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Submaximal Inhibition of Protein Kinase C Restores ADP-induced Dense Granule Secretion in Platelets in the Presence of Ca^{2+}*[S]

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Protein kinase C (PKC) is a family of serine/threonine kinases that play isoform-specific inhibitory and stimulatory roles in platelet activation. We show here that the pan-PKC inhibitor Ro31-8220 can be used to dissect these events following platelet activation by ADP. Submaximal concentrations of Ro31-8220 potentiated aggregation and dense granule secretion to ADP in plasma anticoagulated with citrate, in D-Phe-Pro-Arg-chloromethyl ketone-anticoagulated plasma, which has physiological levels of Ca^{2+}, and in washed platelets. Potentiation was retained on inhibition of cyclooxygenase and was associated with an increase in intracellular Ca^{2+}. Potentiation of aggregation and secretion was abolished by a maximally effective concentration of Ro31-8220, consistent with a critical role of PKC in secretion. ADP-induced secretion was potentiated in the presence of an inhibitor of PKCβ but not in the presence of available inhibitors of other PKC isoforms in human and mouse platelets. ADP-induced secretion was also potentiated in mouse platelets deficient in PKCϵ but not PKCθ. These results demonstrate that partial blockade of PKC potentiates aggregation and dense granule secretion by ADP in association with increased Ca^{2+}. This provides a molecular explanation for the inability of ADP to induce secretion in plasma in the presence of physiological Ca^{2+} concentrations, and it reveals a novel role for PKC in inhibiting platelet activation by ADP *in vivo*. These results also demonstrate isoform-specific inhibitory effects of PKC in platelets.

Platelets play a vital role in the generation of a thrombus or vascular plug, preventing excessive blood loss following vascular injury. Circulating platelets adhere rapidly to exposed subendothelial matrix proteins and undergo activation leading to granule secretion, thromboxane A_2 (TxA_2)[6] formation, and integrin activation. This is followed by aggregation of platelets by binding of fibrinogen to activated integrin αIIbβ3. The conversion of fibrinogen to fibrin and platelet contraction consolidates the growing thrombus (1, 2). Aggregate growth is determined by a synergy between the two feedback mediators, ADP, which is released from dense granules, and TxA_2, which is made *de novo* from liberated arachidonic acid.

ADP is a weak platelet agonist in comparison with other G protein-coupled receptor agonists such as thrombin. ADP is released from platelets following endothelial cell damage, in response to all stimulatory platelet agonists, and acts as a secondary positive feedback mediator of platelet activation (3, 4). ADP signals through two G protein-coupled receptors. The G_q-coupled $P2Y_1$ receptor activates phospholipase Cβ isoforms leading to formation of the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C (PKC) and mobilize Ca^{2+}, respectively. The G_i-coupled $P2Y_{12}$ receptor inhibits adenylyl cyclase and activates PI 3′-kinase (5, 6). The latter is believed to underlie the ability of $P2Y_{12}$ to synergize with other Ca^{2+}-mobilizing receptors, including G_q- and tyrosine kinase receptor-regulated pathways, such as the $P2Y_1$ receptor (7, 8), the TxA_2 receptor, and integrin αIIbβ3 (9, 10).

Paradoxically, it has been observed that ADP is able to stimulate sustained aggregation and marked dense granule secretion in citrated plasma that contains micromolar levels of Ca^{2+}, whereas it induces only transient aggregation and is unable to stimulate dense granule secretion in the presence of physiological and millimolar concentrations of the cation. This difference has been shown to be associated with increased TxA_2 synthesis (11) in citrated plasma suggesting that extracellular Ca^{2+} inhibits ADP-induced TxA_2 formation (9) (12, 13). However, the molecular basis of this paradox is unknown.

PKC is a family of closely related serine/threonine kinases, composed of multiple isoforms that are subdivided into classical (α, βI, βII, and γ), novel (δ, ϵ, η, and θ) and atypical (ξ and ι/λ) families, according to their sensitivity to 1,2-diacylglycerol and Ca^{2+} (14). The classical isoforms are regulated by 1,2-diacylglycerol and Ca^{2+}; the novel isoforms are regulated by 1,2-diacylglycerol, and the atypical isoform is not regulated by either messenger. Robust expression of several isoforms of PKC has been reported in human (α, β, δ, and θ) and mouse (α, β, ϵ, δ, and θ) platelets with evidence of expression of additional isoforms (15–18). Studies using pan-PKC inhibitors have

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[6] The abbreviations used are: TxA_2, thromboxane A_2; PMA, phorbol 12-myristate 13-acetate; MRS, MRS-2179; ARC, ARC-6699331MX.

shown that the PKC superfamily plays a critical, positive role in platelet aggregation to thrombin, although its role downstream of the positive feedback agonist ADP is less well characterized (19–24). Several members of the PKC superfamily are implicated in activation of integrin αIIbβ3 and in dense granule release. For example, PKCα has been shown, in both human and mouse platelets, to play positive roles in the regulation of both α and dense granule secretion (25, 26) and platelet aggregation (27) downstream of several platelet agonists (28). PKCβ, another classical isoform, has been shown to positively regulate outside-in signaling by integrin αIIbβ3 in mouse platelets (29). However, studies investigating individual aspects of platelet signaling pathways have shown that PKC can also function to negatively feedback and limit platelet activation (30–34). Although studies using mice deficient in various isoforms have identified a positive role for PKCϵ in GPVI signaling (18), both positive and negative roles for PKCδ and PKCθ have been described in supporting platelet activation downstream of thrombin and collagen, thus demonstrating that the role of PKC is isotype-dependent (35–38).

As yet, no role for PKC in inhibiting responses induced by ADP has been reported. We have investigated the role of the PKC superfamily downstream of ADP-induced platelet activation in human platelets. Using a range of concentrations of the pan-PKC inhibitor Ro31-8220, we attempted to distinguish between the stimulatory and inhibitory actions of the PKC superfamily in human platelets. We observe a marked potentiation in dense granule secretion and aggregation to ADP in citrated platelet-rich plasma (PRP) by a submaximal but not maximal concentration of the PKC inhibitor. Potentiation is also observed in PRP in the presence of millimolar concentrations of extracellular Ca^{2+} and in ADP-sensitive washed platelets. These results highlight the level of PKC activity as a key regulator of platelet secretion by ADP in plasma, suggesting that the inability of ADP to stimulate secretion in the presence of physiological Ca^{2+} is due to PKC activity. Mouse platelets deficient in the novel isoform PKCϵ show increased dense granule secretion in response to ADP, demonstrating a role for the novel isoform in the inhibitory effect of PKC downstream of ADP in mouse platelets. In addition, isoform-specific inhibitors of PKC indicate a role for the classical isoform PKCβ in the inhibitory effect of PKC downstream of ADP in human and mouse platelets.

