from an injury site. The contribution of P2X1 channels to thrombosis in vivo, and their important role in primary and secondary agonist-induced platelet activation, has been described previously. It has been shown that selective inhibition or desensitization of P2X1 channels reduces the \( \text{Ca}^{2+} \) entry that accompanies platelet activation. Furthermore, the ability of P2X1 receptors to amplify functional responses, particularly at low levels of stimulation, is critical to the development of the platelet degranulation that occurs in response to stimulation of the early tyrosine kinase-dependent steps. P2X1 activity is amplified by P2X1 receptor desensitization, except where stated. Aspirin (100 \( \mu \)M) was also added to inhibit cyclooxygenase for studies of mAb IV.3-induced \( \text{Ca}^{2+} \) increases to allow comparison with previous studies, particularly the relative contribution of P2X1 receptors and effects of endogenous antiplatelet reagents, but was omitted from all other experiments. Platelets were loaded with Fura-2 by incubation with 2 \( \mu \)M Fura-2 AM for 2 minutes before addition of IgG F(ab\(^\prime\))\(_2\). For stimulation by bacteria, platelets were incubated with pooled human IgG (0.1 mg/mL) in the cuvette prior to addition of a 10-fold dilution of the 6 \( \times \) 10\(^8\) CFU/mL (see above) bacteria stock.

### Materials and Methods

#### Reagents

Anti-FcγRIIa monoclonal antibody (mAb) IV.3 was purified in the laboratory from a hybridoma. Goat anti-mouse IgG F(ab\(^\prime\))\(_2\) was purchased from Fisher Scientific (UK). Apyrase (grade VII) from potato, a form of ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), which displays similar properties to human CD39, was from Sigma-Aldrich (Poole, UK). Spermine NONOate was from Enzo Life Sciences Ltd (Exeter, UK). The GPIIb/IIIa inhibitor eptiplatelet was from Source Bioscience (Nottingham, UK). Extracellular ATP measurements were performed using firefly luciferin-luciferase (Chrono-Lume reagent kit #395; Chrono-Log Corporation, Havertown, Pennsylvania, United States). Rabbit anti-human phospho-Syk (Tyr 525/526) mAb and rabbit anti-human phospho-PLCγ2 (Tyr 1217) polyclonal antibody (pAb) were from Cell Signaling Technology (Danvers, Massachusetts, United States). Rabbit anti-human phospho-LAT (Tyr 200) mAb was from Abcam (Cambridge, UK). Mouse anti-human anti-phosphotyrosine (clone 4G10) mAb was from Millipore UK Ltd (Watford, UK). Rabbit anti-human PLCγ2 antibody (Q-20) was from Santa Cruz Biotechnology (Heidelberg, Germany). Unless otherwise stated, all reagents were from Sigma-Aldrich.

#### Bacterial Culture and Preparation

*Streptococcus sanguinis* 133–79 was provided by Prof Mark Herzberg (University of Minnesota). Bacteria were cultured under anaerobic conditions at 37°C overnight and suspensions were prepared as previously described. After washing in phosphate-buffered saline (PBS), the optical density of the suspension was adjusted to 1.5 at a wavelength of 600 nm, which corresponds to 6 \( \times \) 10\(^8\) CFU/mL as previously shown.

#### Preparation of Washed Platelets and FcγRIIa Receptor Stimulation

Blood was collected by venepuncture in accordance with the Declaration of Helsinki from informed consenting volunteers. The study was approved by the University of Leicester College of Life Sciences Committee for Research Ethics concerning human subjects (non-NHS). Acid-citrate-dextrose (ACD; in mM: 85 trisodium citrate, 78 citric acid, 111 glucose) was used as the anticoagulant at a ratio of 6:1 (blood:ACD). After centrifugation at 700g for 5 minutes, platelet-rich plasma was removed and treated with apyrase (grade VII; 0.32 U/mL) to prevent P2X1 receptor desensitization, except where stated. Aspirin (100 \( \mu \)M) was also added to inhibit cyclooxygenase for studies of mAb IV.3-induced \( \text{Ca}^{2+} \) increases to allow comparison with previous studies, particularly the relative contribution of P2X1 receptors and effects of endogenous antiplatelet reagents, but was omitted from all other experiments. Platelets were loaded with Fura-2 by incubation with 2 \( \mu \)M Fura-2 AM for 45 minutes at 37°C, then centrifuged for 20 minutes at 350g followed by resuspension in nominally \( \text{Ca}^{2+} \)-free saline (in mM: 145 NaCl, 5 KCl, 1 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.35 with NaOH), which also contained apyrase (0.32U/mL) except where stated. Platelet responses were measured at the same density as in whole blood. Thirty seconds prior to receptor stimulation, 2 mM CaCl\(_2\) was added to each cuvette. FcγRIIa receptors were stimulated either by cross-linking or bacteria. For cross-linking, platelets were pre-incubated with mAb IV.3 (1 \( \mu \)g/mL) for 2 minutes before addition of IgG F(ab\(^\prime\)). For stimulation by bacteria, platelets were incubated with pooled human IgG (0.1 mg/mL) in the cuvette prior to addition of a 10-fold dilution of the 6 \( \times \) 10\(^8\) CFU/mL (see above) bacteria stock.
Ratiometric [Ca^{2+}] Measurements
Ratiometric [Ca^{2+}] measurements were performed in a Cairn spectrophotometer system (Cairn Research Limited, Faversham, Kent, UK) at 37°C and expressed as [Ca^{2+}] using a dissociation constant for Ca^{2+} of 224nM, as previously described.\(^8\)

