Ca\textsuperscript{2+} Influx through P2X1 Receptors Amplifies P2Y1 Receptor-Evoked Ca\textsuperscript{2+} Signaling and ADP-Evoked Platelet Aggregation

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ABSTRACT

Many cells express both P2X cation channels and P2Y G-protein-coupled receptors that are costimulated by nucleotides released during physiologic or pathophysiologic responses. For example, during hemostasis and thrombosis, ATP-gated P2X1 channels and ADP-stimulated P2Y1 and P2Y12 G-protein coupled receptors play important roles in platelet activation. It has previously been reported that P2X1 receptors amplify P2Y1-evoked Ca\textsuperscript{2+} responses in platelets, but the underlying mechanism and influence on function is unknown. In human platelets, we show that maximally activated P2X1 receptors failed to stimulate significant aggregation but could amplify the aggregation response to a submaximal concentration of ADP. Costimulation of P2X1 and P2Y1 receptors generated a superadditive Ca\textsuperscript{2+} increase in both human platelets and human embryonic kidney 293 (HEK293) cells via a mechanism dependent on Ca\textsuperscript{2+} influx rather than Na\textsuperscript{+} influx or membrane depolarization. The potentiation, due to an enhanced P2Y1 response, was observed if ADP was added up to 60 seconds after P2X1 activation. P2X1 receptors also enhanced Ca\textsuperscript{2+} responses when costimulated with type 1 protease-activated and M1 muscarinic acetylcholine receptors. The P2X1-dependent amplification of G\textsubscript{q}-coupled [Ca\textsuperscript{2+}]i increase was mimicked by ionomycin and was not affected by inhibition of protein kinase C, Rho-kinase, or extracellular signal-regulated protein kinase 1/2, which suggests that it results from potentiation of inositol 1,4,5-trisphosphate receptors and/or phospholipase C. We conclude that Ca\textsuperscript{2+} influx through P2X1 receptors amplifies Ca\textsuperscript{2+} signaling through P2Y1 and other G\textsubscript{q}-coupled receptors. This represents a general form of co-incidence detection of ATP and coreleased agonists, such as ADP at sites of vascular injury or synaptic transmitters acting at metabotropic G\textsubscript{q}-coupled receptors.

Introduction

P2X ligand-gated ion channels and P2Y G-protein-coupled receptors (GPCRs) play important roles in a variety of excitable and nonexcitable tissues (Burnstock and Knight, 2004). In mammals, there are seven P2X receptor subunits that form a range of homotrimeric and heterotrimeric channels (Surprenant and North, 2009), and there are eight genes encoding P2Y receptors. Individual cells normally express more than one P2 receptor subtype, and interactions between their signaling pathways have the potential to regulate cellular responses. For example, platelets express only P2X1, P2Y1, and P2Y12 receptors (Kahner et al., 2006; Hechler and Gachet, 2011; Mahaut-Smith et al., 2011). ATP-gated P2X1 receptors generate significant transient increases in intracellular Ca\textsuperscript{2+} leading to shape change but not aggregation responses (Oury et al., 2001; Rolf et al., 2001; Rolf and Mahaut-Smith, 2002; Hechler et al., 2003). Previous work has demonstrated interactions between platelet P2X1 and P2Y1 receptors at the level of Ca\textsuperscript{2+} mobilization and the activation of P2Y1-evoked nonsensitive cation currents (Vial et al., 2002). In contrast, no evidence was found for interactions between P2X1 receptors and Gi-coupled P2Y12 pathways (Rolf and Mahaut-Smith, 2002). Interestingly, costimulation of G\textsubscript{q}-coupled P2Y1 and Gi-coupled P2Y12 receptor pathways is required for full aggregation responses to ADP (Hechler et al., 1998; Jin et al., 1998; Fabre et al., 1999; Leon et al., 1999; Foster et al., 2001; Cosemans et al., 2006; Jones et al., 2011). Taken together these studies highlight the complex interdependency of P2 receptor signaling.