EXPERIMENTAL PROCEDURES

PKCϵ-deficient mice (39) were bred as heterozygotes on a B6 background, and all results were compared with wild type litter-matched controls. D-Phe-Pro-Arg-chloromethyl ketone, HCl, PKCβ inhibitor (3(-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione), and rottlerin were from Merck. Fura-2-AM was a product of Invitrogen. Anti-phosphoserine (PKC) substrate monoclonal antibody was from Cell Signaling Technology (Beverly, MA). Anti-mouse HRP-conjugated antibody was from Dako. Ro31-8220, Ro31-8425, Gö6983, and other reagents were from Sigma.

Human and Mouse Platelet Preparation—Studies on human platelets were carried out with ethical approval from the Oxford Research Ethic Council (reference number 08/H0605/123). Blood was drawn from aspirin-free, healthy consenting volunteers. For citrated PRP, whole blood was drawn into one-tenth total volume of sodium citrate. For plasma containing extracellular calcium, blood was collected into 60 μM D-Phe-Pro-Arg-chloromethyl ketone. For ADP-sensitive washed platelets, blood was taken into one-sixth total volume of acid citrate dextrose. PRP and washed platelets were prepared as described previously (18). Platelets were resuspended in modified Tyrode's HEPES buffer containing apyrase to prevent ADP receptor desensitization (138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, and 0.05 unit/ml apyrase), pH 7.3, adjusted to a concentration of 2×10^8 platelets/ml, and left to rest for 1 h at 37 °C. Animals were bred, and blood was removed under an approved Home Office License (reference number PPL 30/2721). Blood was drawn either by cardiac puncture or from the vena cavae of terminally CO_2-narcosed mice, anesthetized with gaseous isofluorane. Blood was taken into 100 μl of sodium citrate and centrifuged at $200 \times g$ for 6 min to obtain PRP. Platelets were counted to ensure a platelet count of at least 10^8 platelets/ml.

Aggregometry and ATP Release—Aggregation and ATP secretion were monitored as described previously (18). Platelets were preincubated for 3 min in the presence or absence of Ro31-8220, Ro31-8425, Gö6983, PKCβ inhibitor, Cangrelor/ARC-6699331MX, MRS-2179, indomethacin, or PMA before stimulation with agonist. Each sample was allowed to aggregate for at least 2.5 min. An ATP standard was added to calculate secretion.

Measurement of Intracellular Calcium—Washed platelets were incubated with 3 μM Fura-2-AM for 1 h at 37 °C in the presence or absence of Ro31-8220. ADP was added to platelet suspension at 37 °C under continuous stirring (1200 rpm). Fluorescence changes were monitored using a fluorimeter (340 nm excitation and 510 nm emission). Changes in intracellular calcium concentration were calculated using the Grynkiewicz equation (40).

Western Blot Analysis—Washed platelets (5×10^8/ml) activated as described above were lysed and analyzed by Western blot as described previously (18). Phosphoserine PKC substrate proteins were detected by incubation overnight at 4 °C with a monoclonal anti-phosphoserine PKC substrate antibody.

Statistical Analysis—Statistical analyses were carried on data using unpaired, two-tailed Student's t test, and $p < 0.05$ was considered statistically significant. Values are expressed as mean ± S.E.

RESULTS

Submaximal Ro31-8220 Potentiates Aggregation and Secretion Downstream of ADP but Not PAR-1 Peptide—Studies using mutant mice have revealed isoform-specific inhibitory and stimulatory effects of the PKC superfamily on platelet function downstream of strong platelet agonists such as collagen and thrombin (15, 18, 20, 25, 31, 35, 37, 41–47). In an attempt to dissect this balancing role of PKC in human platelets, we monitored the effect of a range of concentrations of the pan-PKC inhibitor Ro31-8220 on platelet activation in PRP and washed platelets.

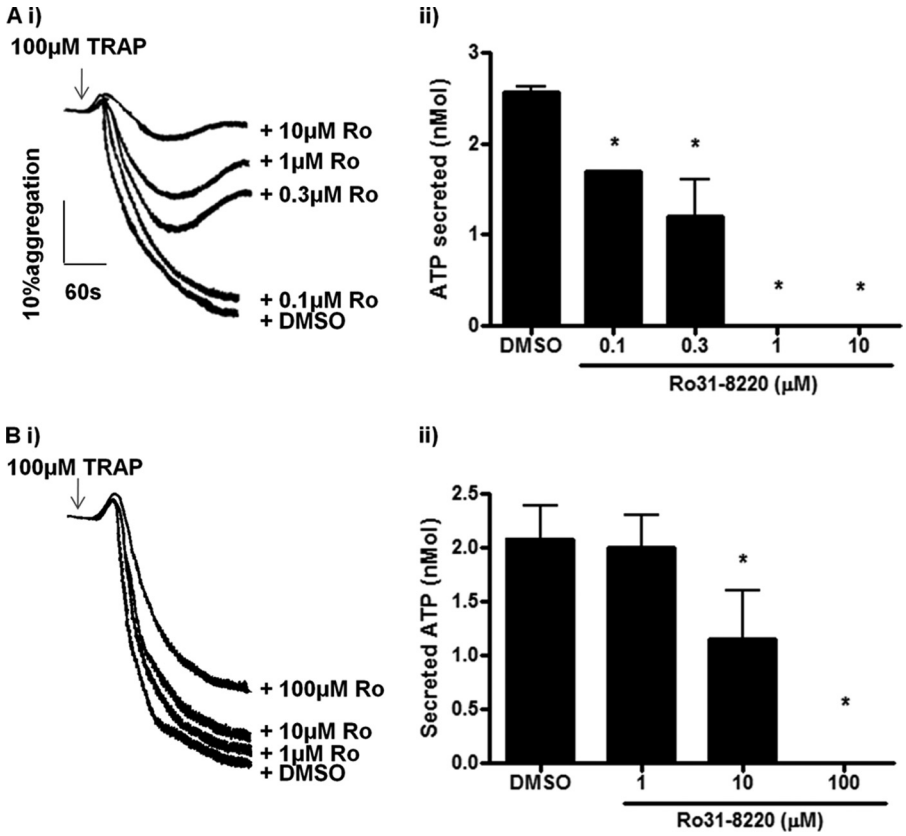

FIGURE 1. **Effect of Ro31-8220 on aggregation and dense granule secretion of human washed platelets and citrated PRP in response to stimulation by PAR-1 peptide.** Human washed platelets (*A*) and citrated PRP (*B*) were stimulated with 100 μM TRAP (a PAR-1 peptide that stimulates the thrombin receptor and is known to be active in both washed platelets and PRP) following incubation with increasing concentrations of Ro31-8220 (*Ro*). *Panel i,* aggregation was measured by optical aggregometry. *Panel ii,* dense granule secretion was measured by monitoring ATP secretion using luminometry ($n = 3$). * indicates $p < 0.05$ in comparison with the DMSO-treated controls.