Aggregometry and Luminescence Measurement of ATP Secretion
Aggregation of washed platelet suspensions was assessed by standard light transmission measurements, with simultaneous luminescence measurements of ATP secretion as required, in a Chrono-Log 400 Lumi-Aggregometer (Chrono-Log Corporation), at 37°C as described in detail elsewhere.\(^8,14\) 100 µg/mL fibrinogen and 2 mM CaCl\(_2\) were added to the cuvette at the start of each experiment. For each batch of luciferin-luciferase, a concentration–response curve across a range of known ATP concentrations (nM) was constructed using the background-corrected peak signal (mV) detected in normal platelet saline containing 0.32 U/mL apyrase (to mimic the conditions used to assess platelet-dependent ATP release).

Protein Phosphorylation
Protein lysates were prepared from 500 µL samples of stirred platelet suspensions, which were pretreated with 9 µM eptifibatide to prevent aggregation. Lysis was achieved by addition of an equal volume of ice-cold 2 x radioimmunoprecipitation assay (RIPA) buffer (including 1 x Roche protease inhibitor tablet, 4 mM sodium orthovanadate and 20 mM sodium fluoride) 60 seconds after FcyRIIa cross-linking using IgG F(ab\(^\prime\))\(_2\) (15 µg/mL). Protein quantification was performed with a Bradford assay. Tyrosine phosphorylation was detected by Western blotting with anti-phosphotyrosine (clone 4G10; 1:1,000), phospho-Syk (Tyr 525/526; 1:500), phospho-LAT (Tyr 1217; 1:250) antibodies. Membranes were re-probed with a pan-anti-PLC\(_6\) antibody (1:200). Lysate (20 µg) was added in each well of a 12-well Bolt 4 to 12% Bis-Tris Plus Gel (Invitrogen, Paisley, UK). Each gel included at least one lane of 10 µL of Color Prestained Protein Standard (11–245 kDa; NEB, Ipswich, Massachusetts, United States). Gels were run at 100V for 90 minutes and semi-dry transfers onto PVDF membranes were performed using a Trans-Blot Turbo Transfer Imaging System (Bio-Rad, Hertfordshire, UK). Bands were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies and an ECL Prime kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. The membranes were placed in a hyperfilm cassette, and hyperfilms (GE Healthcare) were exposed to the membranes in a dark room. Films were developed in a hyperfilm processor and scanned for analysis.

Statistical Analysis
All traces are representative of experiments from at least three separate donors. Average results have been expressed as means ± standard error of the mean (SEM). All statistical analyses were performed on GraphPad Prism 6.0 software (La Jolla, California, United States). Where appropriate, one-way ANOVA followed by Holm-Sidak’s post hoc multiple comparisons, two-way ANOVA followed by Bonferroni’s multiple comparisons tests or two-tailed paired Student’s t-tests were used. p-Values of \(p < 0.05, \quad ^*p < 0.01, \quad ^{*}{*}p < 0.001\) and \(^{**}{*}p < 0.0001\) were considered statistically significant.

Results
P2X1 Receptors Contribute to FcyRIIa Receptor-Evoked Ca^{2+} Responses
To characterize [Ca^{2+}]i increases induced by FcyRIIa receptor stimulation, washed platelets were pre-incubated with mAb IV.3 which permitted cross-linking of FcyRIIa\(^16\) upon addition of IgG F(ab\(^\prime\))\(_2\). This resulted in an increase in [Ca^{2+}]i in all platelet samples tested with a peak value of 440 ± 33 nM. Three different approaches that caused abrogation of P2X1 channel activity\(^4\) (1 µM NF449, omission of apyrase from the platelet saline and 600 nM α,β-meATP added prior to 2 mM external Ca\(^{2+}\); \(►\) Fig. 1 A, B) caused a significant and similar reduction in the peak FcyRIIa-induced [Ca^{2+}] increase (\(►\) Fig. 1 C, E). The average peak FcyRIIa receptor-evoked responses were reduced to 303 ± 31 nM (69% of control; \(p < 0.05, \quad n = 3\)) by NF449; 307 ± 42 nM (70% of control; \(p < 0.05, \quad n = 3\)) without apyrase; and 291 ± 40 nM (66% of control; \(p < 0.05, \quad n = 3\)) after pre-addition of 600 nM α,β-meATP (by one-way ANOVA, followed by Holm-Sidak’s post hoc multiple comparisons). A further reduction of FcyRIIa receptor-evoked responses (to 191 ± 30 nM; 44% of control; \(p < 0.05, \quad n = 3\)) by one-way ANOVA, followed by Holm-Sidak’s post hoc multiple comparisons) was observed in nominally Ca\(^{2+}\)-free saline, indicating that other Ca\(^{2+}\) entry pathways such as store-operated Orai1 channels or TRPC6 also contribute (\(►\) Fig. 1 D, E). As used previously for GPVI and TLR2/1 receptors,\(^8,10\) subsequent experiments used pre-addition of 600 nM α,β-meATP to selectively abrogate P2X1 activity, as it is slightly more effective than 1 µM NF449 and small effects on P2Y receptors have been reported for higher NF449 concentrations or omission of apyrase.\(^8,17\)