P2X receptors are ATP-gated nonsensitive cation channels that generate significant direct Na\textsuperscript{+} and Ca\textsuperscript{2+} entry, leading to membrane depolarization (Surprenant and North, 2009). In excitable tissues, P2X1-induced depolarization can therefore lead to activation of additional Ca\textsuperscript{2+} entry through stimulation of voltage-gated Ca\textsuperscript{2+} channels. Furthermore, in both excitable and nonexcitable cell types, Na\textsuperscript{+} entry has the potential to elevate Ca\textsuperscript{2+} via reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity (Harper et al., 2013), and membrane depolarization has been reported to directly amplify GPCR-evoked Ca\textsuperscript{2+} release (Mahaut-Smith et al., 2008). Of note, it has been shown that P2Y1-evoked Ca\textsuperscript{2+} release...
release is enhanced by membrane depolarization of physiologically relevant magnitude in the platelet precursor cell, the megakaryocyte (Martinez-Pinna et al., 2004; Martinez-Pinna et al., 2005). The ability of P2X1 receptors to modulate Gq-coupled receptor signaling independent of voltage-gated Ca\(^{2+}\) channels could have widespread relevance given the ubiquitous occurrence of ATP as a cotransmitter throughout the peripheral and central nervous system (Burnstock, 2004). Our study used human platelets and a human nonexcitatory cell line expressing P2X1 and P2Y1 receptors to investigate the interactions between these two receptors at the level of signaling and function.

Materials and Methods

Reagents. Fura2-AM and Fluo3-AM were purchased from Invitrogen (Paisley, United Kingdom). GF109203X [2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide], Y-27632 (trans-4-[(1R)-1-aminooethyl]-N-4-pyridinylcyclohexancarboxamide dihydrochloride), MRS2179 [2-deoxy-N(6)-methyl adenosine 3′,5′-diphosphate], and NF449 [4,4′,4″,4‴-carbonylbis(imino-5,1,3-benzotetrazyli)tetraakis-benzene-1,3-disulfonic acid] were purchased from Tocris Bioscience (Avonmouth, United Kingdom). All other reagents were obtained from Sigma-Aldrich (Poole, United Kingdom) unless otherwise stated. ADP was treated with hexokinase to remove contaminating ATP, as reported previously elsewhere (Mahaut-Smith et al., 2000).

Platelet Preparation. Blood was obtained from healthy, aspirin-free, informed, consenting volunteers. The study was approved by the University of Leicester Committee for Research Ethics concerning human subjects (non-NHS) and was performed in accordance with the Declaration of Helsinki. Blood was drawn from the forearm by venipuncture into a syringe containing acid citrate dextrose anticoagulant (ACD, in mM: 85 trisodium citrate, 78 citric acid, 111 glucose) in 700 μl of PRP. For intracellular calcium measurements, PRP was incubated with fura2-AM (2 μM), for 45 minutes at 37°C before addition of agonists. The volume of PRP was equal to twice the volume of PRP. For intracellular calcium measurements, PRP was prepared by centrifugation at 350g for 20 minutes and resuspension in apyrase-containing nominally Ca\(^{2+}\)-free platelet saline (in mM: 145 NaCl, 5 KC1, 1 MgCl2, 10 HEPES, 10 glucose, pH 7.35, 0.32 U/ml apyrase, and 0.02% Pluronic). After 1 hour at room temperature, the loading buffer was replaced with normal saline, and the changes in fluorescence evoked by different agonists were measured using a Flexstation II 96-well fluorometer (Molecular Devices, Sunnyvale, California). When required, NF449 (1 μM), GF109203X (10 μM), Y-27632 (10 μM), or Y-27632 (10 μM) was added to the cells before stimulation.

To examine the role of extracellular Ca\(^{2+}\), experiments were conducted in saline without CaCl2 (nominally Ca\(^{2+}\)-free) or after equimolar substitution with BaCl2. To assess the role of extracellular Na\(^{+}\), NaCl was replaced with N-methyl-D-glucamine chloride (NMDG-Cl). To compare synergy between different batches of cells, responses were normalized to the maximal ADP response (at 100 μM ADP).

Derivation of Predicted Responses and Statistical Analysis. For both platelet suspensions and HEK293 cells, predicted calcium responses for costimulations were calculated by addition of the individual calcium increases induced by the two agonists. The integral of the Ca\(^{2+}\) response was assessed for a period of 60 seconds after agonist addition in the platelet and 90 seconds after agonist addition in HEK cells. Recordings of calcium and aggregation responses are from individual experiments with paired controls, representative of three to eight different donors for platelet responses and three to eight separate batches of cells for experiments with cell lines. Averages are reported as the mean ± S.E.M., and statistical significance was assessed using either paired or unpaired Student’s t-test or one-way analysis of variance (ANOVA), with Bonferroni multiple comparison test (Microsoft Excel, Microsoft, Redmond, WA; GraphPad Prism, GraphPad Software, San Diego, CA). The level of statistical significance is indicated in the figures as *P < 0.05 (**), P < 0.01 (***), and P < 0.001 (****).

