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Protein Kinase C Regulation of 12-Lipoxygenase-Mediated Human Platelet Activation[S]

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ABSTRACT

Platelet activation is important in the regulation of hemostasis and thrombosis. Uncontrolled activation of platelets may lead to arterial thrombosis, which is a major cause of myocardial infarction and stroke. After activation, metabolism of arachidonic acid (AA) by 12-lipoxygenase (12-LOX) may play a significant role in regulating the degree and stability of platelet activation because inhibition of 12-LOX significantly attenuates platelet aggregation in response to various agonists. Protein kinase C (PKC) activation is also known to be an important regulator of platelet activity. Using a newly developed selective inhibitor for 12-LOX and a pan-PKC inhibitor, we investigated the role of PKC in 12-LOX-mediated regulation of agonist signaling in the platelet. To determine the role of PKC within the 12-LOX pathway, a number of biochemical endpoints were measured, including platelet aggregation, calcium mobilization, and integrin activation. Inhibition of 12-LOX or PKC resulted in inhibition of dense granule secretion and attenuation of both aggregation and $\alpha IIb\beta_3$ activation. However, activation of PKC downstream of 12-LOX inhibition rescued agonist-induced aggregation and integrin activation. Furthermore, inhibition of 12-LOX had no effect on PKC-mediated aggregation, indicating that 12-LOX is upstream of PKC. These studies support an essential role for PKC downstream of 12-LOX activation in human platelets and suggest 12-LOX as a possible target for antiplatelet therapy.

Introduction

Platelet activation plays a significant role in hemostasis and thrombosis and a central role in the pathophysiology of cardiovascular disease. Platelet activation can be initiated through a number of different receptor pathways including thrombin and collagen. Reinforcement of the initial activation signal is known to be regulated in part by secondary signaling events mediated by arachidonic acid (AA) released from the phospholipid membrane. Although active metabolites formed by the oxidation of AA by cyclooxygenase-1 (COX-1) are known to regulate platelet reactivity (Brash, 1985), the role of metabolites produced by the oxidation of AA by platelet-type 12-lipoxygenase (12-LOX) is controversial. Some reports have shown that metabolic products of 12-LOX attenuate AA-induced aggregation (Aharony et al., 1982) and also inhibit AA release from membrane phospholipids by blocking PLA_2 (Chang et al., 1985), whereas other studies suggest that 12-LOX activation is prothrombotic and is linked to calcium mobilization (Nyby et al., 1996), regulation of tissue factor activation, and thrombin generation in the

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ABBREVIATIONS: AA, arachidonic acid; COX-1, cyclooxygenase-1; 12-LOX, 12-lipoxygenase; PLA_2, phospholipase A_2; PKC, protein kinase C; 12-HETE, 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; PAR, protease-activated receptor; Ro 31-8220, 3-[1-(3-(amidinothio)propyl-1*H*-indol-3-yl)]-3-(1-methyl-1*H*-indol-3-yl) maleimide (bisindolylmaleimide IX); PMA, phorbol myristoyl acetate; GPCR, G protein-coupled receptor; NCTT-956, *N*-((8-hydroxy-5-nitroquinolin-7-yl)(thiophen-2-yl)methyl)propionamide; ERK, extracellular regulated kinase; AP, activating peptide; FITC, fluorescein isothiocyanate; TxB_2, thromboxane B_2; cPLA2, cytosolic phospholipase A_2; CVX, convulxin; 12-HpETE, 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; MAPK, mitogen-activated protein kinase.

platelet (Thomas et al., 2010). The mechanistic basis for these physiological changes in platelet activity through the 12-LOX pathway is not clear. In particular, the events that occur both upstream and downstream of 12-LOX upon agonist stimulation have not been well characterized.

Protein kinase C (PKC), which is known to play an important role in a number of biochemical activation steps in the platelet (Chari et al., 2009; Konopatskaya et al., 2009), has also been suggested to play a role in 12-HETE regulation in tumor cells (Szekeres et al., 2000). In platelets, similarly to 12-LOX, PKC has been shown to regulate aggregation and play an important role in granule secretion and integrin activation (Harper and Poole, 2010). Furthermore, protease-activated receptor (PAR)-1 and PAR4 signaling in the platelet has been shown to result in Ca^{2+} mobilization and PKC-mediated aggregation and secretion (Fälker et al., 2011). However, the underlying mechanism by which PKC regulates platelet activity is controversial. Kim et al. (2011) reported that PKC inhibition by the pan-PKC inhibitor, 3-[1-(3-(amidinothio)propyl-1H-indol-3-yl)]-3-(1-methyl-1H-indol-3-yl) maleimide (bisindolylmaleimide IX) (Ro 31-8220), potentiated epinephrine-induced platelet aggregation, and Unsworth et al. (2011) showed that PKC inhibition potentiates platelets secretion in the presence of Ca^{2+}. Other reports have shown that PKC inhibition attenuates platelet aggregation (Strehl et al., 2007).

In this study, we investigated the coupling between the activation of 12-LOX and PKC in regulating platelet aggregation and integrin activation. We sought to determine whether PKC acted downstream of 12-LOX upon agonist stimulation. Agonist-mediated platelet aggregation was significantly decreased in the presence of either a 12-LOX or PKC inhibitor. Inhibition of 12-LOX activity by selective small molecule inhibitors (Kenyon et al., 2011), which leads to attenuation of aggregation, was overcome when the PKC activator, PMA, was added together with agonist to the platelets. Furthermore, inhibition of 12-LOX had no effect on PMA-mediated platelet aggregation. Finally, $\alpha IIb\beta_3$ attenuation in the absence of 12-LOX was rescued by addition of PMA. Hence, this is the first report to show that PKC activity occurs downstream of 12-LOX in human platelets and begins to elucidate how this essential pathway mediates normal platelet activation through a number of GPCR and non-GPCR receptors.

Materials and Methods

Materials. 12-LOX inhibitor [*N*-((8-hydroxy-5-nitroquinolin-7-yl)(thiophen-2-yl)methyl)propionamide (NCTT-956)] was synthesized at the National Institutes of Health Chemical Genomics Center (Rockville, MD) and was provided by David Maloney. Baicalein was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERK and total ERK antibodies were purchased from Cell Signaling Technology (Danvers, MA); PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). Fluo-4 AM was from Invitrogen (Carlsbad, CA). Human α-thrombin was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Convulxin was purchased from Centerchem (Stamford, CT). Fluorescein isothiocyanate (FITC)-conjugated PAC1 antibody was purchased from BD Biosciences (San Jose, CA). The C6 flow cytometer was from Accuri (Ann Arbor, MI). Aggregometer, collagen, CHRONO-LUME reagent, and other aggregation supplies were purchased from Chrono-log Corporation (Havertown, PA).

