Blockade of the purinergic P2Y$_{12}$ receptor greatly increases the platelet inhibitory actions of nitric oxide

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Circulating platelets are constantly exposed to nitric oxide (NO) released from the vascular endothelium. This NO acts to reduce platelet reactivity, and in so doing blunts platelet aggregation and thrombus formation. For successful hemostasis, platelet activation and aggregation must occur at sites of vascular injury despite the constant presence of NO. As platelets aggregate, they release secondary mediators that drive further aggregation. Particularly significant among these secondary mediators is ADP, which, acting through platelet P2Y$_{12}$ receptors, strongly amplifies aggregation. Platelet P2Y$_{12}$ receptors are the targets of very widely used antithrombotic drugs such as clopidogrel, prasugrel, and ticagrelor. Here we show that blockade of platelet P2Y$_{12}$ receptors dramatically enhances the antiplatelet potency of NO, causing a 1,000- to 100,000-fold increase in inhibitory activity against platelet aggregation and release reactions in response to activation of receptors for either thrombin or collagen. This powerful synergism is explained by blockade of a P2Y$_{12}$ receptor-dependent, NO/cGMP-insensitive phosphatidylinositol 3-kinase pathway of platelet activation. These studies demonstrate that activation of the platelet ADP receptor, P2Y$_{12}$, severely blunts the inhibitory effects of NO. The powerful antithrombotic effects of P2Y$_{12}$ receptor blockers may, in part, be mediated by profound potentiation of the effects of endogenous NO.

anti-platelet therapy | atherothrombosis | prostacyclin

Activation of platelets in a damaged blood vessel leads to a well-characterized sequence of events, including the important release of secondary mediators of aggregation, notably ADP and thromboxane A$_2$ (1–3). These mediators are the targets of antithrombotic drugs taken by many millions of patients as prophylactic protection against heart attacks and strokes. In particular, inhibition of thromboxane A$_2$ production by aspirin explains the antithrombotic utility of this drug, whereas blockade of the ADP receptor, P2Y$_{12}$, by drugs such as clopidogrel, prasugrel, and ticagrelor provides a second, and possibly even stronger, antithrombotic protection (4). Within the circulation, platelets are constantly exposed to antithrombotic mediators, notably prostaglandin I$_2$ (PGI$_2$), which is derived from the vascular endothelium, and nitric oxide (NO), which can be produced by the vascular endothelium (5) and platelets themselves (6, 7). This leads to the understanding that there is an important interplay between endothelial-derived antiaggregatory mediators and platelet-derived proaggregatory mediators. For example, it has been demonstrated that activation of platelet P2Y$_{12}$ receptors by ADP causes inhibition of platelet adenylyl cyclase, which reduces the ability of PGI$_2$ acting through prostaglandin IP receptors to elevate cAMP, and thus reduces platelet aggregation. Hence, blockade of platelet P2Y$_{12}$ receptors increases the sensitivity of platelets to the antiaggregatory and antisecretory effects of PGI$_2$ (8, 9). This interaction is readily understandable, as both P2Y$_{12}$ receptors and PGI$_2$ have actions on a common signaling pathway, cAMP.

Compared with PGI$_2$, NO is less potent as an inhibitor of platelet activation (10–17). NO acts by directly activating platelet

guanylyl cyclase, causing an increase in intraplatelet cGMP (13). Although this cGMP pathway is distinct from the cAMP pathway that is modulated by PGI$_2$ and P2Y$_{12}$, earlier studies have shown that activation of these two inhibitory pathways produces a synergistic antiplatelet effect (18). Further, it has been shown that a NO donor drug can boost the effectiveness of P2Y$_{12}$ inhibitors against thrombus formation ex vivo (19, 20). Here we report that treatment of platelets with P2Y$_{12}$ receptor blockers dramatically increases the antiplatelet effects of NO, an increase that is very much greater than that already established for PGI$_2$. This reveals that activation of platelet P2Y$_{12}$ receptors by ADP could provide a common switching mechanism by which platelet sensitivity to the antithrombotic effects of NO and PGI$_2$ is rapidly reduced to allow platelet activation, as required for hemostasis. It also raises the important possibility that for individual patients, the effectiveness of P2Y$_{12}$ receptor blockers may vary on the basis of their vascular production of NO and PGI$_2$. This provides a rationale for the commonly reported disconnect between the results of ex vivo testing of platelet responses and thrombotic outcomes.