The relative contribution of P2X1 receptors to collagen-evoked Ca\(^{2+}\) responses compared with other pathways is greater at low compared with high levels of stimulation, ranging from approximately 45 to 92%, across a 10-fold concentration of agonist.\(^8,18\) P2X1 receptors contributed significantly across the entire range of IgG F(ab\(^\prime\))\(_2\) concentrations tested (\(►\) Fig. 2 A–F), with desensitization resulting in a reduction to 71% of control at 30 µg/mL; 67% of control at 15 µg/mL IgG F(ab\(^\prime\))\(_2\); 62% of control at 7.5 µg/mL IgG F(ab\(^\prime\))\(_2\) and 65% of control at 3.75 µg/mL IgG F(ab\(^\prime\))\(_2\) (using two-way ANOVA, followed by Bonferroni’s multiple comparisons tests). Although the largest P2X1 receptor contribution was observed at the intermediate concentrations of IgG F(ab\(^\prime\))\(_2\) (\(►\) Fig. 2 C, D, F), the response was very weak in some donors at or below 7.5 µg/mL. Therefore, 15 µg/mL was selected as a consistent stimulus for further assessment of the contribution of P2X1 receptors to FcyRIIa receptor signalling and function.
FcγRIIa Receptor-Evoked [Ca²⁺]i Increases are Resistant to Elevated Apyrase Levels and Nitric Oxide, but not PGI₂

In the intact circulation, platelets are constantly under the influence of inhibitory molecules, particularly endothelium-derived PGI₂ and NO, and expression of the ectonucleotidase CD39 on the surface of endothelial cells. Elevating the levels of apyrase (from 0.32 to 3.2 U/mL) did not affect the FcγRIIa receptor-evoked Ca²⁺ response and P2X1 receptors were still activated as a substantial reduction in the Ca²⁺ response was observed, from 345 ± 35 nM to 175 ± 12 nM (p < 0.05, n = 3, by one-way ANOVA, followed by Holm-Sidak’s post hoc multiple comparisons), following pre-exposure to 600 nM α,β-meATP (–Fig. 3 A, G). Endothelium-derived NO is known to regulate platelet activation mainly through elevation of cyclic GMP concentrations.¹⁹ Pre-incubation of the platelet suspensions with a maximal concentration of the NO donor spermine NONOate (100 μM; unpublished data, Zeki Ilkan, 2017) caused a 69% reduction in the FcγRIIa receptor-induced Ca²⁺ responses, from 345 ± 35 nM to 107 ± 31 nM.

Fig. 1 Inhibition or desensitization of P2X1 channels reduces [Ca²⁺]i responses induced by FcγRIIa receptor activation. (A) Representative Ca²⁺ responses to P2X1 receptor stimulation with a supramaximal concentration (10 μM) of α,β-meATP, with or without desensitization of P2X1 channels by the pre-addition of α,β-meATP (600 nM) 90 seconds prior to stimulation. (B) Average maximal Ca²⁺ responses to 10 μM α,β-meATP, with and without P2X1 channel inhibition by 1 μM NF449, exclusion of extracellular apyrase, or pre-exposure to 600 nM α,β-meATP. Representative (C, D) and average (E) Ca²⁺ responses to FcγRIIa receptor activation via cross-linking of the receptor-bound mAb IV.3 (1 μg/mL), which was added 2 minutes prior to the cross-linker IgG F(ab’)2 antibody. The effects of the presence of NF449 (1 μM), exclusion of extracellular apyrase and Ca²⁺, and addition of α,β-meATP (600 nM) 90 seconds prior to IgG F(ab’)2 were studied.
The remaining response was virtually eliminated following pre-addition of α,β-meATP (to 60.0 ± 12.4 nM; p < 0.05, compared with control, n = 3, by one-way ANOVA, followed by Holm-Sidak’s post hoc multiple comparisons), demonstrating the major contribution to the NO-resistant component by P2X1 receptors. Previous studies have demonstrated that a maximal concentration of either NO or PGI2 is able to abolish [Ca^{2+}]_i increases evoked by thrombin and thromboxane A2 but only partially reduce responses to collagen and the TLR2/1 agonist Pam3CSK4. In our studies, a concentration of PGI2 (500 nM) that abrogated the Ca^{2+} response to 0.03 U/mL thrombin ([Δ[Ca^{2+}]]_i reduced from 431 ± 56 nM to 15.7 ± 6.2 nM; p < 0.05, n = 3, by paired t-test; Fig. 3C) also virtually eliminated the response via FcγRIIa receptors (345 ± 35 nM under control conditions to 23.0 ± 2.0 nM with PGI2; 7% of control; p < 0.05, n = 3, by one-way ANOVA, followed by Holm-Sidak’s post hoc multiple comparisons; Fig. 3D, G). This was unexpected since both GPVI and FcγRIIa signal through an ITAM-dependent pathway. The collagen-dependent stimulation of P2X1 receptors persists in high PGI2 due to a partial resistance of GPVI-evoked dense granule secretion to elevated cyclic AMP. In contrast, FcγRIIa-induced ATP release was completely blocked by 500 nM PGI2, thus...
Fig. 3 P2X1-mediated Ca\(^{2+}\) responses to FcγRIIa receptor activation are resistant to NO and elevated apyrase levels, but are abolished by PGI2. Representative [Ca\(^{2+}\)]\(i\) responses (A, B, D) induced by FcγRIIa receptor activation by cross-linking of mAb IV.3 in the presence of elevated apyrase levels (3.2 U/ml), spermine NONOate (sNO; 100 μM), and prostacyclin (PGI2; 500 nM). Control traces are representative of the vehicle-treated platelet samples in each panel (saline, 0.01 M NaOH and dH2O, respectively). P2X1 receptor desensitization was achieved by pretreatment with 600 nM α,β-meATP. (C) Effect of a submaximal concentration of PGI2 (500 nM) on [Ca\(^{2+}\)]\(i\) induced by 0.03 U/ml thrombin. (E, F) PGI2 (500 nM) substantially inhibited FcγRIIa-mediated ATP secretion. (G) Average peak [Ca\(^{2+}\)]\(i\), responses obtained in the presence of inhibitors used, compared with the average of control responses (first column).
accounting for its ability to prevent P2X1 receptor activation (►Fig. 3 E, F).