Human Platelets. Human platelets were obtained from healthy volunteers within the Thomas Jefferson University community and the Philadelphia area. These studies were approved by the Thomas Jefferson University Institutional Review Board, and informed consent was obtained from all donors before blood draw. Blood was centrifuged at 200*g* for 13 min at room temperature. Platelet-rich plasma was transferred into a conical tube containing a 10% acid citrate dextrose solution (39 mM citric acid, 75 mM sodium citrate, and 135 mM glucose, pH 7.4) and centrifuged at 2000*g* for 15 min at room temperature. Platelets were resuspended in Tyrode's buffer (12 mM $NaHCO_3$, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES), and the final platelet concentration was adjusted to 3×10^8 platelets/ml after counting with a ZI Coulter particle counter (Beckman Coulter, Fullerton, CA). Reported results are the data obtained using platelets from at least three different subjects. Agonists and inhibitors were used at concentrations indicated in the figures and figure legends.

Gas Chromatography-Mass Spectrometry Analysis of [$^2H_8$]Thromboxane Synthesis in Platelets. [$^2H_4$]TxB_2 (2 ng) was added to the samples as an internal standard. In brief, the sample was prepurified with a C18 Sep-Pak column. [$^2H_8$]TxB_2 was eluted with 10 ml of heptane-ethyl acetate (1:1), dried, and dissolved in acetonitrile, and TxB_2 was converted to pentafluorobenzyl esters. TxB_2 was then purified, and the sample was converted to *O*-trimethylsilyl ether derivatives and analyzed by gas chromatography-

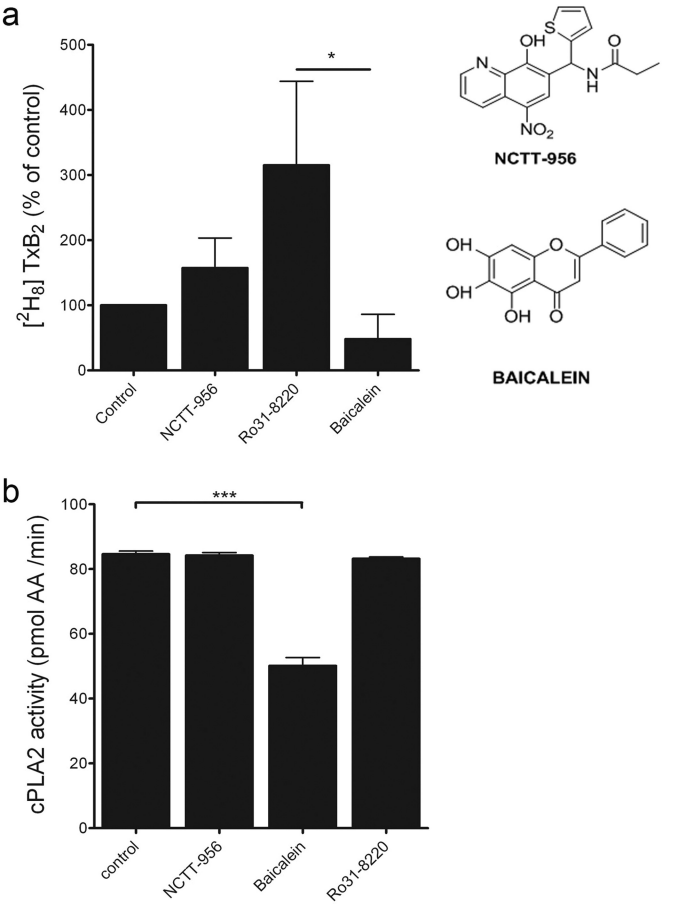

Fig. 1. Inhibitor selectivity. a, washed platelets were stimulated with [$^2H_8$]arachidonic acid, and the level of [$^2H_8$]TxB_2 relative to no inhibitor treatment (control) was measured ($n = 3$). No inhibition of [$^2H_8$]TxB_2 was observed in the presence of 50 μM NCTT-956 or 10 μM Ro 31-2880 relative to the control; 50 μM baicalein induced an inhibition of [$^2H_8$]TxB_2 compared with control levels, and [$^2H_8$]TxB_2 formation in the presence of baicalein was significantly inhibited compared with [$^2H_8$]TxB_2 in the presence of Ro 31-2880. $n = 3$. b, human recombinant $cPLA_2$ activity in the absence or presence of 50 μM baicalein, 50 μM NCTT-956, or 10 μM Ro 31-2880 ($n = 3$). AA, arachidonic acid released from vesicles. *, $P < 0.05$; ***, $P < 0.001$.

electron capture negative chemical ionization mass spectrometry, using an SPB-1 column (15 m), with a temperature gradient from 190 to 300°C at 20°C/min. The ion corresponding to the derivatized TxB_2 was monitored by selected ion monitoring. The signal for TxB_2 is $m/z = 622$. The signal for the internal standard $[^2H_4]TxB_2$ is $m/z = 618$.

Measurement of 12-HETE. Secretion of 12-HETE was measured from platelet supernatants by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry after addition of an internal standard (2 ng of $[^2H_8]$12-HETE) as described previously (Lee et al., 2003). The concentration of 12-HETE was determined by isotopic dilution.

cPLA$_2$ Activation Assay. The effect of the different inhibitors on cPLA$_2$ was tested with recombinant human cPLA$_2$ using the enzymatic activity assay described previously (Reed et al., 2011) with the following differences. The inhibitors were added at a final concentration of 50 μM in dimethyl sulfoxide right before the recombinant enzyme was added to initiate the reaction. After 5 min of incubation, the products of the reaction were analyzed.

Platelet Aggregation. Washed platelets were adjusted to a final concentration of 3×10^8 platelets/ml. Where indicated, platelets were pretreated with 12-LOX inhibitors for 10 min or PKC inhibitor for 1 min. The aggregation response to PAR1-AP, PAR4-AP, or collagen was measured using an aggregometer with stirring at 1100 rpm at 37°C.

Dense Granule Secretion. ATP release was assayed as an indication of dense granule secretion. For ATP studies, washed platelets adjusted to a final concentration of 3×10^8 platelets/ml were pretreated with 12-LOX inhibitors for 10 min or PKC inhibitor for 1 min. ATP release in response to agonist was measured using a lumi-aggregometer at 37°C with stirring at 1100 rpm.

Flow Cytometry. Integrin αIIbβ3 activation on the surface of the platelet was measured by flow cytometry using FITC-conjugated PAC1 (an antibody that only recognizes the active form of αIIbβ3). For these experiments, 40-μl aliquots of washed platelets adjusted to a final concentration of 2.5×10^7 platelets/ml were pretreated with inhibitors for 10 min. After addition of 1 μl of PAC1, platelets were stimulated with agonist for 10 min and then diluted to a final volume of 500 μl using Tyrode's buffer. The fluorescence intensity of platelets was immediately measured using an Accuri flow cytometer.

Western Blotting. Washed platelets adjusted to 10^9 platelets/ml were stimulated with indicated agonists and lysed with 3× Laemmli buffer and then were added to the samples, boiled for 5 min, and subjected to Western blot analysis.

Calcium Mobilization. Platelets were recalcified to a final concentration of 1 mM followed by preincubation with Fluo-4 AM for 10 min. The platelets were then treated with a 12-LOX inhibitor for 10 min before stimulation with the indicated agonist. Calcium mobilization was measured using the Accuri C6 flow cytometer.

Statistical Analysis. Experimental groups was compared using appropriate statistical analyses (paired t test program or analysis of variance with post-test analysis) using Prism software. Differences in mean values (measured as S.E.M.) were considered significant at $P < 0.05$.