Results and Discussion

P2Y$_{12}$ Receptor Blockade Greatly Increases the Inhibition of Platelet Aggregation by NO. To study interactions between P2Y$_{12}$ receptor activation and the inhibitory effect of NO on platelet aggregation, we used thrombin as the stimulus for platelet activation. Thrombin is a physiologically relevant agonist that provides a strong signal for ADP release. Similar to previous studies of this type (8), we found 1 U/mL thrombin to provide a maximal aggregation and secretion response that was relatively insensitive to P2Y$_{12}$ receptor blockade (Fig. S1). By using 1 U/mL thrombin as an agonist, we could therefore study the effects of P2Y$_{12}$ receptor blockade on responses to NO without the confounding effects of a differing baseline. In control conditions, the NO donor diethlyammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA/NONOate), which spontaneously releases NO at physiological pH (half-life ~2 min), produced only weak inhibition of thrombin (1 U/mL)-induced washed platelet aggregation, even at supraphysiological concentrations (Fig. 1A). However, in the presence of the P2Y$_{12}$ receptor blocker prasugrel active metabolite (PAM), both the potency and the maximal effect of DEA/NONOate were dramatically increased (Fig. 1A; logIC$_{50}$ DEA/NONOate, -3.0 ± 0.4; DEA/NONOate +PAM, -7.9 ± 0.2; $P < 0.0001$; $n = 8$). This clearly suggests a powerful interaction between NO and P2Y$_{12}$ blockade.
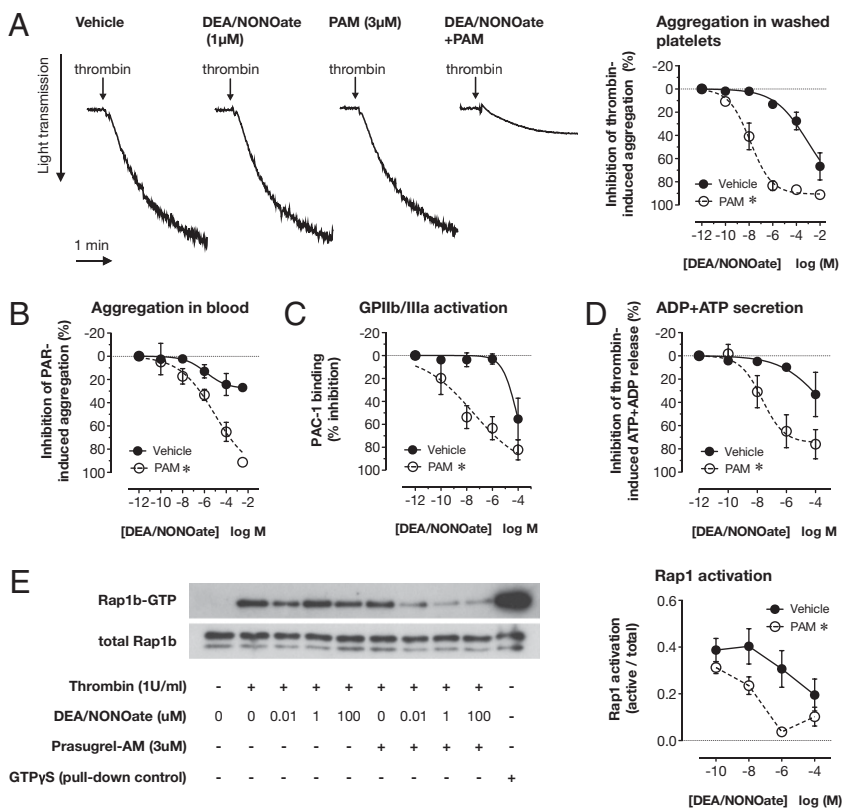
Fig. 1. Blockade of the P2Y$_{12}$ receptor sensitizes platelets to the inhibitory effect of NO against thrombin-induced aggregation, secretion, and Rap1 activation. Thrombin (1 U/mL)-induced aggregation of washed platelets, determined by changes in light transmission, was only weakly inhibited by the NO donor, DEA/NONOate (*A*) (*n* = 8), but this effect was greatly increased in platelets pretreated with the P2Y$_{12}$ receptor blocker PAM (3 μM). Traces show the effects of 1 μM DEA/NONOate in the absence and presence of PAM. Similarly, inhibition by DEA/NONOate of platelet aggregation in hirudin-anticoagulated whole blood stimulated with SFLLRN-amide (10 μM) + AYPGKF-amide (30 μM) and measured by impedance aggregometry (*B*), thrombin (1 U/mL)-induced GPIIb/IIIa activation by flow cytometry (*C*) and ADP+ATP secretion, measured by a modified luminometric method (*D*), were also strongly potentiated by P2Y$_{12}$ receptor blockade (*n* = 4 for each). Thrombin (1 U/mL) also stimulated rapid activation of Rap1, as determined by affinity purification of Rap1-GTP and Western blotting of lysates of platelets stimulated under stirring conditions for 15 s (*E*). Rap1 activation was little altered by DEA/NONOate or P2Y$_{12}$ receptor blockade alone but was strongly inhibited by the combination. Platelet lysates incubated with GTPγS were included on blots as a positive control for affinity isolation of Rap1-GTP (*n* = 4 for each). *$P < 0.05$ by two-way ANOVA vs. vehicle.

We observed a similar potentiation of the inhibitory effect of DEA/NONOate when we examined either aggregation of washed platelets induced by the glycoprotein VI (GPVI) agonist, collagen-related peptide (CRP; 1 μg/mL; Fig. S1), or aggregation in whole blood in response to activating peptides for the thrombin receptors, protease-activated receptor 1 (PAR1) and protease-activated receptor 4 (PAR4) (Fig. 1*B*). Importantly, this demonstrated that the powerful inhibitory interaction between P2Y$_{12}$ receptor blockade and NO exists for another strong platelet agonist and is not an artifact of the method of platelet preparation used (i.e., is not unique for washed platelets).