Results

Stimulation of Platelet P2X1 Receptors Potentiates ADP-Mediated Ca\(^{2+}\) Mobilization and Aggregation. Previous studies have provided clear evidence that P2X1 and P2Y1 receptors synergize at the level of calcium signaling in platelets (Vial et al., 2002), but the impact of this interaction on functional responses has not been determined. It is established that the coupling of P2Y1 receptors to aggregation requires coactivation of P2Y12-stimulated pathways (Kahner et al., 2006; Hechler and Gachet, 2011) and that P2X1 receptors do not directly potentiate P2Y12-evoked aggregation (Rolf and Mahaut-Smith, 2002). Therefore, to explore the consequence of P2X1:P2Y1 interactions on platelet function, we compared responses to the selective P2X1 agonist α,β-meATP, the physiologic P2Y1 and P2Y12 agonist ADP, and both agonists combined.

ADP was used at a concentration (1 μM) shown previously to stimulate ~60% of the maximal Ca\(^{2+}\) response via P2Y receptors (MacKenzie et al., 1996), which in our experiments induced a transient increase in intracellular calcium with an average peak value of 395 ± 20.2 nM (Fig. 1A, i, ii). Maximal stimulation of P2X1 receptors with α,β-meATP (5 μM) evoked a transient Ca\(^{2+}\) response of similar duration and magnitude (peak 277 ± 60.4 nM; Fig. 1A, i, ii) to that seen with ADP.
A major role for extracellular signal-regulated kinase 2 activation (ERK2) has been proposed in the Ca^{2+}-dependent pathways downstream of P2X1 receptor activation that potentiates collagen-evoked aggregation (Oury et al., 2002). Furthermore, Ca^{2+}-dependent ERK2 signaling has been implicated in ADP-stimulated aggregation via the generation of thromboxane \( \Delta_2 \) (TXA2) (Garcia et al., 2007; Stefanini et al., 2009) that reinforces the P2Y-evoked aggregation response (Mustard et al., 1975; Packham et al., 1989). Inhibition of ERK1/2 activation using U0126 (10 \( \mu \)M) did not affect the \( \alpha,\beta\)-meATP-induced potentiation of P2Y1-mediated calcium mobilization (1.68 ± 0.15-fold and 1.62 ± 0.12-fold larger than predicted in the presence of vehicle control and U0126, respectively, \( P < 0.05 \)) (Fig. 2A).

U0126 also had no significant effect on the aggregation response evoked by 1 \( \mu \)M ADP alone (not shown). However, U0126 (10 \( \mu \)M) completely abolished the enhancement of ADP-mediated aggregation by \( \alpha,\beta\)-meATP (aggregation responses 2.1 ± 0.5-fold and 1.0 ± 0.05-fold for vehicle control and U0126, respectively) (Fig. 2B). Consistent with reports that TXA2 generation is downstream of ERK phosphorylation (Garcia et al., 2007; Stefanini et al., 2009), potentiation of intracellular calcium (907 ± 97 nM, denoted “actual” response), which was 1.69 ± 0.18-fold greater (\( P < 0.05 \)) than the response “predicted” by mathematical addition of the Ca^{2+} responses to the individual agonists (536 ± 9 nM; Fig. 1A, i and ii), consistent with previous studies (Vial et al., 2002).