**Streptococcus sanguinis** Induces P2X1-Mediated \([\text{Ca}^{2+}]_i\) Increases

A range of *Streptococci* strains including *S. sanguinis* have been previously shown to induce platelet aggregation in washed platelet suspensions in the presence of human IgGs. Over a period of 25 minutes after addition of *S. sanguinis*, a steady increase in \([\text{Ca}^{2+}]_i\) compared with vehicle control was observed, and the maximal \([\text{Ca}^{2+}]_i\) level reached at the end of the experiment was not significantly altered after desensitization of P2X1 channels using \(\alpha\beta\)-meATP (342 ± 37 nM for control and 344 ± 27 nM after \(\alpha\beta\)-meATP; \(p > 0.05, n = 3\), using paired t-test; ►Fig. 4Ai, Bi). However, a transient \([\text{Ca}^{2+}]_i\) increase was observed approximately 2.5 minutes after addition of bacteria, which was inhibited following P2X1 receptor desensitization (see expanded traces in ►Fig. 4 (Aii) corresponding to the dashed rectangular area indicated in ►Fig. 4Ai). The peak \([\text{Ca}^{2+}]_i\) increase during this initial transient was 69.2 ± 3.2 nM and reduced to 25.8 ± 0.9 nM (\(p < 0.05, n = 3\), using paired t-test) at the same time point in the paired P2X1 inhibited run, which represents a reduction to 37% of control (►Fig. 4Bii). Thus, \([\text{Ca}^{2+}]_i\) entry through P2X1 channels is stimulated following FcγRIIa receptor activation either by bacteria or cross-linking antibodies.

**FcγRIIa Receptor Activation Induces Dense Granule Release and Aggregation that Partially Depends on P2X1 Responses**

Platelet aggregation induced by either IgG F(ab′)\(_2\) antibody or *S. sanguinis* was significantly inhibited by \(\alpha\beta\)-meATP pre-treatment (from 73.8 ± 4.2% to 33.5 ± 13.2%; 45% of control; and from 35.7 ± 1.9% to 23.2 ± 4.4%; 65% of control, respectively; \(p < 0.05, n = 5\) for both, by paired t-test; ►Fig. 5A, Ci). Although the average peak ATP release was smaller after P2X1 desensitization (►Fig. 5B, Cii), this was not statistically significant; the extracellular ATP increase following antibody-induced FcγRIIa activation was 2,108 ± 324 nM and 1,660 ± 732 nM, and following bacteria-induced FcγRIIa activation was 65.8 ± 31.5 nM and 18.6 ± 9.6 nM in the control and \(\alpha\beta\)-meATP pre-treated platelets, respectively (\(p > 0.05, n = 5\) and 6, using paired t-test). Thus, while dense granule secretion is known to be \([\text{Ca}^{2+}]_i\)-dependent and the early FcγRIIa-induced ATP release will stimulate \([\text{Ca}^{2+}]_i\) influx through P2X1 receptors, the amplification of aggregation by this cation channel does not involve a significant enhancement of dense granule secretion.

---

**Fig. 4** P2X1 ion channels contribute to the *S. sanguinis*-induced FcγRIIa receptor \([\text{Ca}^{2+}]_i\) responses (A) Representative \([\text{Ca}^{2+}]_i\) responses measured when *S. sanguinis* was added with and without 600 nM \(\alpha\beta\)-meATP pretreatment, together with the vehicle (PBS) control (i). \(\alpha\beta\)-meATP pretreatment diminished the transient \([\text{Ca}^{2+}]_i\) peak that occurred after bacterial stimulation [shown in dashed rectangular box in (i), which is expanded in (ii)]. (B) Average peak \([\text{Ca}^{2+}]_i\) responses obtained within 25 minutes (i) and 2.5 minutes (ii) after bacterial stimulation, with and without \(\alpha\beta\)-meATP pretreatment.
Amplification of FcγRIIa Receptor Responses Is Independent of Initial Tyrosine Phosphorylation Events

In addition to direct effects of Ca\(^{2+}\) influx on functional events, we also considered the possibility that P2X1 receptors may enhance FcγRIIa receptor-induced responses through modulation of early tyrosine phosphorylation events. FcγRIIa-induced tyrosine phosphorylation was assessed across all sites using a pan-phosphotyrosine antibody (4G10) along with phosphorylation of specific targets using phospho-specific antibodies (Syk: Tyr 525/526; LAT: Tyr 200; PLC\(\gamma\)2: Tyr 1217). Samples were lysed 60 seconds after receptor stimulation, under control and P2X1-desensitizing conditions which represents the time point of maximal \([\text{Ca}^{2+}]_i\) response to cross-linking with 15 \(\mu\)g/mL IgG F(ab\(^{\prime}\))\(_2\). No observable difference in the attained phosphorylation levels after FcγRIIa receptor stimulation was observed following pretreatment with \(\alpha,\beta\)-meATP (►Fig. 6).