Platelets themselves have been previously described to release NO to regulate aggregation responses (6, 7). To study the interaction between P2Y$_{12}$ and platelet-derived NO, we measured thrombin- and collagen-induced platelet aggregation when platelet NO synthase activity was stimulated with L-arginine or inhibited with N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride (L-NAME). In our experimental system, however, we were unable to observe any role for platelet-derived NO, either in the presence or absence of P2Y$_{12}$ blockade (Fig. S2).

Inhibition of Thrombin-Induced Glycoprotein IIb/IIIa Activation, ADP+ATP Secretion, and Activation of the GTPase Rab1b by NO Is Increased by P2Y$_{12}$ Receptor Blockade. In parallel experiments, we confirmed that potentiation of the inhibitory effects of NO on platelet aggregation was reflected at the biochemical level of platelet activation. To do this, we studied glycoprotein IIb/IIIa (GPIIb/IIIa) activation (Fig. 1*C* and Fig. S3) and alpha granule section by flow cytometry (Fig. S3), dense granule secretion by ADP+ATP release (Fig. 1*D*), and Rap1 activation by using Ral guanine nucleotide dissociation stimulator (RalGDS) affinity pull-down for GTP-bound Rap1 (Fig. 1*E*). Thrombin stimulated a robust response in each activation assay, which was only modestly inhibited by DEA/NONOate. However, in each case, the inhibitory potency of DEA/NONOate was greatly increased by P2Y$_{12}$ receptor blockade. For example, 10 nM DEA/NONOate in the presence of PAM caused the same inhibition (31% ± 14%) of

thrombin-induced ADP+ATP secretion as 100 μM DEA/NONOate in the absence of PAM (34% ± 19% inhibition; *n* = 4; Fig. 1*D*). This represents a 10,000-fold increase in the inhibitory potency of DEA/NONOate.

Blockade of P2Y$_{12}$ Receptors Acts Synergistically with NO to Inhibit Platelet Aggregation Induced by Thrombin or CRP. To establish whether the interactions observed between NO and P2Y$_{12}$ blockade for inhibition of thrombin- and CRP-induced platelet aggregation were additive or synergistic, we performed an isobolographic analysis (21). To do this, we performed multiple matched aggregation studies in 96-well microtiter plates, using both thrombin (0.5 and 1 U/mL) and CRP (0.5 and 1 μg/mL) as agonists. Aggregation was measured in the presence of increasing concentrations of two structurally distinct P2Y$_{12}$ receptor blockers, PAM and ticagrelor. From these data, isobolograms were constructed (described in Fig. S4) showing the concentrations of PAM and DEA/NONOate required, in combination, to produce 50% (Fig. S5) or 25% and 75% (Fig. S6) inhibition of platelet aggregation. For a simple additive relationship between two inhibitors, an isobologram displays a straight line in which reducing concentration of one inhibitor requires a linear increase in the concentration of the other to maintain the same effect. However, data displayed here for the interaction between DEA/NONOate and PAM or ticagrelor show a very different pattern (Figs. S5 and S6), with the isoboles curving strongly toward the axes, indicating a powerful synergy between NO and blockade of P2Y$_{12}$ receptors.

P2Y$_{12}$ Activation Limits the Inhibitory Activity of NO Through a Mechanism Requiring Activation of Guanine Nucleotide Binding Protein α$_{i/z}$ but Not Cyclooxygenase. We next wanted to confirm the specific role of P2Y$_{12}$-dependent Gα$_{i/z}$ signaling in regulating the responses of platelets to DEA/NONOate. To do this, we determined the effect of epinephrine (10 μM), which restores Gα$_{i/z}$ activation in the presence of P2Y$_{12}$ blockade (22), on the sensitivity of platelets to DEA/NONOate. As described earlier, DEA/NONOate produced only weak inhibition of platelet aggregation,

but this was not altered by addition of epinephrine (Fig. S7). Similarly, as described earlier, pretreatment of platelets with PAM enhanced the inhibitory potency of DEA/NONOate, but this effect was completely reversed by the addition of epinephrine, indicating that this phenomenon is mediated by $G\alpha_{i/z}$ signaling.

To determine whether the interactions between $P2Y_{12}$ receptor blockade and DEA/NONOate occurred at the level of thromboxane A_2, we repeated experiments in the presence of aspirin (100 μM). Aspirin, however, had no effect on the inhibitory potency of DEA/NONOate or the potentiating effects of $P2Y_{12}$ receptor blockade (Fig. S7).