The integral of the Ca^{2+} response after costimulation by \( \alpha,\beta\)-meATP and ADP was also enhanced compared with the value obtained by summation of the individual agonist responses (Fig. 1A, iii). However, amplification of the peak increase was more pronounced. In turbidimetric measurements, 1 \( \mu \)M ADP evoked a transient aggregation of platelets peaking at 20% transmission increase. 5 \( \mu \)M \( \alpha,\beta\)-meATP generated a transient shape change but failed to stimulate aggregation (Fig. 1B, i), as reported previously by our group and others (Oury et al., 2001; Rolf et al., 2001; Rolf and Mahaut-Smith, 2002; Hechler et al., 2003). However, coaddition of 5 \( \mu \)M \( \alpha,\beta\)-meATP and 1 \( \mu \)M ADP evoked an aggregation response significantly greater than that observed with 1 \( \mu \)M ADP alone (Fig. 1B, i–iii); the amplitude and integral of the aggregation response were increased 1.95 ± 0.20-fold, \( P < 0.05 \), and 2.65 ± 0.63-fold, \( P < 0.01 \), respectively. Under the conditions of these experiments, the ADP-evoked Ca^{2+} response is entirely dependent upon P2Y1 receptors as it is blocked by the P2Y1-selective antagonist MRS2179 and unaffected by the P2Y12 antagonist cangrelor (Fung et al., 2007). Therefore, these results demonstrate a clear synergistic interaction of P2X1 and P2Y1 receptor signaling at the level of intracellular Ca^{2+} mobilization, leading to enhanced platelet ADP-evoked functional responses.
ADP-evoked calcium responses by α,β-meATP was unaffected by treatment with 100 μM aspirin (1.76 ± 0.2-fold increase above predicted Ca²⁺ response, P < 0.05) (Fig. 2A), but the enhanced aggregation was abolished (1.0 ± 0.17-fold change compared with predicted response) (Fig. 2B). This effect was not a result of direct inhibition of the P2Y-evoked response because the aggregation evoked by 1 μM ADP alone was not significantly altered by 100 μM aspirin (not shown).

Use of HEK293 Cells and Recombinant P2X1 Receptor Expression Demonstrates That Ca²⁺ Influx Through P2X1 Receptors Potentiates P2Y1-Evoked Ca²⁺ Responses. To explore further the mechanism whereby P2X1 and P2Y1 receptors interact at the level of Ca²⁺ mobilization, we switched to a HEK293 cell line to avoid the secondary responses that are known to occur in platelets due to release of nucleotides and other agonists. This also allowed us to assess whether the P2X1:P2Y1 synergy is a general phenomenon or specific to platelets. In native HEK293 cells, ADP stimulated a concentration-dependent intracellular Ca²⁺ increase with an EC₅₀ of 0.04 μM (Fig. 3, A and C). ADP-evoked responses were inhibited by the P2Y1-selective receptor antagonist MRS2179 (Fig. 3B) and thus are due to endogenously expressed P2Y1 receptors. In contrast, the P2X1 agonist α,β-meATP failed to induce a Ca²⁺ increase or alter the response to ADP in these native HEK293 cells (not shown). However, when human P2X1 receptors were stably expressed in HEK293 cells (HEK293-P2X1, which did not alter the P2Y1-evoked response) (Fig. 3C), α,β-meATP evoked concentration-dependent inward currents in patch clamp studies (Evans et al., 1996) and increases in intracellular calcium (Fig. 3D).

The HEK293-P2X1 cells therefore provide an ideal model system to investigate the mechanism underlying synergy between P2X1 and P2Y1 receptors. We assessed the magnitude of the interaction between these two receptors through calcium responses to ADP (0.01 μM), α,β-meATP (0.05 μM), or combined application of both agonists at these submaximal concentrations. We reasoned that a lower α,β-meATP concentration was more relevant to study the synergy in the cell line due to its high P2X1 receptor density. Responses to submaximal α,β-meATP concentrations were consistently observed in all batches of HEK293-P2X1 cells, in contrast to platelets where a substantial interdonor variability made it difficult to use low levels of the P2X1 agonist. Figure 4A(i) shows the Ca²⁺ indicator responses from a single experimental run, and Fig. 4A (ii) shows the average peak increase from multiple batches of cells normalized to the response evoked by a maximal ADP concentration (see Materials and Methods for further detail). The “actual” peak Ca²⁺ increase induced by simultaneous addition of these two agonists was an average of 2.55 ± 0.26-fold greater than the response “predicted” by summation of the responses to the individual agonists. This amplification was mediated by P2X1 receptors as 1) α,β-meATP had no effect on ADP responses in native HEK cells that lack P2X1 receptors (Fig. 5C) and 2) 1 μM NF449 (a concentration of this suramin analog that selectively blocks P2X1 receptors; Fung et al., 2007) prevented α,β-meATP-mediated amplification of ADP-evoked P2Y1 Ca²⁺ responses (Fig. 5C). In addition, α,β-meATP (0.05 μM) also caused a similar enhancement of the Ca²⁺ response to the nonhydrolyzable analog ADP₈S (0.1 μM) (2.78 ± 0.18-fold increase above the predicted response) (Fig. 4B, i, ii), demonstrating that the enhanced P2Y1 response does not result from reduced ADP breakdown by inhibition of ectonucleotidase activity (Jones et al., 2011). The integral of the Ca²⁺ response for both ADP and ADP₈S also displayed a superadditive response after costimulation of P2X1:P2Y1 receptors but to a lesser extent than the peak response (Fig. 4A, iii, and Fig. 4B, iii), as observed for platelets.