Many pharmacological reagents have reduced bioavailability in plasma (31). Although 10 μM Ro31-8220 is commonly used for total inhibition of the PKC superfamily in washed platelets, the concentration required to completely inhibit PKC in plasma is not known. Therefore, the effect of different concentrations of Ro31-8220 on platelet aggregation and dense granule secretion was compared in PRP and washed platelets downstream of stimulation of the G protein-coupled PAR-1 receptor using the PAR-1 peptide TRAP, as both of these responses are known to be PKC-dependent (Fig. 1). In washed platelets, increasing concentrations of Ro31-8220 caused dose-dependent inhibition of aggregation and dense granule secretion (Fig. 1*A, panels i* and *ii*). Although 1 μM Ro31-220 was able to achieve full inhibition of dense granule secretion in washed platelets induced by TRAP, a 100-fold higher concentration was required to achieve complete inhibition in PRP, and this concentration was not sufficient to achieve the same level of inhibition of aggregation as seen in washed platelets (Fig. 1*B, panels i* and *ii*). This indicates that in PRP there is markedly reduced bioavailability of Ro31-8220, most likely due to substantial protein binding, which effectively lowers its concentration by more than 2 orders of magnitude.

ADP, a major feedback agonist in platelets, reinforces activation through the $P2Y_1$ and $P2Y_{12}$ receptors. Because the $P2Y_1$ receptor undergoes marked desensitization upon exposure to ADP, special conditions are required to maintain ADP-mediated platelet activation in washed platelets. Therefore, platelet activation by ADP is commonly monitored in citrated PRP, which buffers extracellular Ca^{2+}. Under these conditions, high ADP produces sustained aggregation and dense granule secretion, although at lower concentrations ADP (3 μM) stimulates transient aggregation but not secretion (Fig. 2).

We tested the effect of near maximal and submaximal inhibition of PKC on ADP-induced responses, starting in citrated PRP. Ro31-8220 caused a minor reduction in aggregation to 100 μM ADP in citrated PRP at concentrations of 10 and 100 μM (Fig. 2*A, panel i*), which are submaximal and maximal, respectively, as shown above in TRAP-stimulated platelets. Near maximal Ro31-8220 (100 μM) completely inhibited dense granule secretion to ADP. At a 10-fold lower concentration (10 μM) of Ro31-8220, the onset of secretion was potentiated, although the overall level of secretion was reduced (Fig. 2*A, panel ii*). The same submaximal concentration of Ro31-8220 (10 μM) also converted the transient aggregation response to a low concentration of ADP (3 μM) in PRP to sustained aggregation in association with dense granule secretion (Fig. 2*A, panels iii* and *iv*). This potentiation of the rate and extent of aggregation and secretion was not observed downstream of low concentrations of TRAP peptide (data not shown). These observations demonstrate that partial blockade of PKC potentiates the rate of onset and magnitude of secretion to ADP, which at a submaximal concentration (3 μM ADP) leads to sustained aggregation. At a

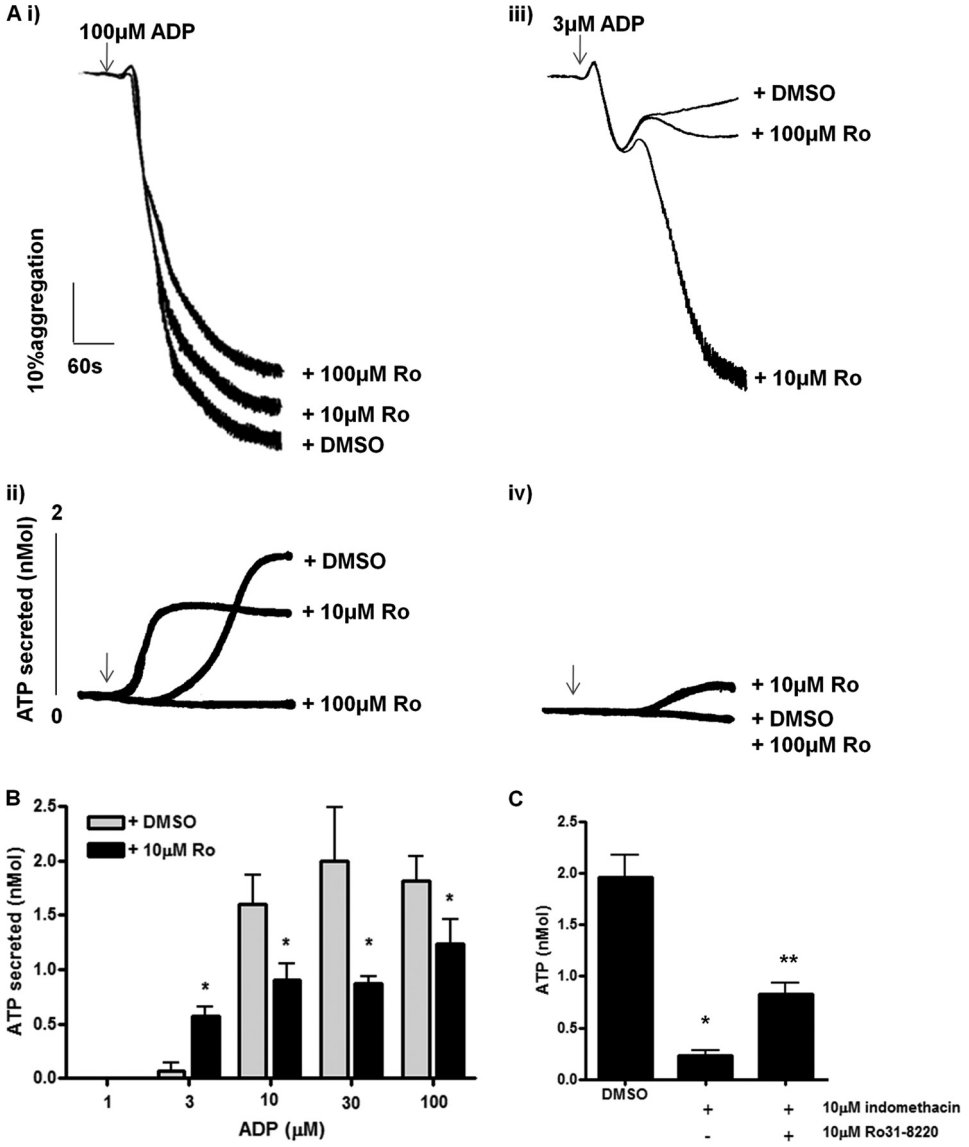

FIGURE 2. **Effect of Ro31-8220 (*Ro*) on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP.** Human citrated PRP was stimulated with either maximal (100 μM) ADP (*A, panels i* and *ii*) or submaximal (3 μM) ADP (*A, panels iii* and *iv*). *A, panels i* and *iii,* aggregation monitored by optical aggregometry. *Panels ii* and *iv*, dense granule secretion, monitored by ATP release. Representative traces of $n = 3$. *B,* dense granule secretion by PRP, monitored by ATP release to a range of ADP concentrations in the absence or presence of Ro31-8220 (10 μM). *C,* effect of Ro31-8220 and indomethacin on dense granule secretion of human PRP treated with or without (10 μM) Ro31-8220 and/or (10 μM) indomethacin, in response to ADP. Platelets were incubated with DMSO, Ro31-8220 (10 μM), or indomethacin (10 μM) for 3 min prior to ADP stimulation. $n \geq 3$. * indicates $p < 0.05$ in comparison with DMSO-treated controls. ** indicates $p < 0.05$ in comparison with platelets treated with 10 μM indomethacin and those treated with DMSO.