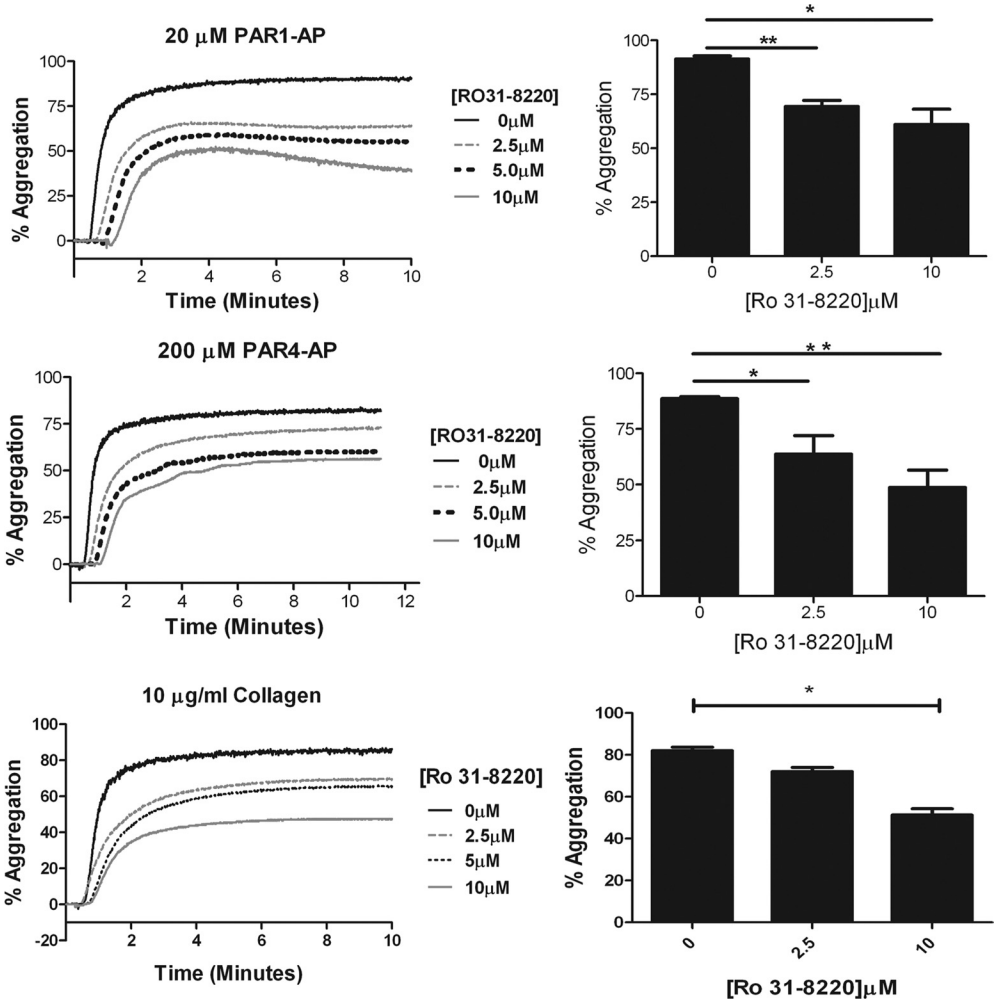

Fig. 2. PKC regulation of platelet aggregation. Platelets were pretreated with increasing concentrations of the pan-PKC inhibitor Ro 31-8220 from 0 to 10 μM for 1 min followed by stimulation with 20 μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen, and platelet aggregation was measured. Pretreatment with increasing concentrations of Ro 31-8220 attenuated agonist-mediated platelet aggregation in a dose-dependent manner. The bars represent means ± S.E.M. (*, $P < 0.05$; **, $P < 0.001$) for aggregation. $n = 3$.

Results

Specificity of 12-LOX Inhibitors. To determine the role of the lipoxygenase pathway in human platelet reactivity, the recently identified and highly selective 12-LOX small molecule inhibitor NCTT-956 (Kenyon et al., 2011) and the less selective commercially available 12-LOX inhibitor (baicalein) were studied. To assess for possible off-target effects on the related cyclooxygenase-1 pathway, which leads to formation of thromboxane A_2 (Fig. 1), platelets were treated with deuterated AA in the presence or absence of NCTT-956 or baicalein, and the deuterated TxB_2 product was measured (Fig. 1a). Values are expressed as a percentage of control to which no inhibitor was added. Deuterated TxB_2 was not inhibited by NCTT-956 or Ro 31-8220, indicating that COX-1 and thromboxane synthase are not directly inhibited by these pharmacological agents. Treatment with baicalein, however, resulted in a significant decrease in the production of $[^2H_8]TxB_2$ suggesting that this drug may have a direct inhibitory effect on COX-1 or thromboxane synthase (Fig. 1a). Because $[^2H_8]TxB_2$ was increased in the presence of the PKC inhibitor, the level of 12-HETE formation was also measured to determine whether Ro 31-2880 directly inhibited 12-LOX. No decrease in 12-HETE formation was observed in the presence of Ro 31-2880 (data not shown). To confirm that NCTT-956, baicalein, and Ro 31-8220 did not inhibit the release of arachidonic acid, $cPLA_2$ activity was measured in the absence or presence of each inhibitor (Fig. 1b). Neither 50 μM NCTT-956 nor 10 μM Ro 31-8220 inhibited $cPLA_2$ activity. However, 50 μM baicalein resulted in almost a 50% decrease in $cPLA_2$ activity ($P < 0.001$), confirming the higher level of selectivity of NCTT-956 toward 12-LOX.

PKC Regulation of Platelet Aggregation and Dense Granule Secretion. Because PKC plays an important role in granule secretion and integrin activation (Harper and Poole, 2010), we investigated PKC involvement in the transduction of 12-LOX signaling in the platelet. First, we confirmed the role of PKC in platelet activation by measuring platelet aggregation in the presence of increasing concentrations of the pan-PKC inhibitor, Ro 31-8220, after stimulation with 20 μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen (Fig. 2). Our data show that inhibiting activation of PKC resulted in a dose-dependent attenuation of platelet aggregation, suggesting that PKC is important for normal platelet aggregation to occur.