Previous work has suggested that many Ca²⁺-dependent functions in platelets require a threshold level of cytoplasmic Ca²⁺ to be achieved, so it is likely that the peak response is more physiologically relevant than the integral (Rink et al., 1982). We therefore used the peak increase in subsequent experiments that explore the mechanism of the synergy.

The P2X1 receptor is a nonselective cation channel, and agonist binding results in channel opening and membrane depolarization that results from Na⁺ and Ca²⁺ influx (Mahaut-Smith et al., 2011; Surprenant and North, 2009). It has previously been shown that P2Y1 receptor–mediated Ca²⁺ mobilization is sensitive to membrane depolarization (Martinez-Pinna et al., 2005) and that sodium influx contributes ~90% to the membrane depolarization upon P2X1 receptor activation.
(Benham, 1989). To test whether sodium influx and membrane depolarization are important for the P2X1-P2Y1 synergy we replaced the Na\(^+\) in the extracellular solution with NMDG\(^+\). NMDG\(^+\) is >30-fold less permeant than Na\(^+\) at P2X1 receptors, resulting in a reversal potential of \(-84\ \text{mV}\) for P2X1 receptors in NMDG\(^+\), Na\(^+\)-free saline, and thus hyperpolarization of the membrane potential when these channels open (Evans et al., 1996). NMDG\(^+\) substitution had no effect on the synergy between P2X1 and P2Y1 receptors (Fig. 5, A and C), which suggests that neither membrane depolarization nor Na\(^+\) influx underlie the synergy. To test whether calcium influx through the P2X1 receptor was responsible, we replaced the external Ca\(^{2+}\) with Ba\(^{2+}\), which is permeable through P2X receptors. Ba\(^{2+}\) substitution prevented the potentiation of ADP responses by \(\alpha\beta\)-meATP (Fig. 5, B and C).

Taken together, these results show that Ca\(^{2+}\) influx through P2X1 receptor cation channels is required to amplify the ADP-evoked calcium responses, with little or no role for influx of Na\(^+\) or membrane depolarization. Inhibition of several Ca\(^{2+}\)-dependent or P2X1 or P2Y1 receptor-dependent pathways, including ERK1/2, Rho kinase, and PKC (using U0126, Y-27632, and GF109203X, respectively, at 10 \(\mu\text{M}\)) had no effect on the synergy between P2X1 and P2Y1 receptors in the HEK293-P2X1 cell line (\(P > 0.05\) for all three conditions). This reinforces the conclusion that an increase in cytosolic Ca\(^{2+}\) is the key signal responsible for the synergy.

To determine whether a general global rise in intracellular calcium could also potentiate P2Y1 receptor-mediated responses we compared the P2X1-induced amplification with effects of the Ca\(^{2+}\) ionophore ionomycin. For these experiments, an ionomycin concentration was selected (0.1 \(\mu\text{M}\)) that produced a similar peak calcium increase to that observed with 0.05 \(\mu\text{M}\) \(\alpha\beta\)-meATP (8.1 \(\pm\) 1.1\% and 10.2 \(\pm\) 1.7\% of the maximal ADP response, respectively). Costimulation with 0.1 \(\mu\text{M}\) ionomycin and ADP (0.01 \(\mu\text{M}\)) resulted in a response that was significantly greater (1.77 \(\pm\) 0.33-fold, \(P < 0.05\)) than that predicted from the sum of the individual responses (see sample traces in Fig. 5D). This potentiation by ionomycin was not significantly different from that observed by P2X1 receptors (\(P < 0.05\)).

Together, these experiments suggest that amplification of P2Y1 receptor–mediated Ca\(^{2+}\) mobilization by P2X1 receptors requires an increase in intracellular Ca\(^{2+}\), which is likely to occur through a global increase in intracellular calcium concentration rather than via a microdomain specific to the ionotropic receptor.