higher concentration of ADP, the increase in rate of onset of secretion associated with partial blockade of PKC is followed by a diminished overall response, presumably due to a balance between the inhibitory and stimulatory actions of PKC, as maximal inhibition of PKC abolishes secretion.

ADP-induced aggregation and secretion in citrated plasma have been attributed to an increase in TxA_2 formation (9). In confirmation of this, significant inhibition of dense granule secretion to 100 μM ADP is observed in the presence of the cyclooxygenase inhibitor indomethacin in citrated plasma (Fig. 2*C*). Treatment with submaximal Ro31-8220 (10 μM) in the presence of indomethacin caused a partial restoration of this secretion. These results confirm a critical role for TxA_2 formation in mediating ADP-induced secretion but indicate the potentiation induced by submaximal Ro31-8220 concentrations is not dependent on TxA_2 formation.

The physiological significance of this potentiation is unclear because the studies were performed in plasma containing micromolar concentrations of extracellular calcium, although it should be noted that these conditions are commonly used for platelet testing in the clinic. We therefore investigated the effect of PKC inhibition in PRP containing physiological levels of extracellular Ca^{2+} where a maximally effective concentration of ADP (100 μM) induces reversible aggregation and no dense granule secretion (Fig. 3). In the presence of submaximal Ro31-8220 (10 μM), ADP (100 μM) stimulates sustained aggregation and dense granule secretion (Fig. 3), although the level of secretion was much lower than that observed in citrated plasma

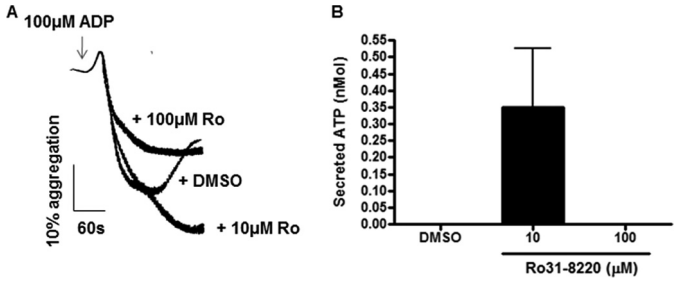

FIGURE 3. **Effect of Ro31-8820 on aggregation and dense granule secretion of human PRP in the presence of extracellular calcium in response to stimulation by ADP.** Human PRP was prepared using D-Phe-Pro-Arg-chloromethyl ketone as an anticoagulant to maintain physiological levels of extracellular calcium and incubated in the presence or absence of 10 or 100 μM Ro31-8220. Aggregation (*A*) and dense granule secretion (*B*) were monitored following stimulation by ADP (100 μM). Traces are representative of $n = 3$.

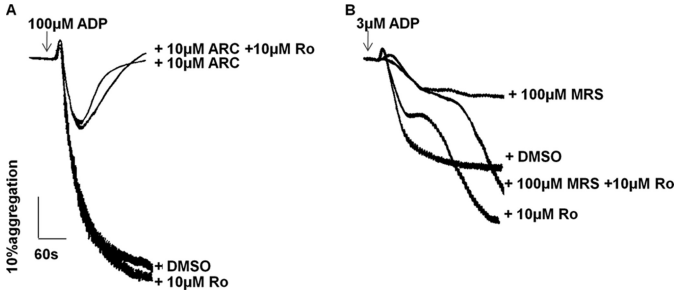

FIGURE 4. **Effect of $P2Y_1$ and $P2Y_{12}$ inhibition on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP in the presence and absence of Ro31-8220.** *A,* human citrated PRP was stimulated with maximal (100 μM) ADP in the absence or presence of ARC (10 μM) and/or submaximal Ro31-8220 (*Ro*) (10 μM). *B,* human citrated PRP was stimulated with submaximal (3 μM) ADP in the absence or presence of MRS (100 μM) and/or submaximal Ro31-8220 (10 μM). Aggregation was monitored by optical aggregometry. *Traces* are representative of $n = 3$. Platelets were incubated with DMSO, Ro31-8220 (10 μM), ARC (10 μM), or MRS (100 μM) for 3 min prior to ADP stimulation ($n \geq 3$).

(Fig. 2). In the presence of 100 μM Ro31-8220, the aggregation response to ADP was decreased and secretion abolished. Therefore, submaximal inhibition of the PKC superfamily by Ro31-8220 potentiates ADP-induced secretion in the presence of physiological concentrations of Ca^{2+}. This raises the possibility that inability of ADP to stimulate sustained aggregation and secretion in Ca^{2+}-containing PRP is due to PKC exerting a constitutive feedback effect and highlights the level of PKC activity as a key regulator of platelet activation by ADP in low or normal Ca^{2+} plasma.

Role of $P2Y_1$ and $P2Y_{12}$ in Potentiation of ADP Induced Platelet Activation—To address which of the two ADP receptors are required for the potentiation by submaximal Ro31-8220, human PRP was treated with either the $P2Y_1$ inhibitor MRS-2179 (MRS) or the $P2Y_{12}$ inhibitor cangrelor/ARC-6699331MX (ARC) and stimulated by a maximal (100 μM) or submaximal (3 μM) ADP. Pretreatment with ARC blocked dense granule secretion induced by ADP (100 μM) and reduced aggregation to a diminished, transient response (Fig. 4*A*) (data not shown). Significantly, neither response was rescued in the presence of submaximal Ro31-8220 (10 μM) (Fig. 4*A*) (data not shown). Inhibition of the $P2Y_1$ receptor using MRS (100 μM) (Fig. 4*B*) inhibited shape change, aggregation, and secretion to a lower concentration of ADP (3 μM). This inhibition, however, could be rescued, and potentiation was observed in the presence of submaximal Ro31-8220, despite $P2Y_1$ inhibition (Fig. 4*B*) (data not shown). This therefore indicates that potentiation of aggregation is mediated by the $P2Y_{12}$ receptor or the synergy between $P2Y_1$ and $P2Y_{12}$.