NO-Induced cGMP Formation and Activity Is Not Affected by $P2Y_{12}$ Receptor Blockade. It has been recently demonstrated that physiologically relevant concentrations of DEA/NONOate and other NO donors act in platelets exclusively through activation of soluble guanylyl cyclase, driving the production of cGMP (13). cGMP is an important signaling molecule with its own downstream effectors but may also act in part by influencing cAMP signaling. For example, it has been previously suggested that cGMP can elevate cAMP levels, possibly by competitive inhibition of phosphodiesterase (PDE) 3 (23, 24). To determine whether platelet cGMP and/or cAMP was affected by the interaction between NO and $P2Y_{12}$ receptor activation, we measured platelet cGMP and cAMP levels, as well as vasodilator-stimulated phosphoprotein (VASP) phosphorylation at serine 239,

Fig. 2. $P2Y_{12}$ receptor blockade does not alter the ability of NO to stimulate cAMP or cGMP formation or VASP phosphorylation. cGMP content, determined by immunoassay in lysates of washed platelets stimulated with thrombin (1 U/mL) under stirring conditions, was increased by DEA/NONOate (*A*). This response was not different in platelets pretreated with the $P2Y_{12}$ receptor blocker PAM (3 μM; *n* = 9). cAMP content, measured in the same way, was not altered by either DEA/NONOate or $P2Y_{12}$ receptor blockade (*B*) (*n* = 9). The level of phospho(Ser239)-VASP, measured by flow cytometry in washed platelets stimulated with thrombin under nonstirring conditions, was also increased by DEA/NONOate, and this response was not altered by $P2Y_{12}$ receptor blockade (*C*). Representative histograms show the effect of 100 μM DEA/NONOate in the presence and absence of PAM (*n* = 4).

a composite cGMP and cAMP response (25). $P2Y_{12}$ blockade alone had no detectable effect on platelet cGMP or cAMP content or on VASP phosphorylation (Fig. 2). DEA/NONOate caused a concentration-dependent increase in the platelet content of cGMP (Fig. 2*A*) without affecting cAMP (Fig. 2*B*), but with a concomitant increase in VASP phosphorylation (Fig. 2 *C* and *D*). These actions were unaffected by blockade of $P2Y_{12}$ receptors, suggesting that the interaction between DEA/NONOate and $P2Y_{12}$ is downstream of the formation of cAMP and cGMP.

Blockade of $P2Y_{12}$ Receptors Does Not Interact with Inhibitors of PDE2, PDE3, or PDE4 in Affecting Platelet Aggregation. To further exclude any role for regulation of phosphodiesterase activity as underlying the interactions between NO and $P2Y_{12}$ receptors, we performed experiments using selective inhibitors of the 3 major platelet PDE isoforms: PDE2, PDE3, and PDE5 (26). Inhibition of PDE2 by *erythro*-9-2-hydroxy-3-nonyladenine, or PDE3 by milrinone, produced no change in the effects of DEA/NONOate on platelet aggregation, cAMP formation, or cGMP formation (Fig. S8). In contrast, inhibition of PDE5 by sildenafil enhanced the inhibitory activity of DEA/NONOate against thrombin (1 U/mL)-induced platelet aggregation, which was associated with an increased platelet content of cGMP but not cAMP (Fig. S8). The addition of PAM increased the inhibitory effects of DEA/NONOate; this increase was not altered by either milrinone or *erythro*-9-2-hydroxy-3-nonyladenine but was further increased by sildenafil. The results of these experiments are consistent with the well-established role for PDE5, but not PDE2 or PDE3, as a limiter of cGMP accumulation in platelets. More relevantly, these results indicate that neither PDE inhibition nor cAMP play a specific role in the interaction between $P2Y_{12}$ receptor blockade and NO. Indeed, there were no detectable changes in platelet cAMP content even when platelet cGMP content was elevated more than 150-fold by the combination of DEA/NONOate and sildenafil.

$P2Y_{12}$ Blockade Increases the Ability of cGMP-Analogs to Inhibit Platelet Aggregation, but Not VASP Phosphorylation. Having shown that $P2Y_{12}$ receptor blockade was not influencing the formation or metabolism of cAMP and cGMP, we next sought to determine whether $P2Y_{12}$ receptor blockade alters the sensitivity of platelets to exogenous cGMP. We found that PAM enhanced the ability of a cell-permeable analog of cGMP, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP), to inhibit aggregation (Fig. 3*A*), but it did not alter the ability of cGMP to stimulate VASP phosphorylation (Fig. 3*B*). This confirmed that the interaction between $P2Y_{12}$ receptor blockade and NO occurs downstream of cGMP.

To further explore the role for cGMP, we performed experiments using two structurally distinct inhibitors of cGMP regulated protein kinase (cGK), which is a major downstream effector of cGMP. We found that neither 8-(4-chlorophenylthio)guanosine-3′,5′cyclic monophosphorothioate, Rp-isomer (Rp-8-pCPT-cGMPS) (200 μM) nor KT5823 (5 μM) affected the inhibition of platelet aggregation following the combination of DEA/NONOate and PAM (Fig. S8). This may indicate that the inhibition of platelet aggregation occurs through a cGK-independent mechanism. Alternatively, and possibly more likely, this may reflect the limitations of these experimental compounds (27–29).