**Prestimulation of P2X1 Receptors Enhances P2Y1 Responses.** After activation, P2X1 receptors rapidly desensitize, but the Ca\(^{2+}\) increase they generate extends beyond the period of channel opening because of the time taken for homeostatic mechanisms to restore the cytoplasmic Ca\(^{2+}\) concentration to resting levels. Therefore, we investigated whether synergy also occurred if P2Y1 receptors were stimulated at various times after \(\alpha\beta\)-meATP in HEK293-P2X1 cells. Application of ADP (0.01 \(\mu\text{M}\)) 15, 30, and 60 seconds after \(\alpha\beta\)-meATP (0.05 \(\mu\text{M}\)) resulted in calcium responses significantly greater than the predicted levels, but of a magnitude that decreased as the interval was prolonged, consistent with the time course of the P2X1-dependent Ca\(^{2+}\) response (amplifications of 1.77 \(\pm\) 0.2, 1.84 \(\pm\) 0.07, and 1.52 \(\pm\) 0.14-fold, respectively, compared with 2.3 \(\pm\) 0.18 for costimulation) (Fig. 6A). In contrast, synergy was absent if ADP was added 5 minutes after \(\alpha\beta\)-meATP. As with P2X1-mediated synergy, ionomycin was capable of potentiating the P2Y1 response if...
ADP was added up to 1 minute later, but this was lost at 5 minutes (Fig. 6B). Therefore, P2X1-dependent amplification of P2Y1 Ca²⁺ responses display a “memory,” allowing synergy between these two pathways in situations where the GPCR is activated subsequent to the ATP-gated channel.

**P2X1 Receptors Potentiate Other Gq-Coupled Receptors.** To determine whether P2X1 receptors have a more general synergistic effect on GPCR-evoked Ca²⁺ signaling, we examined the effect of α,β-meATP in combination with activation of other endogenous Gq-coupled GPCRs in HEK293 cells. Type 1 protease activated receptors (PAR1) and M1 muscarinic acetylcholine receptors (M1AChR) were activated by submaximal concentrations of thrombin (Fig. 7, A) or carbachol (Fig. 7, B), established from concentration response curves (data not shown), along with P2X1 receptors by 0.05 μM α,β-meATP. Thrombin-stimulated Ca²⁺ responses were amplified 1.38 ± 0.05-fold and carbachol 2.27 ± 0.14-fold relative to the predicted values. These studies establish the principle that P2X1 receptors can amplify Ca²⁺ signals downstream of a range of Gq-coupled receptors.

**Discussion**

It is well established that ADP-evoked P2Y1 and P2Y12 receptor signals interact to enhance downstream functional responses in the platelet (Hechler and Gachet, 2011). We now demonstrate that costimulation of ATP-gated P2X1 receptors and ADP-stimulated P2Y receptors also potentiates platelet aggregation. It is also worth noting that ATP is a partial agonist at P2Y1 and P2Y12 receptors and unable to generate GPCR-evoked responses in the platelet due to low levels of receptor expression (reviewed in Mahaut-Smith et al., 2011). Furthermore, ADP is not an agonist at P2X1 receptors (Mahaut-Smith et al., 2000). Therefore, in the platelet, P2X1 and P2Y receptors are selectively activated by ATP and ADP, respectively, and the synergy we demonstrate here provides a means of coincidence detection of these two nucleotides during hemostasis and thrombosis. Regarding the underlying mechanism of the synergy, P2X1 receptor-evoked Ca²⁺ influx clearly potentiates P2Y1-evoked Ca²⁺ responses, but additionally ERK2 and TXA2 generation are required for amplification of aggregation responses.

These effects are consistent with the essential involvement of a cytosolic Ca²⁺ increase in ADP-evoked aggregation (Jin and Kunapuli, 1998; Garcia et al., 2007; Varga-Szabo et al., 2009), and also the importance of Ca²⁺/CalDAG-GEFI-dependent ERK2 in release of TXA2 that reinforces the P2Y-evoked aggregation response (Mustard et al., 1975; Packham et al., 1989; Garcia et al., 2007; Stefanini et al., 2009). Although previous work has shown that P2X1 does not directly synergize with P2Y12 to amplify aggregation (Rolf and Mahaut-Smith, 2002), Ca²⁺-dependent ERK2-activation and thus TXA2 release depend upon both P2Y1 and P2Y12 receptors (Garcia et al., 2007). Therefore, P2X1 receptors can be considered to indirectly enhance P2Y12-dependent aggregation and thrombosis via an action on P2Y1 receptor-evoked Ca²⁺ responses.
to platelets; it also occurs between P2X1 and other Gq-coupled receptors. Therefore, this interaction may have relevance in the placement of the major permeating ion under physiologic conditions (Na\(^+\)) with impermeant NMDG\(^-\) had no effect on the amplification of P2Y1 responses. This experiment also rules out a role for an increase in cytosolic Na\(^+\) or reverse Na\(^+\)/Ca\(^{2+}\) exchange (Sage et al., 1991; Harper et al., 2013). Increases in intracellular Ca\(^{2+}\) delivered by ionomycin mimic the synergistic effect of P2X1 on P2Y1 receptors, further supporting the conclusion that the P2X1-induced increase in cytosolic Ca\(^{2+}\) is responsible for the synergy. Inhibition of a number of other pathways (ERK2, Rho kinase, or PKC) had no effect.