Submaximal Inhibition of PKC Potentiates Aggregation, Secretion, and Ca^{2+} in Washed Platelets—We investigated whether the potentiation following partial blockade of PKC could be observed in washed platelets, using a lower range of concentrations of Ro31-8220 to those in plasma, where it has limited bioavailability (see Fig. 1). ADP (100 μM) stimulated weak, transient aggregation in washed platelets in the absence of granule secretion (Fig. 5*A*). In the presence of submaximal Ro31-8220 (0.3 and 1 μM), ADP (100 μM) stimulated maximally sustained aggregation and dense granule secretion, with both responses being inhibited in the presence of a maximally effective concentration of the PKC inhibitor (10 μM) (Fig. 5*A*). Potentiation is therefore not dependent on the presence of plasma.

ADP-induced platelet activation is dependent on the mobilization of intracellular Ca^{2+} that plays a key role in mediating aggregation and dense granule secretion (48, 49). It is known that PKC has both inhibitory and stimulatory effects on Ca^{2+} mobilization in platelets (15) so inhibition of the former could underlie Ro31-8220-mediated potentiation. To address this, washed platelets were loaded with the Ca^{2+} reporter dye Fura-2 (40) before stimulation by ADP. Ro31-8220 caused a concentration-dependent increase in intracellular Ca^{2+} induced by ADP, with a threshold at 0.1 μM and a peak effect at 1 μM, which parallels the potentiation of aggregation and secretion (Fig. 5*B*). The increase in Ca^{2+} was sustained at a maximally effective Ro31-8220 (10 μM).

Potentiation Is Working through PKC—One concern was that the effects of Ro31-8220 were due to an alternative target, rather than the well characterized inhibition of PKC, so we used a second pan-PKC inhibitor Ro31-8425 (45, 50). In washed platelets, the presence of Ro31-8425 (0.3 and 1 μM) stimulated maximally sustained aggregation and dense granule secretion to ADP (100 μM), whereas both responses were inhibited in the presence of a maximally effective concentration of the pan-PKC inhibitor (10 μM) (supplemental Fig. 1). Furthermore, ADP-stimulated Ca^{2+} mobilization was potentiated over the same concentration of Ro31-8425 (0.3–10 μM). These results are essentially the same as those with Ro31-8220.

We also investigated PKC-dependent substrate phosphorylation. As shown in Fig. 6*A*, Ro31-8220 inhibited phosphorylation of several PKC substrates in platelets downstream of ADP and PMA, a direct activator of PKC (51), with the same concentration-response relationships as seen for aggregation on washed platelets. Weak inhibition of phosphorylation was observed between 0.1 and 0.3 μM Ro31-8220 with full inhibition at 10 μM. The blot shows inhibition to all concentrations of Ro31-8220 that have been used most likely due to the combined inhibition of multiple isoforms. Thus, the potentiation of platelet activation by ADP by Ro31-8220 occurs over the same concentration range as those for inhibition of PKC-dependent phosphorylation (Fig. 6).

We investigated whether activation of PKC using the phorbol ester PMA had the opposite effect on ADP-induced dense

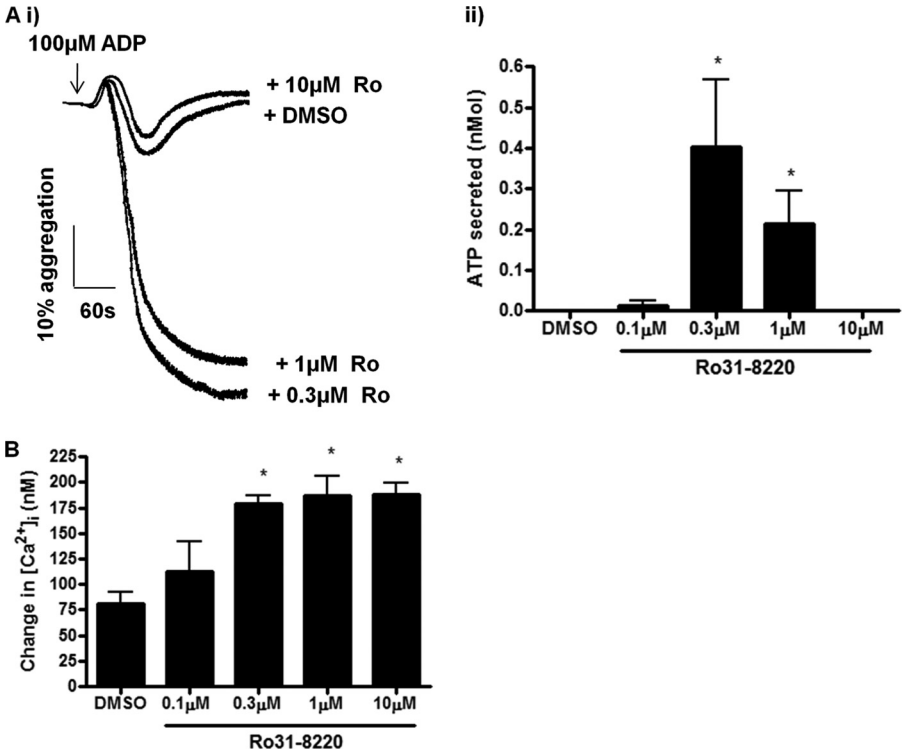

FIGURE 5. **Mechanisms of potentiation by submaximal concentrations of Ro31-8220 in human washed platelets.** *A,* effect of Ro31-8220 (*Ro*) on aggregation and dense granule secretion in response to stimulation by ADP. Human ADP-sensitive washed platelets were stimulated with 100 μM ADP in the presence and absence of varying concentrations of Ro31-8220 (0.1, 0.3, 1, and 10 μM). Aggregation (*panel i*) and dense granule secretion (*panel ii*) were monitored as described previously. *B,* effect of Ro31-8220 on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with Fura-2-AM and pretreated with or without varying concentrations of Ro31-8220 before stimulation with 100 μM ADP. Fluorescence was measured before and after (1 min) ADP addition, and the increase in intracellular calcium concentration was calculated using the Grynkiewicz equation. Platelets were incubated for 3 min with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces are shown. Data are presented as mean ± S.E., $n \geq 3$. * indicates $p < 0.05$ in comparison with DMSO-treated controls.

granule secretion. Pretreatment of platelets with increasing concentrations of PMA led to a decrease in dense granule secretion relative to control platelets following activation by ADP (Fig. 6*B*). These results demonstrate that low level activation of platelets by PMA mediates platelet inhibition in response to ADP, consistent with a model in which antagonism of constitutive signaling by the PKC superfamily underlies the potentiation of ADP-induced aggregation and supporting the conclusion that potentiation is a PKC-specific effect.