Inhibition of Thrombin-Induced Intracellular Calcium Mobilization by NO Is Not Limited by $P2Y_{12}$ Receptor Activation. Current evidence indicates that the inositol triphosphate receptor associated cGK target (IRAG) is a primary mediator of the inhibitory effects of cGMP in platelets. IRAG exists in a complex with cGK-I and the type 1 $InsP_3$ receptor in the membrane of the dense tubular system and regulates $InsP_3$-mediated calcium release, a major component of aggregation and secretion responses to many agonists, including thrombin. IRAG deletion (14), or mutation to prevent its association with the $InsP_3$ receptor, strongly blunts the inhibitory effects of NO donors on platelets (12). In comparison,
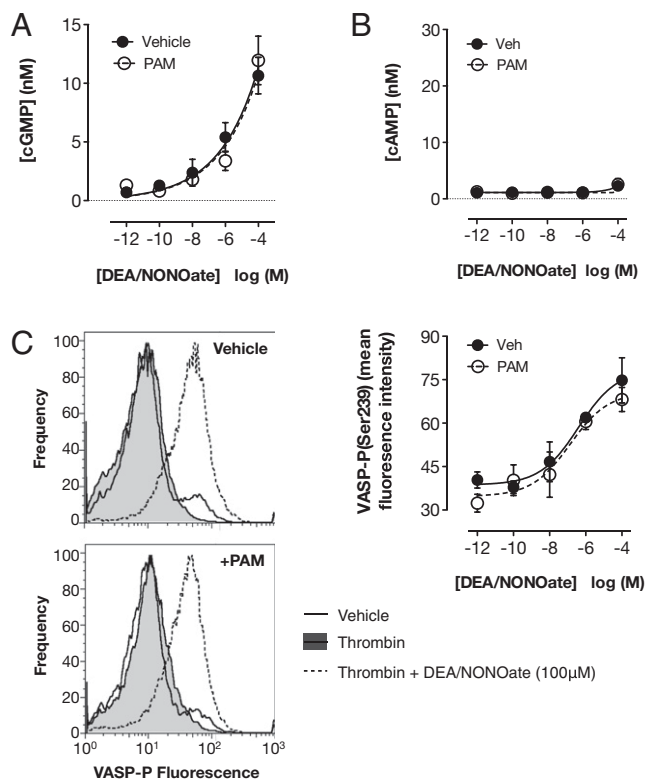
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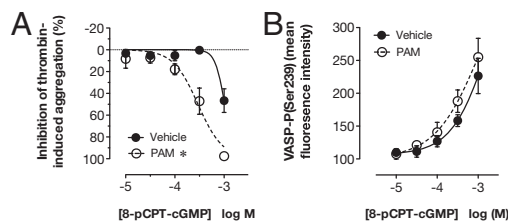**Fig. 3.** P2Y$_{12}$ receptor blockade potentiates inhibition of platelet aggregation to exogenous cGMP but does not potentiate phosphorylation of VASP. Thrombin (1 U/mL)-induced aggregation of washed platelets, determined by changes in light transmission, was inhibited by the cell-permeable cGMP analog, 8-pCPT-cGMP (A), and this action was enhanced in platelets pretreated with the P2Y$_{12}$ receptor blocker PAM (3 μM). An increase in platelet phospho(Ser239)-VASP levels also was produced by 8-pCPT-cGMP, as determined by flow cytometry, but this was not altered by P2Y$_{12}$ receptor blockade (B). *$P < 0.05$ by two-way ANOVA vs. vehicle ($n = 3–4$).

deletion of other putative targets of platelet cGMP, such as VASP, causes more modest reductions in sensitivity to NO and cGMP (30). We therefore examined how P2Y$_{12}$ blockade and NO interacted at this level. Stimulation of washed platelets with thrombin led to a rapid rise in free intracellular calcium concentration (Fig. 4A); DEA/NONOate produced concentration-dependent, but incomplete, inhibition of this calcium release (12). This inhibitory effect of DEA/NONOate was unaffected by PAM and occurred at concentrations of DEA/NONOate very much below those that inhibited platelet aggregation and secretion (Fig. 4A).

The Ability of P2Y$_{12}$ Receptor Activation to Limit the Inhibitory Effect of NO Depends on P2Y$_{12}$ Receptor-Dependent Phosphoinositide-3 Kinase Activation. These data demonstrate that P2Y$_{12}$ receptor activation drives a cGMP-insensitive pathway of platelet activation that supports platelet aggregation, even in the presence of NO. To investigate this hypothesis, we studied the effect of DEA/NONOate and P2Y$_{12}$ receptor blockade on phosphoinositide-3-kinase (PI3K) activation, a principle signaling mechanism downstream of the P2Y$_{12}$ receptor. To do this, we measured phosphorylation of the PI3K substrate, protein kinase B (Akt), at serine 473. Thrombin (1 U/mL)-induced phosphorylation of Akt (Fig. 4B) was partially inhibited by PAM and strongly inhibited by the specific PI3K inhibitor, wortmannin. This is consistent with the current understanding that Akt phosphorylation is primarily dependent on activation of P2Y$_{12}$ receptors by secreted ADP (31). By itself, DEA/NONOate had little inhibitory effect on thrombin (1-U/mL)-induced Akt phosphorylation, but in the presence of PAM, it produced a much greater inhibitory effect (Fig. 4C). This suggests that P2Y$_{12}$ receptor-dependent PI3K activation could constitute a cGMP-insensitive pathway that supports aggregation in the presence of NO.