Discussion

ATP-gated P2X1 channels are the only ligand-gated Ca\(^{2+}\)-permeable ion channels conclusively shown to be expressed on the platelet surface and provide a rapid route for Ca\(^{2+}\) entry
following ATP release from damaged vascular cells or from activated platelets and other blood cells.\(^{2,21,22}\) These channels have been shown to increase the platelet \([Ca^{2+}]_i\) responses observed in vitro following stimulation by several major haemostatic agonists, including ADP, collagen, thrombin and thromboxane \(A_2\), and to exacerbate thrombosis in vivo.\(^{8-11}\) In addition to their role in haemostasis, platelets are recognized to contribute to the immune system, assisting for example in the opsonization and thus clearance of bacteria.\(^{23-27}\) Platelets have been shown to secrete cytokines, chemokines and antimicrobial substances such as \(\beta\)-defensin, which help facilitate leukocyte recruitment and assist in the elimination of invading pathogens through the formation of neutrophil extracellular traps (NETs).\(^{26-28}\) The present study demonstrates that P2X2 channel activation also amplifies the platelet \([Ca^{2+}]_i\) and aggregation responses following stimulation of FcγRIIA, the only receptor in this cell type that recognizes the Fc region of IgG antibodies. Another immune receptor expressed on platelets is the TLR2/1 complex, which likewise uses ATP release and P2X1 receptors to enhance \(Ca^{2+}\) responses and aggregation.\(^{10}\) Therefore, by contributing to signalling downstream of both TLR2/1 receptors and FcγRIIA, P2X1 ion channels represent a route for \(Ca^{2+}\) entry that can amplify platelet-dependent immune responses.

An increase in \([Ca^{2+}]_i\) provides an essential signal that universally links surface receptors to inside-out activation of the main receptor for fibrinogen, GPIIb/IIIa, through activation of the Ca\(^{2+}\)-sensor CalDAG-GEF1.\(^{29-31}\) Arman and colleagues have demonstrated that various IgG-coated bacterial strains, including \(S.\) sanguinis 133–79, induce GPIIb/IIIa-dependent tyrosine phosphorylation of FcγRIIA receptors.\(^5\) P2X1 desensitization resulted in a more substantial reduction of FcγRIIA-evoked aggregation (by \(~55\%\)) than peak \(Ca^{2+}\) increase (\(~34\%\)), although this likely reflects the fact that \([Ca^{2+}]_i\) activates functional events in a highly nonlinear manner and with a threshold above the resting concentration of approximately 100 nM.\(^{32}\) However, the substantial proportion of the bacteria-induced aggregation that was dependent upon P2X1 receptors was surprising given the small size of the initial \(Ca^{2+}\) increase induced by these cation channels following bacterial stimulation. It is possible that the timing of this early signalling event is crucial in the chain of events leading to inside-out activation of GPIIb/IIIa following bacteria-dependent engagement of FcγRIIA. A similar large inhibition of both \(Ca^{2+}\) responses and aggregation is observed in human platelets at a low concentration of collagen, despite the small changes in \([Ca^{2+}]_i\).\(^{9,12,18}\) An additional explanation could be that P2X1-dependent bacteria-induced inside-out activation of GPIIb/IIIa via FcγRIIA occurs within microdomains that display higher local \([Ca^{2+}]_i\) increases. In support of this concept, single cell recordings from both platelets and megakaryocytes reveal that both ATP release and P2X1 receptor activation occur as a series of discrete transient events reflecting spatially restricted foci of dense granule content release and channel activation.\(^{33}\) This spatial organization may result from the reported organization of P2X1 receptors, components of the secretory pathway and FcγRIIA-dependent signalling events into lipid rafts.\(^{34-36}\)