Role for the Novel PKCε Isoform in the Regulation of ADP-induced Platelet Activation in Mice—Our present results suggest novel, opposing regulatory roles for members of the PKC superfamily in platelet activation downstream of ADP, namely an inhibitory role sensitive to low concentrations of Ro31-8220 and an activatory role sensitive to high concentrations of Ro31-8220. There is increasing evidence that the novel isoforms of the PKC superfamily have many negative roles in the processes of platelet activation compared with the classical isoforms, which have mainly positive roles (15, 18, 25, 27, 35–37, 41, 42, 44, 45). To determine whether one of the novel PKC isoforms was responsible for potentiation, ADP-induced responses were determined in mouse PRP. As for human platelets, submaximal Ro31-8220 caused potentiation of dense granule secretion in wild type (WT) mouse-citrated PRP (Fig. 7). We therefore investigated ADP-induced responses in PRP from mice lacking either the novel PKC isoforms PKCε or PKCθ. Interestingly, although no difference in aggregation was observed in PRP harvested from PKCε$^{-/-}$ mice following activation by a high concentration of 100 μM ADP, dense granule secretion is potentiated in PKCε$^{-/-}$ mice in comparison with WT controls to a much greater extent than that seen with Ro31-8220. This potentiation is reduced following treatment with Ro31-8220 indicating a positive role for PKC in ADP-induced platelet activation in mouse platelets. However, dense granule secretion is not completely inhibited and can still be observed suggesting involvement of an additional PKC isoform (Fig. 7). No significant increase in ADP-induced secretion was observed in platelets from PKCθ$^{-/-}$ mice (data not shown). These results demonstrate that the potentiation observed in mouse platelets is mediated, at least in part, through inhibition of PKCε.

Role for the Classical Isoform PKCβ in the Regulation of ADP-induced Platelet Activation in Human Platelets—Although PKCε clearly plays a role in mouse platelets, the lack of expression of PKCε in human platelets points to the involvement of a different isoform. Therefore, we tested the ability of inhibitors of the PKC superfamily, which are reported to be selective to various PKC isoforms, to show potentiation. These inhibitors included Gö6983 that primarily targets the classical PKC isoforms but also inhibits PKCδ, a PKCβ inhibitor (3-(1-(3-imidazol-1-yl-propyl)-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione) that selectively inhibits the β isoform over PKCα, -γ and -ε, a PKCθ inhibitor (52), and rottlerin, which has been used as a PKCδ inhibitor. The frequently used classical isoform inhibi-

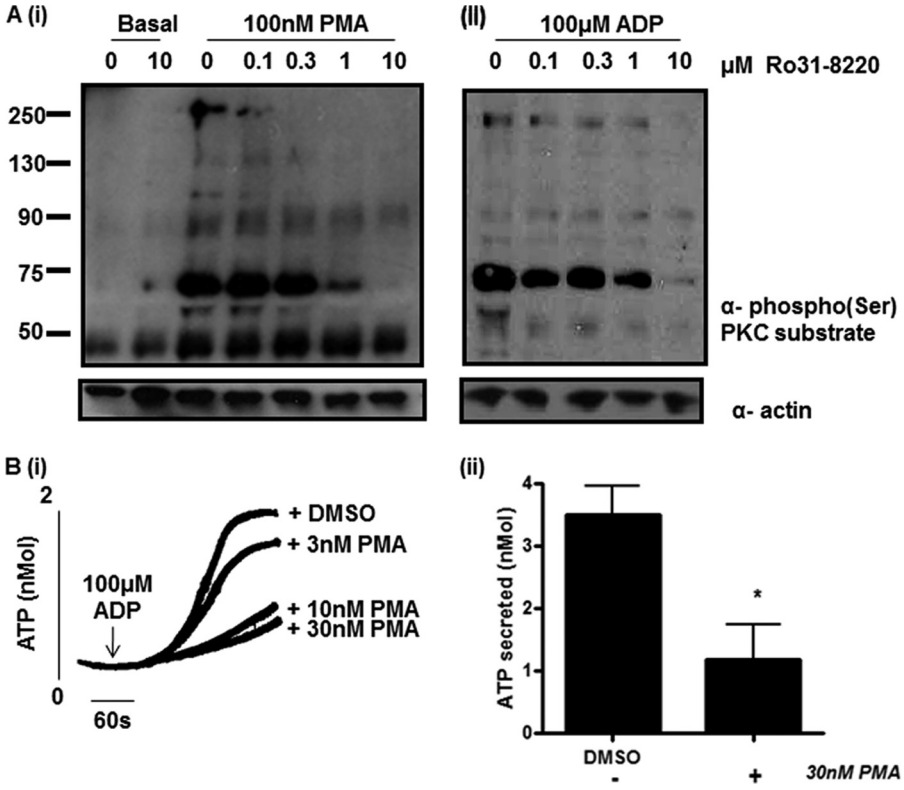

FIGURE 6. **Effect of Ro31-8220 on PKC-mediated phosphorylation and ADP-induced platelet aggregation.** *A,* effect of Ro31-8220 on PKC substrate phosphorylation in human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subjected to Western blot analysis. Phosphorylation by PKC in washed platelet lysates was detected using α-phospho(Ser)-PKC substrate antibody, following activation by PMA (100 nM) (*panel i*) and ADP (100 μM) (*panel ii*) in the presence of Ro31-8220 (*Ro*) (0.1, 0.3, 1, and 10 μM). Blots were stripped and reprobed for actin to determine equal loading. *B,* effect of PKC activation by PMA on dense granule secretion from human citrated PRP in response to stimulation by ADP. Human citrated PRP was prepared and incubated with or without increasing concentrations of PMA (nM), and dense granule secretion was monitored by ATP release, following stimulation by 100 μM ADP. *Panel i,* representative trace. *Panel ii,* data are presented as mean ± S.E., $n \geq 3$. Platelets were incubated with DMSO, PMA (30 nM), or Ro31-8220 (10 μM) for 3 min prior to ADP stimulation. $n = 3$. * indicates $p < 0.05$ in comparison to DMSO-treated controls.