One likely mechanism for the synergy is enhanced inositol 1,4,5-trisphosphate (IP\(_3\)) receptor activation because these intracellular Ca\(^{2+}\) release channels are known to be potentiated by cytosolic Ca\(^{2+}\) in the range of 10 nM to 1 \(\mu\)M (Bezprozvanny et al., 1991; Foskett et al., 2007). Indeed, evidence for amplification of IP\(_3\)-dependent Ca\(^{2+}\) release after P2X receptor stimulation has been provided in renal arterial smooth muscle (Povstyan et al., 2011), although depolarization leading to activation of voltage-gated Ca\(^{2+}\) channels is responsible for a substantial component of the P2X-evoked Ca\(^{2+}\) influx; thus, caution should be taken in directly comparing with our study on nonexcitable cells.

Another mechanism by which P2X1 could enhance P2Y1 receptor Ca\(^{2+}\) responses is via potentiation of phospholipase-C (PLC) because the activity of this enzyme has been reported to be Ca\(^{2+}\)-dependent, including in platelets (Eberhard and Holz, 1988; Watson et al., 1995). However, a \(^{3}H\)IPX assay was unable to distinguish whether P2Y1-evoked PLC responses were amplified due to the very low levels of IP\(_3\) generated in HEK293 cells at agonist concentrations matching those within the Ca\(^{2+}\) studies (R. Mistry, S. Jones, and R.A.J. Challiss, unpublished observations).

Our studies in HEK293 cells show that the P2X1-dependent potentiation of P2Y1 receptor Ca\(^{2+}\) increases is not restricted to platelets; it also occurs between P2X1 and other Gq-coupled receptors. Therefore, this interaction may have relevance in a range of cell types given both the widespread expression of P2X1 and the common use of ATP as an extracellular signaling molecule (Burnstock and Knight, 2004; Surprenant and North, 2009). Ca\(^{2+}\) influx through P2X1 receptors is necessary and sufficient to explain the enhancement of P2Y1 receptor-dependent Ca\(^{2+}\) signals as the synergy was abolished in Ca\(^{2+}\)-free salines or after treatment with NF449 at a concentration that is selective for P2X1 (Fung et al., 2007). Although activation of P2Y1 and other Gq-coupled receptors leads to enhanced P2X1 receptor responses in cell lines, most likely via phosphorylation of an accessory protein (Vial et al., 2004), this effect requires a delay of at least 30 seconds and thus was not responsible for the P2X1:P2Y1 synergy observed in our present work.

We have previously shown that P2Y1 receptors are directly enhanced by depolarization, and cation influx through P2X1 receptors will exert a depolarizing influence; however, replacement of the major permeating ion under physiologic conditions (Na\(^+\)) with impermeant NMDG\(^-\) had no effect on the amplification of P2Y1 responses. This experiment also rules out a role for an increase in cytosolic Na\(^+\) or reverse Na\(^+\)/Ca\(^{2+}\) exchange (Sage et al., 1991; Harper et al., 2013). Increases in intracellular Ca\(^{2+}\) delivered by ionomycin mimic the synergistic effect of P2X1 on P2Y1 receptors, further supporting the conclusion that the P2X1-induced increase in cytosolic Ca\(^{2+}\) is responsible for the synergy. Inhibition of a number of other pathways (ERK2, Rho kinase, or PKC) had no effect.

One likely mechanism for the synergy is enhanced inositol 1,4,5-trisphosphate (IP\(_3\)) receptor activation because these intracellular Ca\(^{2+}\) release channels are known to be potentiated by cytosolic Ca\(^{2+}\) in the range of 10 nM to 1 \(\mu\)M (Bezprozvanny et al., 1991; Foskett et al., 2007). Indeed, evidence for amplification of IP\(_3\)-dependent Ca\(^{2+}\) release after P2X receptor stimulation has been provided in renal arterial smooth muscle (Povstyan et al., 2011), although depolarization leading to activation of voltage-gated Ca\(^{2+}\) channels is responsible for a substantial component of the P2X-evoked Ca\(^{2+}\) influx; thus, caution should be taken in directly comparing with our study on nonexcitable cells.