To identify whether PKC regulates dense granule secretion, washed platelets were stimulated with 20 μM PAR1-AP, 200 μM PAR4-AP, 10 μg/ml collagen, or the PKC activator, PMA (Fig. 3). ATP, which is secreted from the dense granule, was measured by luminescence in the absence or presence of increasing concentrations of Ro-31 8220. As the concentration of the Ro 31-8220 was increased, the level of

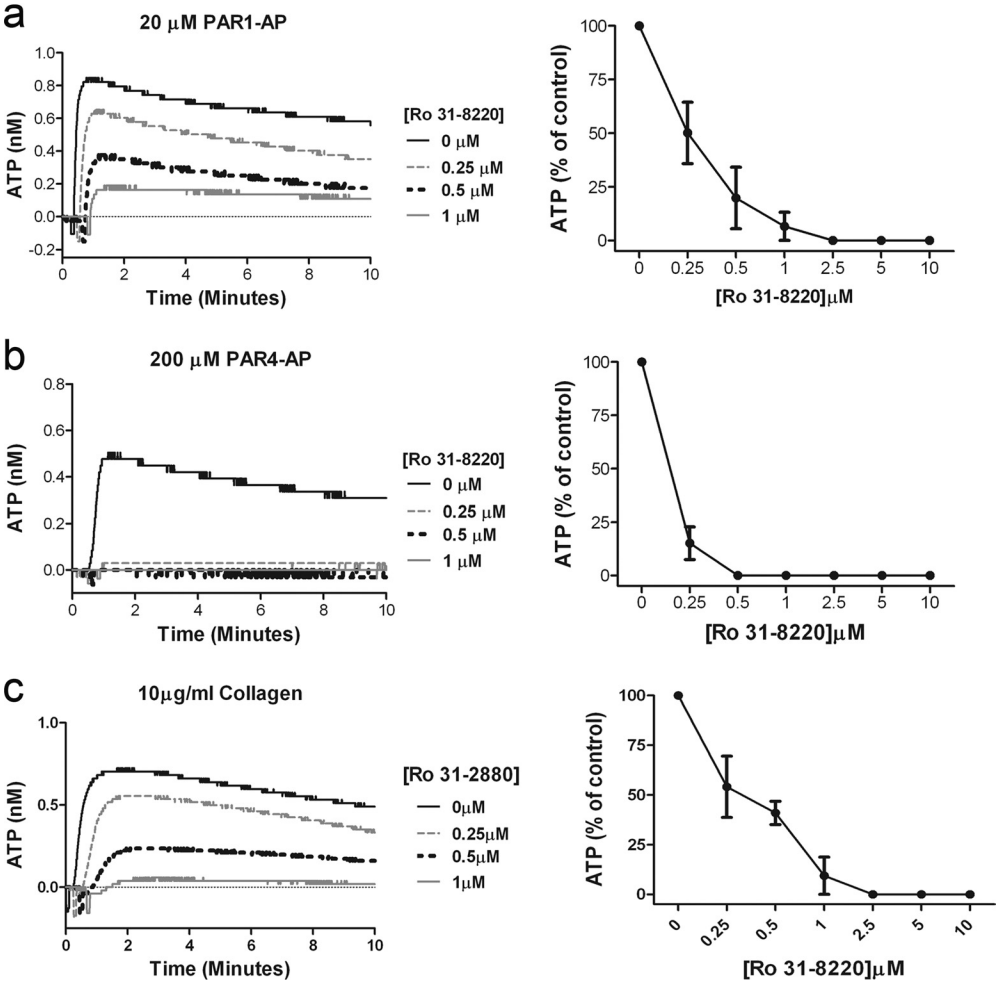

Fig. 3. PKC is an important determinant of platelet ATP secretion. Washed platelets were treated with or without increasing concentrations of the pan-PKC inhibitor Ro 31-8220 for 1 min, and platelet ATP secretion was measured after stimulation with 20 μM PAR1-AP (a), 200 μM PAR4-AP (b), or 10 μg/ml collagen (c). The right panel graphs represent normalized ATP secretion ($n = 3$).

ATP secreted from the dense granule was decreased with full inhibition observed at 1 μM Ro-31 8220 (Fig. 3, a–c).

To determine whether PKC activation can rescue platelet activation in the absence of 12-LOX activation, washed platelets were stimulated with the diacylglycerol mimetic (PMA) in the absence or presence of the 12-LOX inhibitors baicalein or NCTT-956, and platelet aggregation was measured for 15 min (Fig. 4). Stimulation of washed platelets with 250 nM PMA resulted in full platelet aggregation, which was sustained over time. PMA-mediated aggregation was not affected by either 12-LOX inhibitor, indicating that 12-LOX activation is not required for PMA-mediated platelet aggregation (Fig. 4a). Although stimulation with PMA results in aggregation of washed platelets, we had previously shown that PMA does not induce calcium mobilization and neither thromboxane nor 12-hydroxyeicosatetraenoic acid was produced, giving evidence that PKC activation alone does not liberate arachidonic acid from the plasma membrane of the platelet (Holinstat et al., 2011). To determine whether PKC activation acts downstream of 12-LOX, we assessed whether PMA might rescue the aggregation defect observed in the presence of the 12-LOX inhibitors. Washed platelets were stimulated with either 50 μM PAR4-AP or 10 μg/ml collagen in the absence or presence of 12-LOX inhibitors (Fig. 4, b and c). Treatment with either baicalein or NCTT-956 significantly inhibited PAR4-AP and collagen-induced platelet aggregation. However, addition of PMA fully rescued agonist-mediated platelet aggregation in the presence of 12-LOX