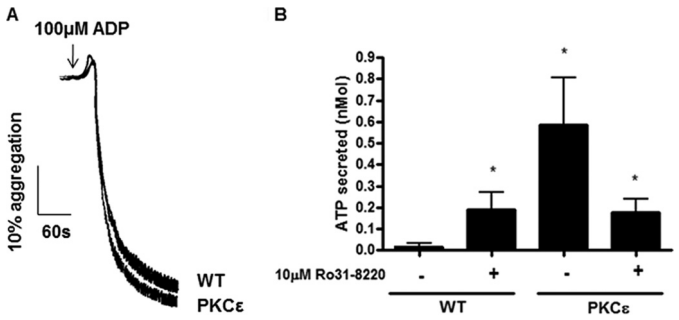

FIGURE 7. **Potentiation of dense granule secretion by ADP in PKCε null mice.** Mouse PRP from WT and PKCε-deficient mice was prepared using citrate as an anticoagulant and incubated in the presence or absence of 10 μM Ro31-8220 (*Ro*). Platelets were then stimulated with 100 μM ADP. *A,* aggregation of WT and PKCε null mice following activation by ADP monitored by optical aggregometry. *B,* dense granule secretion monitored by ATP release to 100 μM ADP. Data are presented as mean ± S.E., $n = 3$. * indicates $p \leq 0.05$ in comparison with WT controls.

tor Gö6976 has recently been found to inhibit the tyrosine kinase Syk in human platelets, and so it was not used (53).

Potentiation of aggregation and secretion to ADP (100 μM) were observed in the presence of Gö6983 and the PKCβ inhibitor (10 μM) in human washed platelets (supplemental Fig. 2 and Fig. 8). A concentration-dependent increase in intracellular Ca^{2+} by ADP was also observed in the presence of the PKCβ inhibitor (0.3–10 μM) (Fig. 8*A, panel iii*). In contrast, neither the PKCθ inhibitor (0.1–30 μM) nor rottlerin (0.3–30 μM) potentiated the response to ADP (data not shown). The PKCβ inhibitor (10 and 30 μM) also potentiated ADP-induced responses in mouse PRP (Fig. 8*B*). Together, these results support a role for PKCβ in inhibiting activation by ADP in human and mouse platelets.

DISCUSSION

The PKC superfamily is a key regulator of platelet activation downstream of a range of platelet agonists, including ADP as shown in this study. Studies of the individual isoforms of PKC have highlighted both positive and negative regulatory roles for the kinase in several processes required for platelet activation and thrombus formation (15, 18–21, 23, 25–27, 30–32, 35–37, 42–45, 47, 50). In an attempt to differentiate the net effect of the PKC superfamily on ADP activation, we investigated the effect of a range of concentrations of the pan-PKC inhibitors Ro31-8220 and Ro31-8425 on platelet activation by ADP. The relative affinities of Ro31-8220 and Ro31-8425 for the classical PKC isoforms have been reported (50, 51) and both are believed to be pan-PKC inhibitors that do not discriminate between isoforms at the concentrations required to inhibit PKC in intact cells.

Many pharmacological reagents have a reduced bioavailability in plasma (31). Comparison of PAR-1-dependent responses in PRP and washed platelets confirmed that Ro31-8220 had reduced bioavailability in PRP in comparison with washed

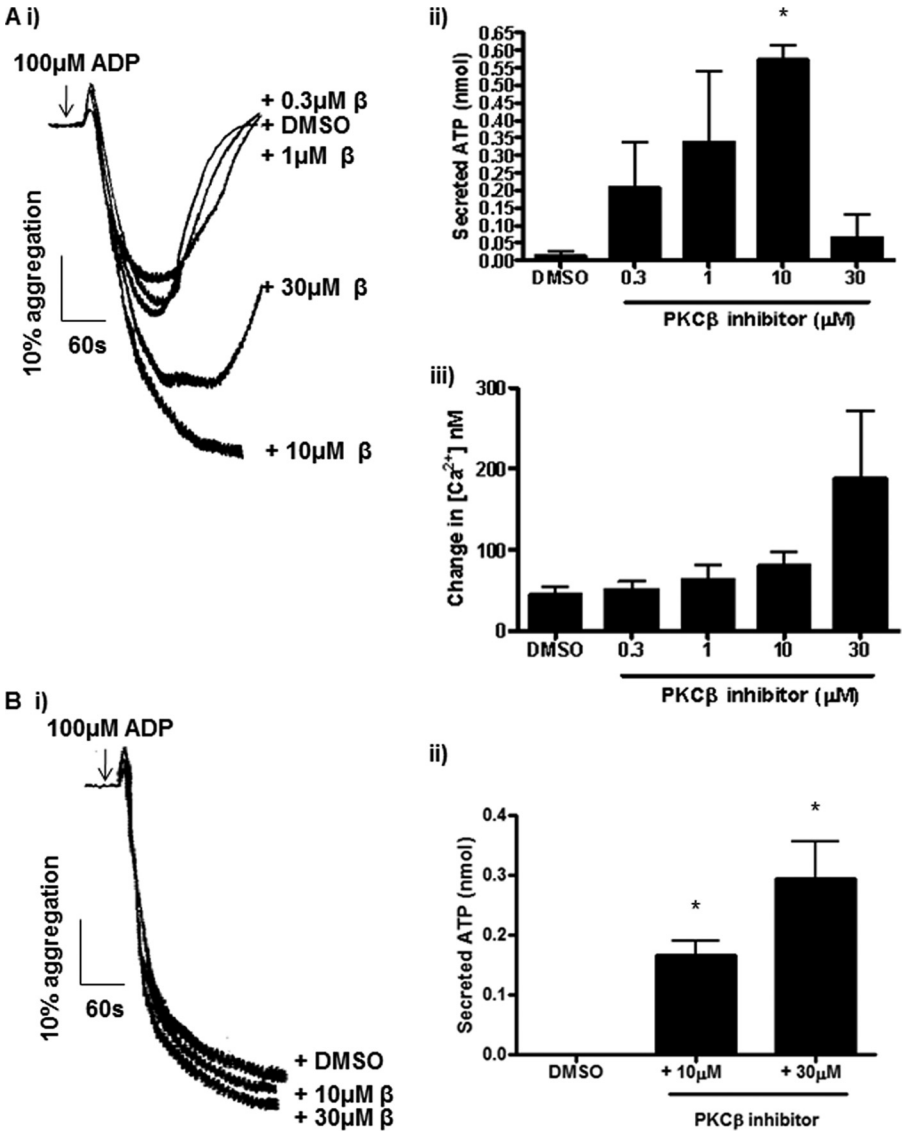

FIGURE 8. **Effect of PKCβ inhibitor on aggregation, dense granule secretion, and levels of intracellular calcium of human washed platelets and mouse PRP in response to stimulation by ADP.** *A,* effect of PKCβ inhibitor on aggregation and dense granule secretion in response to stimulation by ADP. Human ADP-sensitive washed platelets were stimulated with 100 μM ADP in the presence and absence of varying concentrations of PKCβ inhibitor (0.3, 1, 10, and 30 μM). Aggregation (*panel i*) and dense granule secretion (*panel ii*) were monitored as described previously. *Panel iii,* effect of PKCβ inhibitor on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with Fura-2-AM and pretreated with or without varying concentrations of PKCβ inhibitor before stimulation with 100 μM ADP. Fluorescence was measured before and after (1 min) ADP addition, and the increase in intracellular calcium concentration was calculated using the Grynkiewicz equation. *B,* mouse PRP from WT mice was prepared as described previously and incubated in the presence or absence of 10 or 30 μM PKCβ inhibitor. Platelets were then stimulated with 100 μM ADP. *Panel i,* aggregation following activation by ADP monitored by optical aggregometry. *Panel ii,* dense granule secretion monitored by ATP release induced by 100 μM ADP. Platelets were incubated for 3 min with inhibitor(s) or DMSO prior to ADP stimulation. Data are presented as mean $\pm$ S.E., representative traces shown, $n = 3$. * indicates $p < 0.05$ in comparison with DMSO-treated controls.

platelets. A 100-fold higher concentration was required to inhibit dense granule secretion in PRP, yet this concentration was not sufficient to achieve the same level of inhibition of aggregation as seen in washed platelets.