The efficiency with which P2X1 can amplify \(Ca^{2+}\) and aggregation responses downstream of tyrosine kinase-coupled receptors such as FcγRIIA and GPVI receptors also raises the question of whether the cation channel enhances early kinase activation and/or granule secretion. However, no significant changes in FcγRIIA tyrosine phosphorylation events or bulk phase ATP secretion could be detected following inhibition of P2X1 receptors. It follows, therefore, that activation of P2X1 channels by FcγRIIA receptors and subsequent contribution to aggregation occur downstream of both PLCγ activation and initial release of ATP from dense granules. Maximal P2X1 receptor-dependent \(Ca^{2+}\) influx is not able to independently cause aggregation,\(^{37}\) thus must synergize with other cytosolic signals to enhance inside-out activation of GPIIb/IIIa. However, P2X1 receptors may enhance FcγRIIA-stimulated \(Ca^{2+}\) mobilization via potentiation of IP3 receptors, as shown for P2Y1 receptors.\(^{39}\) Pathways for \(Ca^{2+}\) entry other than P2X1 also contribute to the FcγRIIA-induced \([Ca^{2+}]_i\) increase (\(\sim \)) which likely include Orai1 store-operated \(Ca^{2+}\) channels\(^{39,40}\) and/or TRPC6 stimulated by either diacylglycerol or a decrease in PIP2.\(^{41}\) Nevertheless, the autocrine activation of P2X1 by secreted ATP permits a very efficient early mechanism for \(Ca^{2+}\) influx that contributes distinctly and separately from other \(Ca^{2+}\) entry pathways.\(^{10}\)
Platelet FcγRIIa represents the largest pool of these receptors in the body due to the large number of circulating platelets compared with other immune-competent cells expressing such receptors.\textsuperscript{1,42,43} FcγRIIa can directly activate platelets following the binding of IgG-containing complexes, certain strains of IgG-coated bacteria and unidentified ligand(s) on cancer cells.\textsuperscript{1,4} Innate pentraxins such as C-reactive protein can also activate FcγRIIa receptors, at least in leukocytes.\textsuperscript{44} FcγRIIa receptors play a particularly important role in heparin-induced thrombocytopenia (HIT).\textsuperscript{45,46} Despite the reduced platelet count, thrombosis occurs in HIT due to direct activation of FcγRIIa receptors on platelets in combination with monocytic-dependent generation of thrombin and tissue factor.\textsuperscript{47} It has also been shown that FcγRIIa receptors amplify platelet activation by weak levels of haemostatic agonists such as thrombin and thromboxane A2, which may contribute to the prothrombotic phenotype in HIT.\textsuperscript{47} It is therefore interesting to speculate that P2X1 receptor inhibition could serve as a useful antithrombotic therapy during immune thrombocytopenia, particularly since P2X1\textsuperscript{−/−} mice display no significant bleeding phenotype.\textsuperscript{9}

An important property of the \([\text{Ca}^{2+}]_{\text{i}}\) increases evoked by both GPVI and TLR2/1 is their partial resistance to inhibition by NO and PGI\(_2\) even at low levels of stimulation, since this permits activation even in the intact circulation.\textsuperscript{10,20} This contrasts with an ability of both these cysC nucleotide-elevating endogenous platelet inhibitors to totally abolish \([\text{Ca}^{2+}]_{\text{i}}\) responses mediated by several G-protein–coupled receptors. This can be explained by a difference in the cyclic nucleotide sensitivity of secretion downstream of GPCRs compared with tyrosine kinase-linked receptors such as GPVI and TLR2/1.\textsuperscript{10,20} P2X1 receptors remain unaffected by cyclic nucleotide elevation and continue to be activated by the remaining amounts of ATP released. Like GPVI, FcγRIIa receptors signal through an immunoreceptor-based tyrosine ITAM motif and activation of the Src/Syk/LAT/PLC\(_\gamma2\) signalling cascade.\textsuperscript{2,48} Thus, it is not surprising that \([\text{Ca}^{2+}]_{\text{i}}\) responses downstream of FcγRIIa receptors were also partially resistant to NO in part due to continued P2X1 receptor involvement (\textsuperscript{\*}Fig. 3B, G). However, in contrast, PGI\(_2\) completely abolished FcγRIIa-evoked secretion and \([\text{Ca}^{2+}]_{\text{i}}\) responses (\textsuperscript{\*}Fig. 3D–G). Furthermore, this was not due to a weaker secretory response for FcγRIIa, since antibody-induced ATP release was substantially larger than observed previously for GPVI under the same conditions (\textsuperscript{\*}Fig. S1B).\textsuperscript{10} The large ATP release in combination with efficient autocrine P2X1 activation can explain why FcγRIIa-evoked \([\text{Ca}^{2+}]_{\text{i}}\) increases were insensitive to elevated apyrase compared with a significant ability of this ectonucleotidase to reduce P2X1-stimulated \([\text{Ca}^{2+}]_{\text{i}}\) increases after GPVI engagement.\textsuperscript{10} At present, we cannot explain the difference in PGI\(_2\) sensitivity of GPVI versus FcγRIIa secretion and \([\text{Ca}^{2+}]_{\text{i}}\) mobilization other than to speculate that the downstream signalling events are sufficiently distinct in their susceptibility to cyclic AMP or protein kinase A. This may result from the differences in coupling of the external ligand to ITAM domain activation, since the FcγRIIa receptor contains the tyrosine motif within its intracellular tail, whereas GPVI needs to associate with FcRy to achieve ITAM-induced signalling. The FcγRIIa and FcRy ITAM domains also differ in structure. A further site for differential cyclic AMP/PKA-dependent inhibition could be at the level of exocytosis. It is becoming clear that different modes of exocytosis exist in platelets, such as single and multigranular fusion events, which could allow specificity amongst individual agonists.\textsuperscript{49,50}

In conclusion, the present study provides evidence that ATP-gated P2X1 channels amplify the intracellular \([\text{Ca}^{2+}]_{\text{i}}\) and aggregation responses in human platelets following stimulation of FcγRIIa receptors. FcγRIIa-induced P2X1 activation was resistant to increased ectonucleotidase activity and persisted following an increase in the presence of the endogenous platelet inhibitor NO, which may allow immune complexes or opsonized bacteria to stimulate platelets in the intact circulation. This raises the possibility that inhibition of P2X1 receptors could represent an antithrombotic target during immune thrombocytopenia or inflammatory situations such as IE.