Another mechanism by which P2X1 could enhance P2Y1 receptor Ca\(^{2+}\) responses is via potentiation of phospholipase-C (PLC) because the activity of this enzyme has been reported to be Ca\(^{2+}\)-dependent, including in platelets (Eberhard and Holz, 1988; Watson et al., 1995). However, a \(^{3}H\)IPX assay was unable to distinguish whether P2Y1-evoked PLC responses were amplified due to the very low levels of IP\(_3\) generated in HEK293 cells at agonist concentrations matching those within the Ca\(^{2+}\) studies (R. Mistry, S. Jones, and R.A.J. Challiss, unpublished observations).

We previously observed an increased activation of a Gq-activated cation channel by P2X1 during whole-cell recordings in the megakaryocyte (Vial et al., 2002). The underlying channel is most likely canonical transient receptor channel (TRPC) 6 (Hassock et al., 2002; Carter et al., 2006), which could be enhanced by the P2X1-induced increase in [Ca\(^{2+}\)]\(_i\) (Shi et al., 2004), by stimulation of diacylglycerol (DAG) production (Hofmann et al., 1999; Hassock et al., 2002; Ramanathan et al., 2012), or by a decrease in membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) levels (Tolhurst et al., 2005). Several TRPC channels are endogenously expressed in HEK293 cells, including TRPC3, TRPC6, and TRPC7, which have been suggested to be DAG activated (Wu et al., 2000; Zagranichnaya et al., 2005) and may also contribute to the synergy in this cell line. Greater release of IP\(_3\)-dependent Ca\(^{2+}\) stores could also lead to increased Ca\(^{2+}\) entry through Orai1 store-operated Ca\(^{2+}\) channels (Varga-Szabo et al., 2009). It was interesting to note that the peak of the Ca\(^{2+}\) response was enhanced more than the integral, which may be explained by an acceleration of one or more of these Ca\(^{2+}\)-dependent events by the P2X1-induced Ca\(^{2+}\) entry. We have previously demonstrated an essential role for lipid raft location for P2X1 function in platelets and cells lines (Vial and Evans, 2005; Vial et al., 2006), raising the possibility that a close association with Gq-coupled receptors in these microdomains may contribute to the synergy that we describe. However, the comparable amplification of P2Y1 receptor Ca\(^{2+}\) responses by ionomycin and P2X1 receptors argues against any role for microdomains (Fig. 6). Furthermore, in our previous work...
we found no role for lipid rafts in P2Y1 function (Vial et al., 2006). Figure 8 summarizes the mechanism(s) whereby P2X1 receptors can interact to enhance P2Y1- and P2Y12-dependent amplification of P2Y1 responses, this effect decreased over a time course that mirrored the α,β-metATP-stimulated Ca2+ response (Fig. 3D, 6A), and a similar time course was observed for ionomycin-evoked enhancement of the P2Y1 response (Fig. 6B). This time dependence of P2Y1 amplification has significance in vivo because ATP released at the site of injury is converted to ADP by ectonucleotidases present on endothelial cells and leukocytes, and also soluble in plasma, thereby providing sequential delivery of ATP followed by ADP. Furthermore, when ATP is released from nerve endings at tissues that coexpress P2X1 and P2Y1 receptors, ectonucleotidases will generate ADP subsequent to P2X1 stimulation (Burnstock and Knight, 2004; Robson et al., 2006).

In conclusion, our study demonstrates for the first time that synergy occurs between platelet P2X1 and P2Y1 receptors at the level of functional responses. The underlying mechanism involves enhanced Ca2+ responses due to P2X1-dependent Ca2+ influx, which amplifies aggregation through ERK2 and release of TXA2. This represents a form of coincidence detection of ATP and ADP released at sites of vascular injury and may contribute to the reportable ability of P2X1 receptors to amplify thrombosis in small arteries and arterioles (Mulyaran et al., 2000). Experiments presented here also provide evidence that P2X1 receptors can amplify Ca2+ mobilization evoked via several Gq-coupled receptors, which may lead to amplification of responses at neuronal or neuromuscular junctions when ATP is released as a cotransmitter.

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