We have made the novel observation that submaximal PKC inhibition causes a marked potentiation in the extent of dense granule secretion and converts reversible to sustained aggregation to a low concentration of ADP (3 μM). At high ADP concentrations, a partial blockade of PKC increases the rate of onset of secretion, although this is followed by a diminished overall secretory response, consistent with both inhibitory and stimulatory actions of the PKC superfamily. This potentiation effect was not seen downstream of PAR-1 receptor stimulation possibly because it initiates activation through a G_q-dependent pathway. Consistent with this, potentiation was also absent in platelets solely activated through the $P2Y_1$ ADP receptor, which also signals through a G_q-dependent pathway. Our results suggest the PKC superfamily has opposing regulatory roles in platelet activation downstream of ADP, an inhibitory role sensitive to low concentrations of Ro31-8220 and an activatory role sensitive to high concentrations. This is the first indication that pan-PKC inhibitors can be used to selectively block one of these opposing roles.

These results raise the question of why these broad spectrum PKC inhibitors have differential and opposing effects at different concentrations. It is possible that this is due to differential sensitivities of the different PKC isoforms to Ro31-8220 and Ro31-8425 or differential dose-response relationships for substrate phosphorylation. It has been reported that PKCε is one of the most highly expressed novel isoforms of PKC in mouse platelets even though it is absent in human platelets. In contrast, mice platelets express relatively low levels of PKCδ, which is expressed at a high level in human platelets (18). Using PRP from mice deficient in the novel isoforms, we have highlighted a role for PKCε in the potentiation. Treatment of PKCε-deficient mice with Ro31-8220 prior to ADP stimulation partially reduced dense granule secretion demonstrating that the PKC component that activates secretion is mediated by other PKC isoforms. Because PKCε is not expressed in human platelets, potentiation must be mediated by another PKC isoform. We therefore extended our study to the use of various PKC isoform-selective inhibitors that are currently available. The range of concentrations of the inhibitors used included concentrations that were lower than previously described in human platelets (15) in an attempt to ensure that observations made were a result of isoform selectivity of the inhibitor rather than nonspecific inhibition of the PKC superfamily. Treatment of human washed platelets with the PKCβ inhibitor potentiated aggregation and dense granule secretion. This potentiation was also observed following treatment with Gö6983, which primarily inhibits the classical isoforms PKCα and PKCβ, further implicating inhibition of PKCβ in the potentiation.

Treatment of platelets with indomethacin confirmed a critical role for TxA_2 in the ability of ADP to stimulate sustained aggregation and marked dense granule secretion in citrated plasma. However, submaximal Ro31-8220 partially rescued this, indicating that, although it may contribute, enhanced TxA_2 formation is not essential for potentiation. Use of inhibitors of the two ADP G protein-coupled receptors, ARC that inhibits $P2Y_{12}$ and MRS that inhibits $P2Y_1$, indicates that signaling through $P2Y_{12}$ but not $P2Y_1$ is essential for the potentiation, although this may be mediated through potentiation of the synergy of the two receptors as the synergy is associated with an increase in intracellular Ca^{2+}. Initially, it seems that these results are at odds with previous reports that $P2Y_{12}$ potentiates thrombin-induced calcium mobilization and that inhibition of the $P2Y_{12}$ receptor inhibits ADP-induced calcium mobilization (54, 55). However, these observations refer to two different mechanisms of calcium regulation. There is synergy between $P2Y_{12}$ and G_q-coupled receptors upstream of phospholipase C activation, and therefore calcium responses are increased as a result of increased inositol 1,4,5-trisphosphate production. The PKC superfamily, however, has both inhibitory and stimulatory effects on platelet Ca^{2+} mobilization. For example, the novel isoform PKCθ negatively regulates intracellular Ca^{2+} following GPVI signaling (47). We observed an elevation in intracellular Ca^{2+} following treatment with both submaximal and maximal concentrations of Ro31-8220 supporting published data that PKC suppresses agonist-induced Ca^{2+} signaling (31). This increase in Ca^{2+} has been attributed to inhibition of the plasma membrane Ca^{2+}-ATPase pump (56, 57), which is inhibited by PKC, and this mechanism to increase Ca^{2+} potentially underlies the increase in platelet activation observed here following submaximal PKC inhibition.

The inhibition of dense granule secretion by high Ro31-8220 has been reported downstream of multiple platelet agonists suggesting a common target (21, 58). Multiple proteins, including components of the soluble NSF attachment protein receptors complex and the vesicular trafficking machinery, which are essential for secretion, are phosphorylated in a PKC-dependent manner, including SNAP-23, syntaxin 4, and Munc18c (59–63). Inhibition of one or more of these events may underlie the positive role for PKC.

The data presented here identify both positive and negative regulatory roles for the PKC superfamily in the regulation of activation by ADP in human and mouse platelets. The data also support previous reports that suggest differential regulatory roles for the individual isoforms of PKC, indicating a role for the classical isoform PKCβ in human platelets and PKCβ and the novel isoform PKCε in the regulation of ADP-induced platelet activation in mouse platelets. This is the first report of an inhibitory role for a classical PKC isoform in platelets.

It is essential that powerful inhibitory pathways exist to prevent unwanted platelet activation within the intact circulation. ADP is a key feedback mediator in platelet activation and thrombus formation. The release of low levels of ADP from damaged cells is therefore potentially very dangerous as this could give rise to unwanted thrombus formation. The work described here demonstrates a previously unrecognized role for members of the PKC superfamily in inhibiting platelet activation by low concentrations of ADP and therefore presents a new pathway of prevention of unwanted platelet activation in plasma. PKC activity is a key regulator of many signal transduction pathways in a variety of cell types, and it is therefore considered as a possible target for therapy for treatment of cancer and various other diseases in addition to being a putative target for antithrombotic therapies (64). The potentiation of platelet aggregation downstream of low concentrations of ADP therefore has implications for the use of PKC-targeted antithrombotic and anti-cancer therapies.

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