**What is known about this topic?**
- Platelets express FcγRIIa receptors which signal through tyrosine kinases.
- An increase in intracellular \([\text{Ca}^{2+}]_{\text{i}}\) is an essential signalling event during platelet activation; however, the relative importance of different \([\text{Ca}^{2+}]_{\text{i}}\) entry pathways to FcγRIIa receptor signalling is unknown.

**What does this paper add?**
- Stimulation of FcγRIIa receptors by antibodies or bacteria leads to \([\text{Ca}^{2+}]_{\text{i}}\) mobilization and aggregation, both of which are amplified by activation of P2X1 receptors following ATP release.
- P2X1 receptors enhance FcγRIIa-evoked responses downstream of tyrosine phosphorylation.
- FcγRIIa-dependent P2X1 receptor platelet signals may enhance innate immune responses in vivo.

**Funding**
This work was supported by the British Heart Foundation grants PG/11/56, PG/05/014 and RG/13/18/30563. Z.I. was supported by a Medical Research Council Doctoral Training Award.

**Conflict of Interest**
None declared.

**Acknowledgements**
The authors thank Dr. Mónica Arman for providing the *S. sanguinis* and information on its growth conditions, Dr. Sara Sandrini for assistance with bacterial cultures and Dr. Lorenza Francescut for excellent technical support.

**References**
1. Arman M, Krauel K. Human platelet IgG Fc receptor FcγRIIa in immunity and thrombosis. J Thromb Haemost 2015;13(06):893–908
Role for P2X1 in FcγRIIα Signalling

Ilkan et al.

2 Watson SP, Gibbins J. Collagen receptor signalling in platelets: extending the role of the ITAM. Immunol Today 1998;19(06):260–264

3 Zhi H, Rauova L, Hayes V, et al. Cooperative integrin/ITAM signalling in platelets enhances thrombus formation in vitro and in vivo. Blood 2013;121(10):1858–1867

4 Kerrigan SW, Clarke N, Loughman A, Meade G, Foster TJ, Cox D. Molecular basis for Staphylococcus aureus-mediated platelet aggregate formation under arterial shear in vitro. Arterioscler Thromb Vasc Biol 2008;28(02):335–340

5 Arman M, Krauel K, Tilley DO, et al. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. Blood 2014;123(20):3166–3174

6 Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. Nat Rev Microbiol 2006;4(06):445–457

7 Mahaut-Smith MF. The unique contribution of ion channels to platelet and megakaryocyte function. J Thromb Haemost 2012;10(09):1722–1732

8 Fung CY, Cendana C, Farndale RW, Mahaut-Smith MP. Primary and secondary agonists can use P2X1 receptors as a major pathway to increase intracellular Ca²⁺ in the human platelet. J Thromb Haemost 2007;5(05):910–917

9 Hechler B, Lenain N, Marchese P, et al. Role of the fast ATP-gated P2X1 cation channel in thrombosis of small arteries in vivo. J Exp Med 2003;198(04):661–667

10 Fung CY, Jones S, Ntrakwah A, Naseem KM, Farndale RW, Mahaut-Smith MF. Platelet Ca²⁺ responses coupled to glycoprotein VI and Toll-like receptors persist in the presence of endothelial-derived inhibitors: roles for secondary activation of P2X1 receptors and release from intracellular Ca²⁺ stores. Blood 2012;119(15):3613–3621

11 Erhardt JA, Toomey JR, Douglas SA, Johns DG. P2X1 stimulation promotes thrombin receptor-mediated platelet aggregation. J Thromb Haemost 2006;4(04):882–889

12 Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermeylen J, Hoylaerts MF. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. Thromb Haemost 2001;86(05):1264–1271

13 Pampolina C, McNicol A. Streptococcus sanguinis-induced platelet activation involves two waves of tyrosine phosphorylation mediated by FcgammaRIIa and alphaIIbβ3 receptors. Thromb Haemost 2005;93(05):832–839

14 Jones S, Evans RJ, Mahaut-Smith MP. Extracellular Ca²⁺ modulates ADP-evoked aggregation through altered agonist degradation: implications for conditions used to study P2Y receptor activation. Br J Haematol 2011;153(01):83–91

15 Tilley DO, Arman M, Smolenski A, et al. Glycoprotein Ibα and FcγRIIa play key roles in platelet activation by the colonizing bacterium, Streptococcus oralis. J Thromb Haemost 2013;11(05):941–950

16 Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. J Biol Chem 1996;271(30):18095–18099

17 Hechler B, Magenstretz S, Zighetti ML, et al. Inhibition of platelet functions and thrombosis through selective or nonselective inhibition of the platelet P2 receptors with increasing doses of NF449 [4,4′,4′′-carbonylbis(mino-5,1,3-benzotriylibs-(carbonyli)-minol][tetakis-benzene-1,3-disulfonic acid octasodium salt]. J Pharmacol Exp Ther 2005;314(01):232–243

18 Fung CY, Brearley CA, Farndale RW, Mahaut-Smith MP. A major role for P2X1 receptors in the early collagen-evoked intracellular Ca²⁺ responses of human platelets. Thromb Haemost 2005;94(01):37–40

19 Schwarz UR, Walter U, Eigenthaler M. Taming platelets with cyclic nucleotides. Biochem Pharmacol 2001;62(09):1153–1161

20 Rink TJ, Sanchez A. Effects of prostaglandin I2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester and exogenous diacylglycerol. Biochem J 1984;222(03):833–836