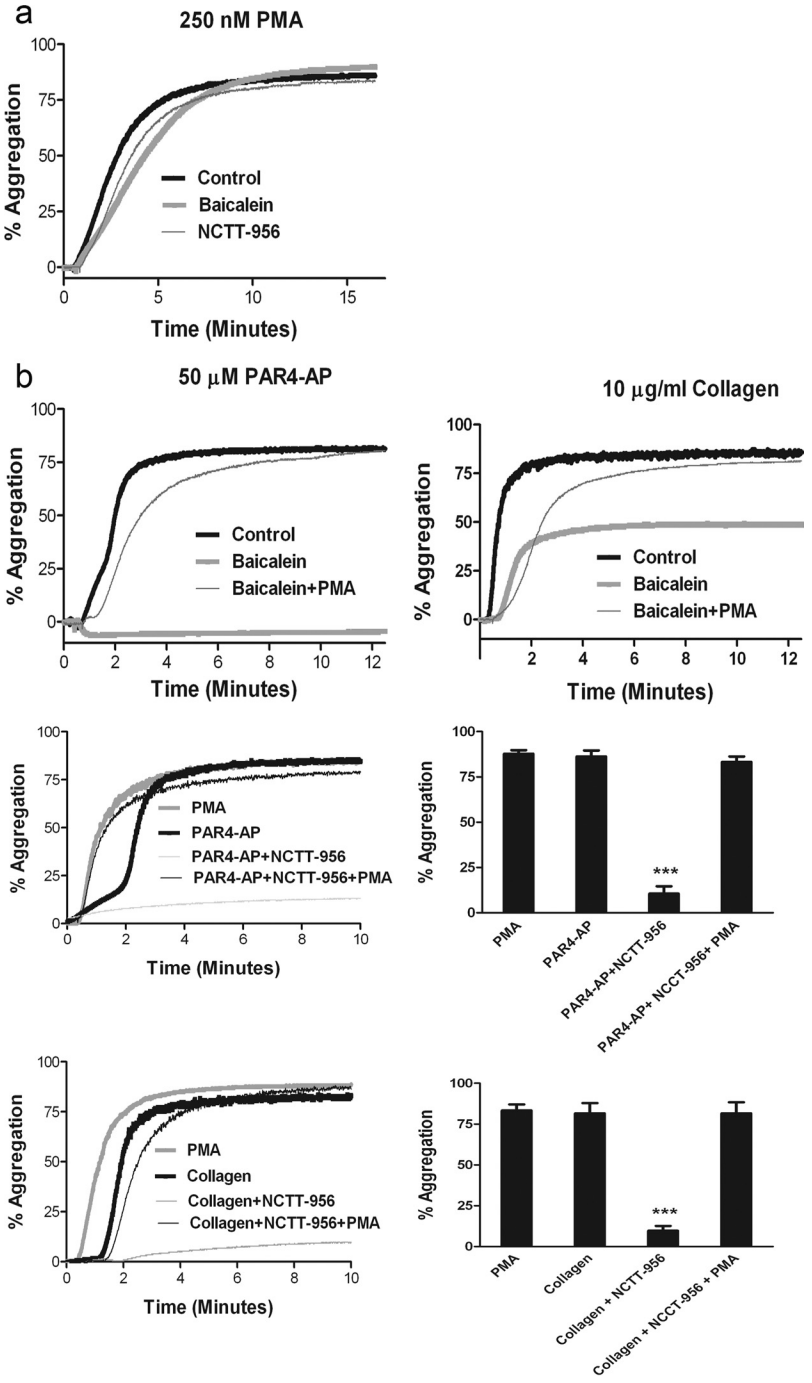

Fig. 4. PKC activation rescues platelet aggregation downstream of 12-LOX. a, washed human platelets were treated with or without 25 μM NCTT-956 or 50 μM baicalein followed by 250 nM PMA stimulation. Platelet aggregation was then measured for 16 min. b, washed platelets were treated with or without baicalein, followed by receptor agonist alone, 50 μM PAR4-AP (left panel), or 10 μg/ml collagen (right panel) or receptor agonist plus PMA, and platelet aggregation was measured for 12 min. c, washed platelets were treated with or without NCTT-956, followed by stimulation with PMA, PAR4-AP, collagen, or a combination of PMA and PAR4-AP (or collagen), and platelet aggregation was measured. ***, $P < 0.001$ for aggregation. $n = 3$.

inhibitors. Although these data suggest that PKC activation is playing a positive role in 12-LOX-mediated platelet aggregation through a number of signaling pathways including PAR4 and collagen, a concept supported by previous studies on 12-LOX (Khan et al., 1993; Chari et al., 2009), it is also possible that PMA causes activation of platelets in a 12-LOX-independent manner. Although plausible, this alternative hypothesis is not as likely, considering that both PAR1 and PAR4 signal PLCβ, result in strong activation of PKC.

Role of PKC in PAR-Mediated Integrin αIIbβ3 Activation. The primary adhesive receptor mediating platelet aggregation is the integrin αIIbβ3. Because there is a direct correlation between activation levels of αIIbβ3 and platelet aggregation, the role of PKC in this pathway was investigated (Fig. 5). Washed platelets were stimulated with PAR1-AP, PAR4-AP, or convulxin (a snake venom known to activate the collagen GPVI receptor) in the absence or presence of two concentrations of the PKC inhibitor, and active αIIbβ3 was assessed. For all agonists, αIIbβ3 activation was partially blocked in the absence of PKC activity (Fig. 5a). To confirm that PKC could rescue agonist-mediated αIIbβ3 activation in the absence of 12-LOX activity, platelets were pretreated with or without 25 μM NCTT-956 and stimulated with PAR1-AP, PAR4-AP, or convulxin in the presence or absence of PMA (Fig. 5, b–d). NCTT-956 inhibited more than 50% of PAR4-AP-mediated αIIbβ3 activation ($P = 0.02$), and this inhibition was significantly overcome in the presence of PMA ($P = 0.04$), further supporting a role for PKC after 12-LOX activation in regulating agonist-mediated platelet activation. In contrast, inhibition of 12-LOX by NCTT-956 did not affect PAR1-AP-induced αIIbβ3 activation either alone or in combination with PMA, suggesting another pathway of transduction through PAR1 requiring PKC activation but not 12-LOX (Fig. 5a). Of interest, the effect of NCTT-956 on αIIbβ3 activation in platelets stimulated with convulxin was not rescued by PMA (Fig. 5d). These results clearly indicate a very complex agonist-dependent mechanism of regulation of integrin activation in platelets.

12-LOX Regulation of PKC and Phospho-ERK. Previous work suggested that PKC activation is not upstream of 12-LOX because PMA does not result in formation of 12-HETE in human platelets (Holinstat et al., 2011). To deter-