To determine the functional consequences of P2Y$_{12}$ receptor-mediated PI3K activation, we assessed how specific PI3K inhibition by wortmannin influenced the ability of DEA/NONOate to reduce platelet aggregation. PI3K inhibition increased the sensitivity and maximal inhibition of aggregation produced by DEA/NONOate (Fig. 4D), confirming that PI3K can limit the antiplatelet effect of NO. Further, addition of wortmannin to PAM produced no further increase in the sensitivity of platelets to DEA/NONOate, indicating that this NO-limiting PI3K pathway is driven by P2Y$_{12}$ receptor activation.

The Synergy Between P2Y$_{12}$ Blockade and NO Is Very Much Greater than That Between P2Y$_{12}$ Receptor Blockade and Prostacyclin. It has been previously demonstrated that P2Y$_{12}$ blockade can also potentiate the inhibitory effects of prostacyclin (8). To compare the magnitude of the interaction between P2Y$_{12}$ receptor activation and prostacyclin with that described earlier for NO, we determined in paired experiments the effect of PAM on the

inhibitory potency of prostacyclin and DEA/NONOate against thrombin (1 U/mL)-induced aggregation and secretion responses (Fig. S9). We found that although PAM potentiated the inhibitory effect of both agents, its effects on DEA/NONOate were very much greater than those on prostacyclin.
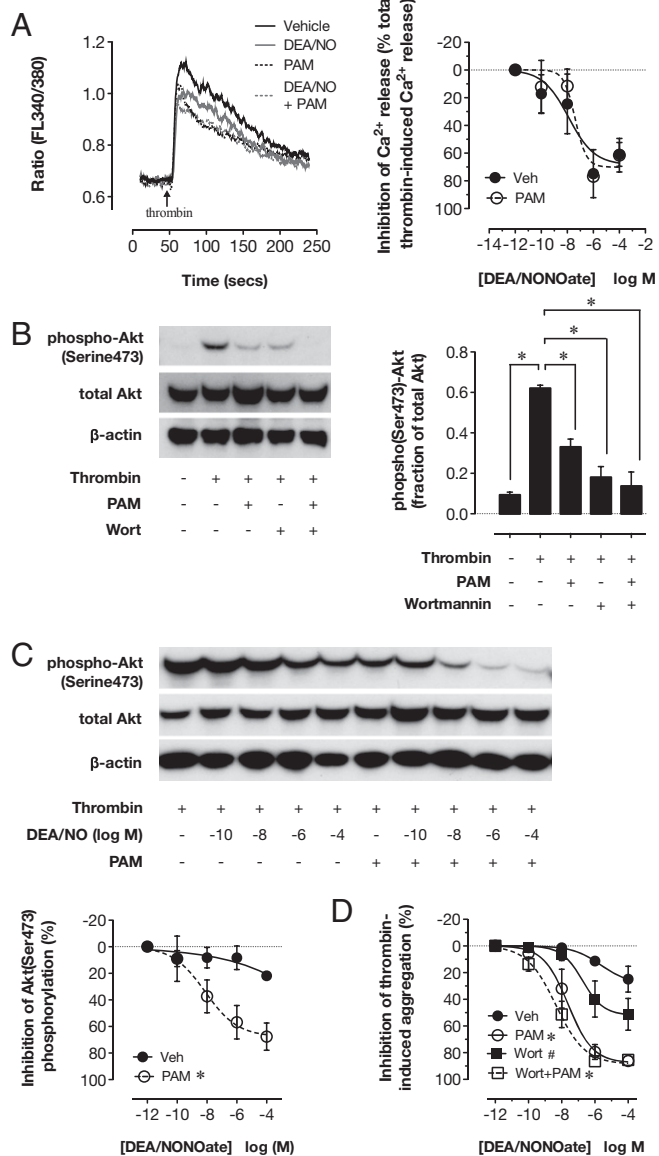

Fig. 4. P2Y$_{12}$ receptor blockade does not alter the ability of NO to inhibit intracellular Ca^{2+} mobilization but facilitates its ability to inhibit PI3K signaling. Thrombin (1 U/mL)-induced mobilization of intracellular calcium in Fura2-loaded washed platelets was inhibited by DEA/NONOate, and this inhibition was not altered by the P2Y$_{12}$ receptor blocker PAM (3 μM) (A). Thrombin (1 U/mL) also stimulated phosphorylation of Akt(Ser473) under stirring conditions measured in washed platelets lysed 2 min after agonist addition (B). Akt (Ser473) phosphorylation was strongly inhibited by P2Y$_{12}$ receptor blockade (PAM; 3 μM) or the PI3K inhibitor, wortmannin (Wort; 200 nM) (B). DEA/NONOate only weakly inhibited thrombin-induced Akt(Ser473) phosphorylation, but this effect was greatly potentiated in the presence of PAM (C). In parallel studies, specific inhibition of PI3K by wortmannin enhanced the ability of DEA/NONOate to inhibit thrombin (1 U/mL)-induced aggregation of washed platelets in the absence, but not the presence, of P2Y$_{12}$ blockade (D). (A, C, and D) *$P < 0.05$ by two-way ANOVA vs. vehicle. (B) *$P < 0.05$ by one-way ANOVA vs. thrombin. (D) #$P < 0.05$ by two-way ANOVA vs. the same treatment in the absence of wortmannin ($n = 4$).