21 Wang L, Ostberg O, Wihlborg AK, Brogren H, Jern S, Erlinge D. Quantification of ADP and ATP receptor expression in human platelets. J Thromb Haemost 2003;1(02):330–336

22 MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1, not P2T purinoceptors in human platelets. J Biol Chem 1996;271(06):2879–2881

23 Semple JW, Aslam R, Kim M, Speck ER, Freedman J. Platelet-bound lipopolysaccharide enhances Fc receptor-mediated phagocytosis of IgG-opsonized platelets. Blood 2007;109(11):4803–4805

24 Maugeri N, Rovere-Querini P, Evangelista V, et al. Neutrophils phagocytose activated platelets in vivo: a phosphatidylserine, P-selectin, and beta2 integrin-dependent cell clearance program. Blood 2009;113(21):5254–5265

25 Huang ZY, Chien P, Indik ZK, Schreiber AD. Human platelet FcγRIIA and phagocytes in immune-complex clearance. Mol Immunol 2011;48(04):691–696

26 Cox D, Kerrigan SW, Watson SP. Platelets and the innate immune system: mechanisms of bacterial-induced platelet activation. J Thromb Haemost 2011;9(06):1097–1107

27 Kapur R, Zufferey A, Boilard E, Semple JW. Nouvelle cuisine: platelets served with inflammation. J Immunol 2015;194(12):5579–5587

28 Kraemer BF, Campbell RA, Schwertz H, et al. Novel anti-bacterial activities of β-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. PLoS Pathog 2011;7(11):e1002355

29 Critten JG, Bergmeier W, Zhang Y, et al. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 2004;10(09):982–986

30 Stefanini L, Roden RC, Bergmeier W. CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. Blood 2009;114(12):2506–2514

31 Eto K, Murphy R, Kerrigan SW, et al. Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. Proc Natl Acad Sci U S A 2002;99(20):12819–12824

32 Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of P2X1, purinoceptors with alpha, beta-methylene ATP. Thromb Haemost 2001;85(02):303–308

33 Isgro M, Vial C, Leon C, Gachet C, Evans RJ, Mahaut-Smith MP. Interplay between P2Y1, P2Y12, and P2X1 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. Blood 2005;106(05):1644–1651

34 Salaün C, James DJ, Chamberlain LH. Lipid rafts and the regulation of exocytosis. Traffic 2004;5(04):255–264

35 Bodin S, Viala C, Ragab A, Payrastre B. A critical role of lipid rafts in the organization of a key FcgammaRIIa-mediated signaling pathway in human platelets. Thromb Haemost 2003;89(02):318–330

36 Vial C, Fung CY, Goodall AH, Mahaut-Smith MP, Evans RJ. Differential sensitivity of human platelet P2X1, and P2Y1 receptors to disruption of lipid rafts. Biochem Biophys Res Commun 2004;324(02):415–419

37 Rolf MG, Mahaut-Smith MP. Effects of enhanced P2X1 receptor Ca²⁺ influx on functional responses in human platelets. Thromb Haemost 2002;88(03):495–502

38 Jones S, Evans RJ, Mahaut-Smith MP. Ca²⁺ influx through P2X1 receptors amplifies P2Y1 receptor-evoked Ca²⁺ signaling and ADP-evoked platelet aggregation. Mol Pharmacol 2014;86(03):243–251

39 Braun A, Varga-Szabo D, Kleinschnitz C, et al. Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. Blood 2009;113(09):2056–2063
40 Tolhurst G, Carter RN, Amisten S, Holdich JP, Erlinge D, Mahaut-Smith MP. Expression profiling and electrophysiological studies suggest a major role for Orai1 in the store-operated Ca$^{2+}$ influx pathway of platelets and megakaryocytes. Platelets 2008; 19(04):308–313

41 Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS. Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. Blood 2002; 100(08):2801–2811

42 Karas SP, Rosse WF, Kurlander RJ. Characterization of the IgG-Fc receptor on human platelets. Blood 1982;60(06):1277–1282

43 Tomiyama Y, Kunicki TJ, Zipf TF, Ford SB, Aster RH. Response of human platelets to activating monoclonal antibodies: importance of Fc gamma RII (CD32) phenotype and level of expression. Blood 1992;80(09):2261–2268

44 Lu J, Marnell LL, Marjon KD, Mold C, Du Clos TW, Sun PD. Structural recognition and functional activation of FcgammaR by innate pentaxins. Nature 2008;456(7224):989–992

45 Tutwiler V, Madeeva D, Ahn HS, et al. Platelet transactivation by monocytes promotes thrombosis in heparin-induced thrombocytopenia. Blood 2016;127(04):464–472

46 Qiao J, Al-Tamimi M, Baker RI, Andrews RK, Gardiner EE. The platelet Fc receptor, FcγRIIa. Immunol Rev 2015;268(01):241–252

47 Canobbio I, Stefanini L, Guidetti GF, Balduini C, Torti M. A new role for FcγRIIIA in the potentiation of human platelet activation induced by weak stimulation. Cell Signal 2006;18(06):861–870

48 Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C-gamma 2 but not phospholipase C-gamma 1 in human platelets. FEBS Lett 1994;353(02):212–216

49 Eckly A, Rinckel JY, Proamer F, et al. Respective contributions of single and compound granule fusion to secretion by activated platelets. Blood 2016;128(21):2538–2549

50 Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. Blood 2012;120(26):5209–5216