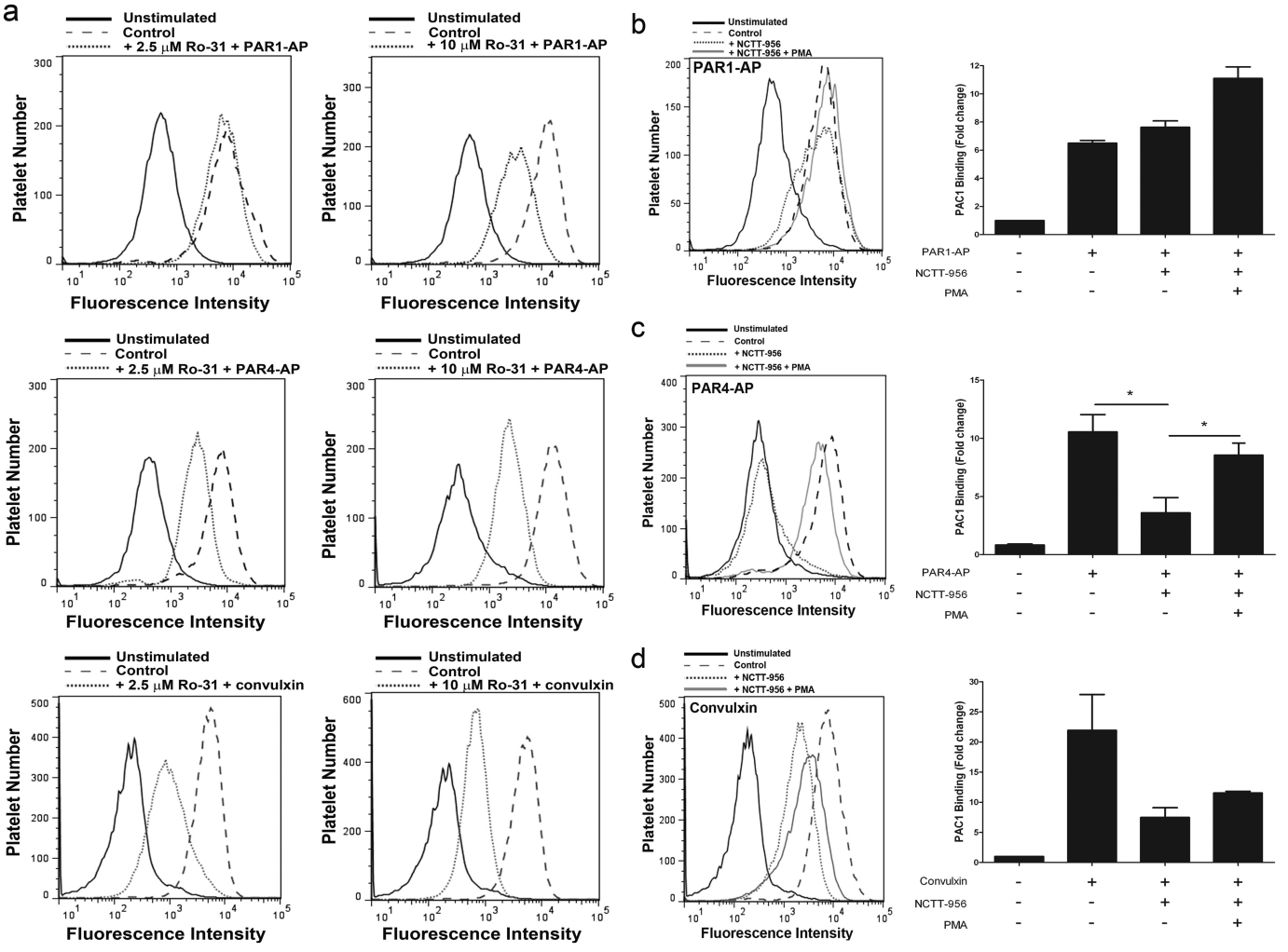

Fig. 5. Role of PKC in PAR-mediated integrin αIIbβ3 activation. a, washed platelets were preincubated with PAC1 antibody and treated with 2.5 or 10 μM pan-PKC inhibitor, Ro 31-8220, for 1 min. αIIbβ3 activation by flow cytometry was measured after stimulation with 5 μM PAR1-AP, 25 μM PAR4-AP, or 0.1 μg/ml convulxin for 10 min. The histograms shown are representative of three different experiments. Washed platelets were preincubated with PAC1 antibody and treated with the 25 μM NCTT-956 for 10 min followed by stimulation with PAR1-AP (b), PAR4-AP (c), or convulxin (d) in the presence or absence of 1 μM PMA. αIIbβ3 activation was measured by flow cytometry with FITC-PAC1. The bars represent means ± S.E.M. for fluorescence. $n = 3$. *, $P < 0.05$.

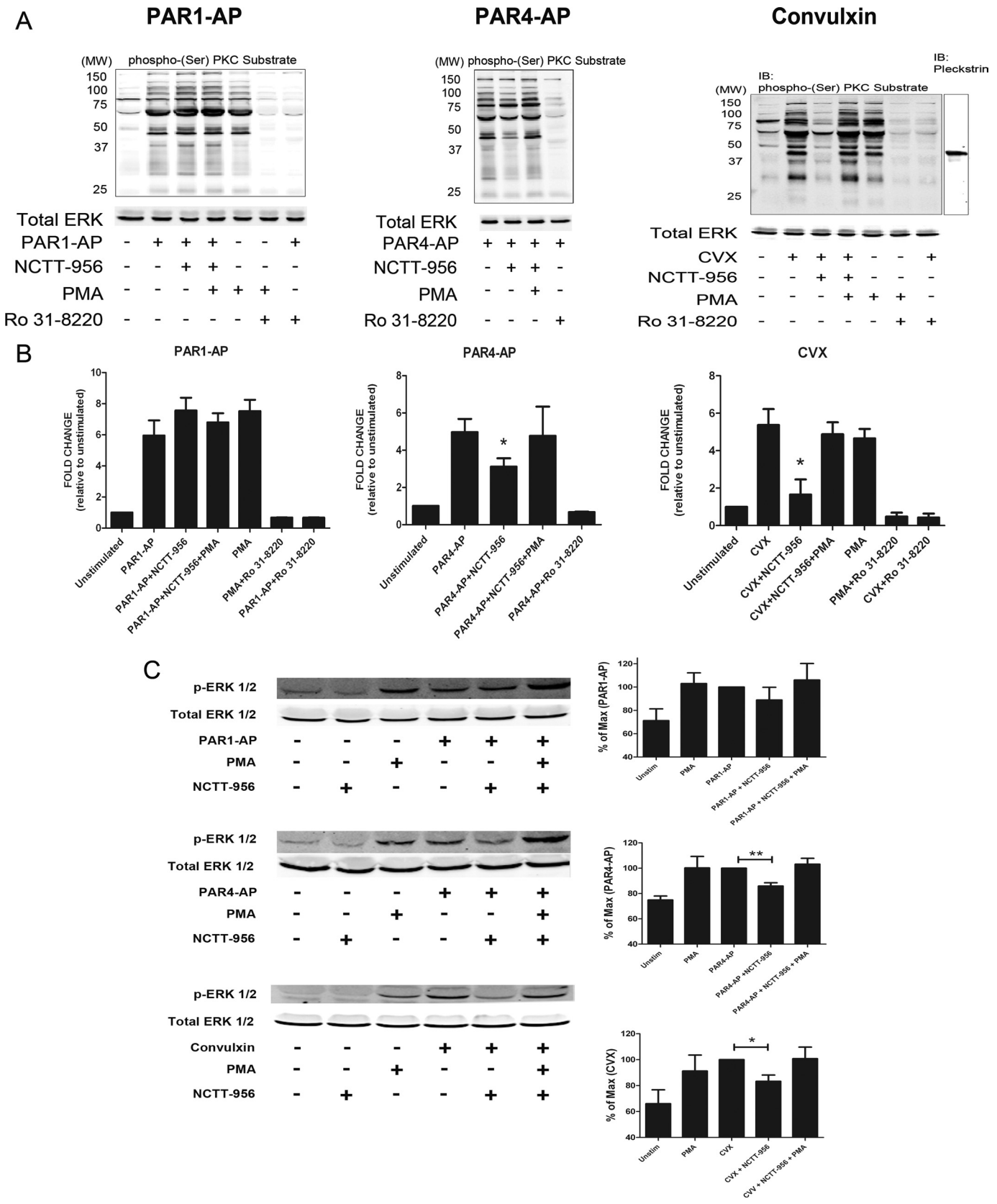

Fig. 6. 12-LOX regulates agonist-mediated regulation of pleckstrin and ERK phosphorylation. a, washed platelets were treated with or without NCTT-956 or Ro 31-8220 for 15 min followed by stimulation with 5 μM PAR1-AP (left panel), 50 μM PAR4-AP (middle panel), or 0.1 μg/ml CVX (right panel) alone or in combination with 1 μM PMA for 1 min under stirring conditions. The cytosolic fraction was assessed by Western blot with a phospho-(Ser) PKC substrate antibody. The pleckstrin band was identified by Western blotting with anti-pleckstrin antibody (to the right of the convulxin panel), and representative blots are shown for each condition ($n = 3$). All samples were normalized to total ERK for each lysate ($n = 3$). b, bar graphs showing fold changes in pleckstrin phosphorylation relative to the unstimulated condition for each lane in a ($n = 3$). $*, P < 0.05$. c, washed