Summary and Conclusions. Here we report the unique observation that P2Y$_{12}$ receptor activation strongly limits the antiplatelet effects of NO. The power of this limiting pathway is illustrated by the finding that blockade of P2Y$_{12}$ receptors increases the inhibitory potency of the NO donor, DEA/NONOate, by many orders of magnitude. We observed this synergy between NO and two chemically unrelated blockers of P2Y$_{12}$ receptors, PAM and ticagrelor, on multiple platelet responses after activation of thrombin or collagen receptors. The interplay between P2Y$_{12}$ receptor blockade and NO was not related to altered synthesis or degradation of either cGMP or cAMP. Instead, it appears to reflect the ability of P2Y$_{12}$ receptors to activate PI3K and other cGMP-insensitive signaling pathways that can sustain aggregation responses even in the presence of very high concentrations of NO (Fig. 5A). Thus, although platelets can sense nanomolar concentrations of NO, as determined by cGMP formation, VASP phosphorylation, or inhibition of Ca^{2+} mobilization, millimolar concentrations are required to inhibit platelet functional markers such as aggregation and secretion after strong activation. Only when P2Y$_{12}$ receptors are blocked is the true inhibitory activity of NO revealed (Fig. 5B).

In conclusion, previously published data (8) have shown the ability of P2Y$_{12}$ receptor activation to limit the antiplatelet effect of prostacyclin (Fig. S9). Our observations on the interaction between NO and P2Y$_{12}$ receptor signaling indicate that in the presence of a strong stimulus, P2Y$_{12}$ receptor activation is a key mechanism by which platelets can overcome the effects of

inhibitory autacoids released by the vascular endothelium (18). Overcoming the effects of these inhibitory autacoids allows the platelet activation essential for hemostasis. As such, potentiating the inhibitory actions of NO and prostacyclin in vivo may provide a key mechanism through which P2Y$_{12}$ receptor blockers, such as clopidogrel, prasugrel, and ticagrelor, exert antithrombotic protection in man. The effectiveness of P2Y$_{12}$ receptor blockers as antithrombotic agents in individual patients may, therefore, vary markedly as a function of endothelial activity. This raises many interesting questions as to how antithrombotic therapies can best be used relative to the vascular health of particular individuals or patient groups.

Materials and Methods

Additional methodological detail can be found in the *SI Materials and Methods*.

Preparation of Platelets. Blood was collected by venipuncture from healthy volunteers, who had provided written consent (St. Thomas's Hospital, London, Research Ethics Committee, Reference 07/Q0702/24), into trisodium citrate (0.32% final). Washed platelets were prepared by centrifugation and resuspended in Tyrodes-Hepes buffer at pH 7.4 at 3×10^8 platelets/mL.

Light Transmission Aggregometry. Washed platelet suspensions were incubated (30 min, room temperature) with the P2Y$_{12}$ receptor blocker PAM (3 μM; AstraZeneca) or vehicle (0.5% DMSO). In some experiments, additional pretreatments were performed as detailed. Aggregation to thrombin (1 U/mL; Sigma) or CRP [1 μg/mL (a kind gift of Richard Farndale University of Cambridge, Cambridge, United Kingdom) in the presence of 1 mg/mL human fibrinogen (Sigma)] was measured in a Bio/Data PAP-8E turbidimetric aggregometer after preincubation (1 min, 37 °C) with the NO donor DEA/NONOate (Sigma) or vehicle.

96-Well Plate Aggregometry. In some experiments, platelet aggregation was assessed in 96-well plates, as we have previously reported (32). Washed platelets were treated as described earlier, before aggregation was stimulated by the addition of thrombin (0.5 or 1 U/mL) or CRP (0.5 or 1 μg/mL in the presence of 1 mg/mL human fibrinogen) and shaking on a microplate shaker (BioShake IQ, QUANTIFOIL Instruments). Absorbance was then determined using a microplate reader and percentage platelet aggregation calculated with reference to the absorbance of Tyrodes-Hepes buffer, taken as a surrogate for 100% aggregation.

Isobolographic Analysis. Using data from 96-well platelet aggregation experiments, IC$_{25}$, IC$_{50}$, and IC$_{75}$ values for DEA/NONOate in the presence of increasing concentrations of PAM or ticagrelor were calculated. These were used to generate isobolograms, as detailed in Fig. S4.

Whole-Blood Aggregometry. Whole blood, collected into lepirudin (25 μg/mL final; Celgene), was treated with P2Y$_{12}$ receptor blockers and DEA/NONOate, as described earlier, before platelet aggregation to the thrombin receptor activating peptides SFLLRN amide (10 μM, Bachem) and AYPGKF-amide (30 μM; Bachem) was monitored by impedance aggregometry (Multiplate; Dynabyte).

ADP+ATP Secretion. Washed platelets, treated with P2Y$_{12}$ inhibitors and DEA/NONOate as described earlier, were stimulated with thrombin (1 U/mL), and ADP+ATP secretion was measured using ChronoLUME reagent (Chronolog).

P-Selectin Expression and GPIIb/IIIa Activation. Washed platelets, treated with P2Y$_{12}$ inhibitors and DEA/NONOate as described earlier, were activated with thrombin (1 U/mL) for 2 min before staining with anti–CD61-allophycocyanin (eBioscience), PAC-1-FITC (BD Bioscience), and anti–P-selectin-PE (eBioscience) for 15 min at 4 °C. PAC-1 and anti–P-selectin binding were determined by flow cytometry after gating of CD61+ events (Fig. S10).

Akt Phosphorylation. Light transmission aggregometry was performed as described earlier, and platelets were lysed after 2 min. Platelet lysates were separated by Western blotting, using antiphospho(Ser473)-Akt (New England Biolabs) or anti–pan-Akt primary antibodies (New England Biolabs).

Rap1b Activation. Light transmission aggregometry was performed as described earlier, and platelets were lysed after 15 s. Active Rap1-GTP was purified by RalGDS affinity pull-down, using a commercial available kit (Thermo Scientific). Rap1-GTP and total Rap1 were then separated and quantified by Western blotting as described earlier, using the supplied antibodies.
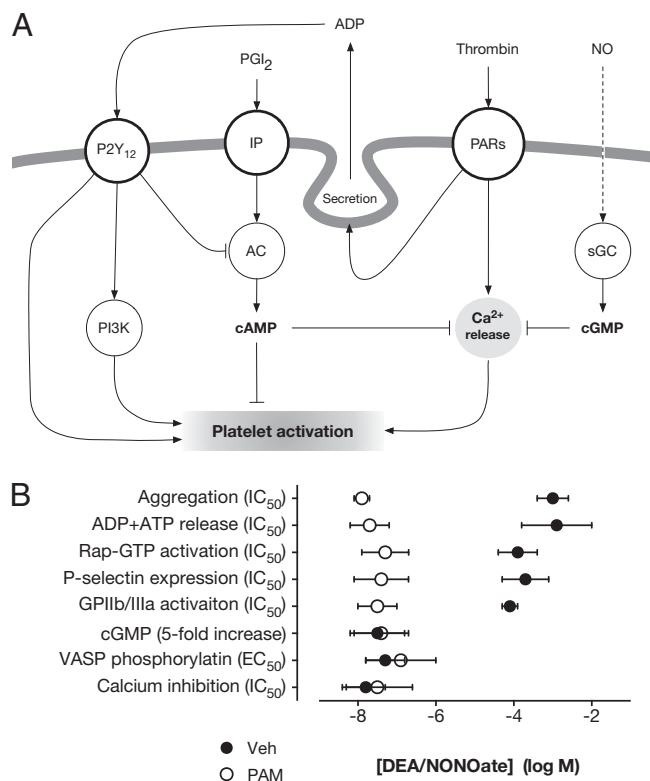

Fig. 5. Schematic and summary for interaction between P2Y$_{12}$ receptors and NO signaling in platelets. Schematic showing proposed mechanism by which P2Y$_{12}$ receptor activation by secreted ADP limits the antiplatelet effect of NO by providing a cGMP-insensitive pathway for platelet aggregation, which depends partly on PI3K signaling (A). Relative potencies for DEA/NONOate on platelet responses (B) highlight that although DEA/NONOate is active on platelets at nanomolar concentrations (cGMP, VASP, Ca^{2+}), inhibition of strong activation (aggregation, secretion, etc.) requires millimolar concentrations of DEA/NONOate. However, when P2Y$_{12}$ receptors are blocked, the inhibitory potency of NO against activation markers closely correlates with its potency on cGMP production, VASP, and calcium.

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cAMP and cGMP Measurements. Light transmission aggregometry was performed as described earlier, and platelets were lysed after 4 min. cAMP and cGMP concentrations were determined by homogenous time-resolved fluorescence-based competitive immunoassays (Cisbio).

VASP Phosphorylation. Washed platelets, treated with P2Y$_{12}$ inhibitors and DEA/NONOate as described earlier, were activated with thrombin (1 U/mL) for 4 min before staining with anti–VASP-P(Ser239) primary antibody (Enzo Lifesciences), Alexa647-conjugated secondary antibody (Invitrogen), and anti–CD42b-FITC (eBiosciences). VASP-P-associated fluorescence was quantified in CD42b+ events by flow cytometry (Fig. S10).

Calcium Mobilization. Washed platelets, loaded with Fura2 (Sigma), were treated with P2Y$_{12}$ receptor blockers and DEA/NONOate as described earlier.

Thrombin (1 U/mL)-induced intracellular calcium mobilization was recorded using a Perkin-Elmer LS940B fluorometer.

Statistics and Data Analysis. Unless otherwise stated, data were analyzed by two-way ANOVA, using GraphPad Prism 5 for Mac OS X, and differences were considered significant if $P < 0.05$. Summary data (IC$_{50}$, EC$_{50}$) were obtained by fitting of data to a logistic equation and tested by Student t test (2 groups) or one-way ANOVA (>2 groups).

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