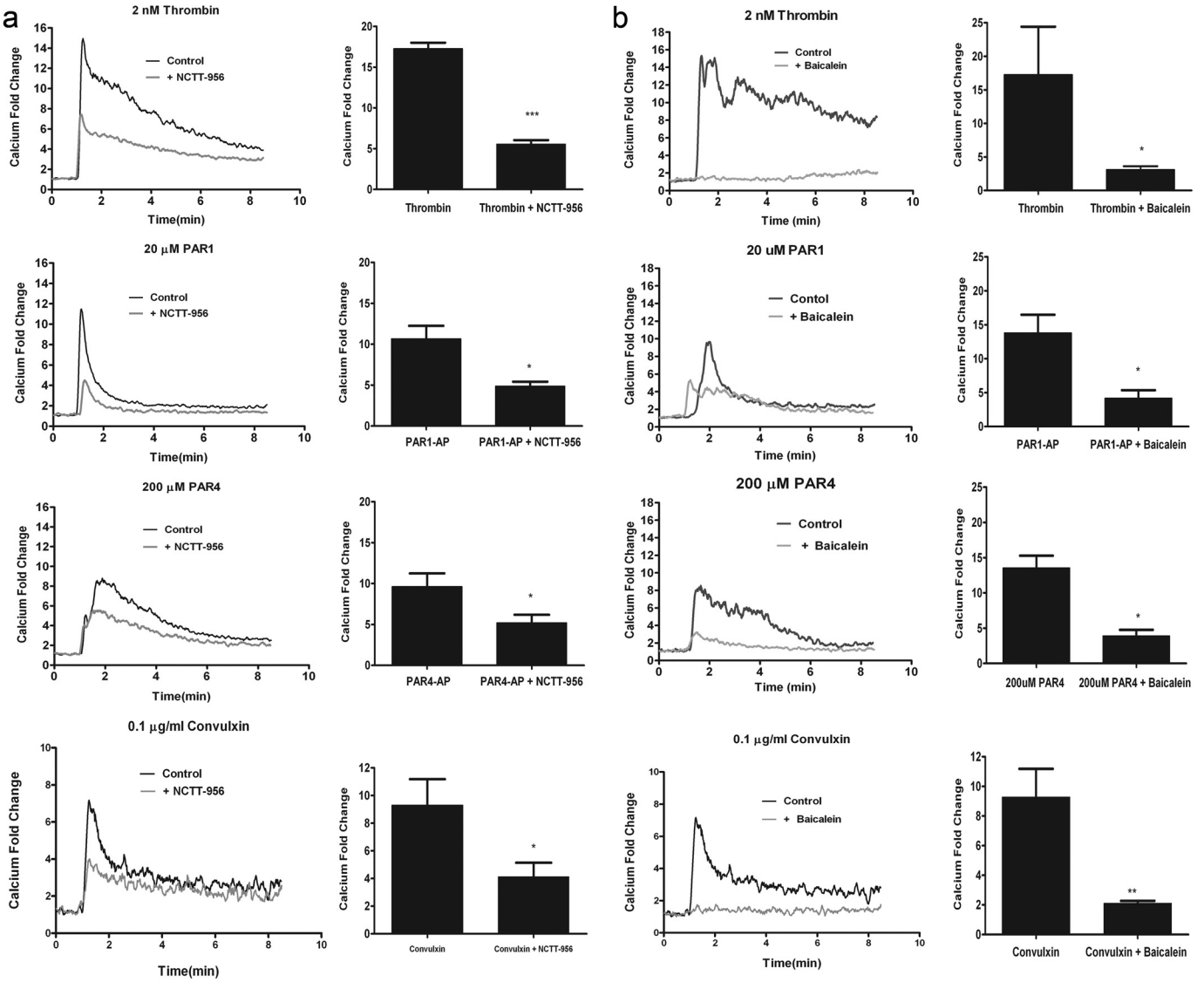

Fig. 7. 12-LOX inhibition attenuates calcium mobilization in human platelets. Calcium mobilization was measured in recalcified washed platelets in the presence or absence of 12-LOX inhibitors after stimulation with 2 nM thrombin, 20 μM PAR1-AP, 200 μM PAR4-AP, or 0.1 μg/ml convulxin. a, platelets loaded with Fluo-4 AM for 10 min were incubated with NCTT-956 for an additional 10 min followed by stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin, and calcium mobilization was monitored for 8 min after stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 min. The bar graphs on the right indicate the maximal increase in calcium mobilization ($n = 3$). b, platelets incubated with 50 μM baicalein were stimulated with thrombin, PAR1-AP, PAR4-AP, or convulxin, and calcium mobilization was monitored for 8 min after stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 min. The bar graphs on the right indicate the maximal increase in calcium mobilization ($n = 3$). Composite bar graphs are calculated as means ± S.E.M. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mine whether 12-LOX is an upstream regulator of PKC activation, washed platelets were stimulated with PAR1-AP, PAR4-AP, or CVX in the absence or presence of NCTT-956 and assayed for phosphorylation of PKC substrates (Fig. 6a). Stimulation with PAR1-AP, PAR4-AP, CVX, or PMA resulted in a significant increase in phosphorylation of a number of PKC substrates. Because pleckstrin is a well established phosphorylated substrate for active PKC, the pleckstrin band was determined by immunoblot in the platelet samples to be approximately 47 kDa (Fig. 6a, right panel; Supplemental Fig. 1). The phospho-(Ser) PKC band corresponding to pleckstrin was blocked after treatment with the pan-PKC inhibitor, Ro-31-8220 (Fig. 6, a and b), confirming that inhibition of PKC blocks pleckstrin phosphorylation. In the presence of the 12-LOX inhibitor, the level of pleckstrin phosphorylation, as well as that of other PKC substrates, was significantly decreased after stimulation with PAR4-AP and CVX (middle and right panels) compared with control (no inhibitor). PAR1-AP-induced pleckstrin phosphorylation, however, was not inhibited in the presence of NCTT-956 (left panel). Equal

platelets were treated with 25 μM NCTT-956 for 10 min followed by stimulation with 5 μM PAR1-AP, 50 μM PAR4-AP, or 0.1 μg/ml convulxin in the presence or absence of 1 μM PMA for 3 min under stirring conditions. The cytosolic fraction was assessed by Western blot for phosphorylation of ERK ($n = 4$). Representative data for each condition stimulated with PAR1-AP, PAR4-AP, or convulxin are presented on the left. Total ERK antibody was used as a loading control. The bar graphs on the right indicate the mean phosphorylation (± S.E.M.) of each condition as a percentage of maximal phosphorylation with agonist alone. Conditions were compared using a paired t test ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

protein loading was confirmed by measuring total ERK for each condition. Figure 6b shows that NCTT-956 reproducibly attenuates PAR4 and CVX-mediated pleckstrin phosphorylation in the platelet ($n = 3$). Treatment with PMA was able to rescue NCTT-956-mediated attenuation of pleckstrin phosphorylation in both PAR4-AP and CVX-stimulated conditions (Fig. 6, a and b).

As further evidence that 12-LOX is an upstream regulator of PKC activation, ERK phosphorylation was measured in washed platelets stimulated with PAR1-AP or PAR4-AP in the absence or presence of NCTT-956 with or without PMA (Fig. 6c). ERK phosphorylation was measured because ERK has been shown to be partially regulated by PKC in the platelet (Yacoub et al., 2006). Stimulation with PAR-AP or PMA resulted in significant phosphorylation of ERK. In the presence of NCTT-956, PAR4-AP-mediated ERK phosphorylation was significantly reduced and was partially rescued with the addition of PMA. Of interest, PAR1-AP-mediated ERK phosphorylation was not significantly affected by treatment with NCTT-956. To determine whether ERK phosphorylation was regulated downstream of 12-LOX solely through the PAR-4 pathway, platelets were treated with convulxin, the snake venom known to specifically activate the collagen receptor. Convulxin alone induced phosphorylation of ERK, and this phosphorylation event was significantly attenuated in the presence of NCTT-956. However, similar to PAR4-AP, the presence of PMA fully rescued NCTT-956-induced inhibition of convulxin-mediated ERK phosphorylation, supporting a proximal role for 12-LOX in regulating platelet activation upstream of PKC and ERK.

12-LOX Inhibition Attenuates Calcium Mobilization in Human Platelets. It has been reported that inhibition of 12-LOX attenuates calcium entry into platelets (Nyby et al., 1996). Because calcium mobilization also plays a role in regulation of eicosanoid production and platelet activation, calcium levels were monitored after stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin in the absence or presence of 12-LOX inhibitors (Fig. 7). Agonist stimulation induced a significant and transient increase in free calcium in the platelet. In the presence of NCTT-956, free calcium in the platelet was significantly diminished after stimulation with thrombin, PARs, or convulxin (Fig. 7a). Inhibition with baicalein more severely attenuated platelet mobilization compared with that with NCTT-956, which may be due to the higher level of selectivity toward 12-LOX exhibited with NCTT-956 relative to baicalein (Fig. 7b) (Kenyon et al., 2011).

Discussion

Platelet reactivity plays a critical role in hemostasis and thrombosis. Much attention has been given to limiting unwanted platelet activation and vessel occlusion through inhibition of the ADP receptor ($P2Y_{12}$) and cyclooxygenase-1(Mahanonda, 1998; Varon and Spectre, 2009). These therapies, although successful in decreasing the morbidity due to myocardial infarction and stroke (Diener et al., 2004; Durand-Zaleski and Bertrand, 2004), have significant shortcomings including genetic variability (Gurbel et al., 2010) and aspirin resistance (Patrono et al., 2005). Furthermore, all of these approaches result in a significant increase in bleeding, which can be more deleterious than the clot itself. Therefore, alternative approaches with fewer side effects are warranted. Targeting 12-LOX, which metabolizes AA in a stereospecific manner to generate 12(*S*)-HpETE, may be one such target (Pidgeon et al., 2007). 12-LOX and its metabolites have been shown to promote cancer progression and metastasis through a MAPK-dependent pathway (Szekeres et al., 2000; Ding et al., 2001). Inhibition of 12-LOX in tumor cells was shown to induce apoptosis and was blocked by either overexpression of 12-LOX or addition of 12-HETE (Szekeres et al., 2000; Chen et al., 2008). In platelets, however, the underlying signaling mechanisms regulating 12-LOX-mediated platelet reactivity have not been well characterized. Our data demonstrate that inhibition of 12-LOX significantly attenuates agonist-mediated platelet aggregation. This is in line with the prothrombotic actions attributed to production of 12(*S*)-HETE in the platelet (Thomas et al., 2010). Furthermore, our data suggest that 12-LOX-mediated regulation of platelet activity may be regulated, at least in part, through activation of PKC.

PKC has been shown to regulate a number of biochemical pathways in platelets, affecting platelet physiology by modulating aggregation and dense granule secretion (Chari et al., 2009; Konopatskaya and Poole, 2010). Although some studies have indicated that arachidonic acid may activate PKC directly through 12-HETE (McPhail et al., 1984; Shearman et al., 1989), others have proposed an indirect mechanism for eicosanoid regulation of PKC activation (Liu et al., 1995; Seth et al., 2001). Our present work showed that inhibition of 12-LOX resulted in attenuation of platelet aggregation. Likewise, we found that PKC inhibition attenuated PAR-induced platelet aggregation. Therefore, we hypothesized that 12-LOX regulation of platelet reactivity may in some way be coupled to that of PKC. Through a number of approaches, PKC was determined to be downstream of 12-LOX activation, because activation with PMA was able to rescue 12-LOX-mediated inhibition of platelet aggregation, αIIbβ3 activation, and ERK phosphorylation. Together with our earlier work, which showed that PMA activation of platelet aggregation did not result in calcium mobilization or formation of 12-HETE (Holinstat et al., 2011), these data are suggestive of a signaling cascade in which PKC is downstream of 12-LOX activation. Although the present study supports a role for PKC activation downstream of 12-LOX, the specific isoform(s) of PKC involved in this process are unclear. Observations in the current study coupled with published work investigating regulation through PKC and 12-LOX (McPhail et al., 1984; Shearman et al., 1989; Liu et al., 1995; Szekeres et al., 2000; Seth et al., 2001; Nadal-Wollbold et al., 2002; Yacoub et al., 2006; Chari et al., 2009; Konopatskaya and Poole, 2010) are highly suggestive of a signaling cascade involving 12-LOX regulation of a conventional PKC either directly or through positive feedback via activation of a GPCR in the platelet. Future investigations will focus on elucidating the mechanisms by which PKC regulates platelet reactivity and stability after activation of 12-LOX and the isoform(s) of PKC involved in this process.

Platelet aggregation requires activation of the integrin αIIbβ3. 12(*S*)-HETE has been linked to regulation of integrin activation in other cells (Rásó et al., 2001), and we observed partial inhibition of αIIbβ3 activation in platelets in the absence of PKC activity. Similar attenuation of PAR4-induced integrin activation was observed in the absence of

12-LOX activity, attenuation that was also rescued by addition of PMA. These results support a role for 12-LOX-dependent PKC regulation of integrin activity after activation of platelets by PAR4-AP. Of interest, we found that the integrin activity induced by PAR1 and by convulxin was not significantly affected by the 12-LOX inhibitor. Together with our observation that the PKC inhibitor attenuates αIIbβ3 activation induced by these two agonists, our results indicate that, contrary to activation by PAR4, activation of the integrin by PAR1 and GPVI agonists is mediated by a 12-LOX-independent but PKC-dependent mechanism.

Although several MAPKs have been identified in platelets including ERK1/2, p38MAPK and c-Jun NH_2-terminal kinase (Samiei et al., 1993; Kramer et al., 1995; Bugaud et al., 1999; Yacoub et al., 2006), their role in mediating platelet function downstream of 12-LOX activation is unclear. 12-HETE has been reported to induce ERK activation in human epidermal carcinoma cells, and this activation could be inhibited by pertussis toxin, suggesting the potential involvement of a G protein-coupled receptor (Szekeres et al., 2000). ERK has also been shown to be regulated after protein kinase A activation (Borsch-Haubold et al., 1996; Nadal-Wollbold et al., 2002). Our data support a role for 12-LOX regulation of ERK in human platelets as well, because inhibiting 12-LOX activation resulted in a partially attenuated activation of ERK by PAR4-AP or convulxin. The mechanism by which 12-LOX regulates ERK, whether it be through 12-HpETE, 12-HETE, or some other bioactive metabolite, is currently under current investigation, and understanding its regulation will significantly aid our understanding of 12-LOX metabolite regulation of platelet function. The observation that attenuation of ERK phosphorylation in the absence of 12-LOX activation was partially rescued by PMA (Fig. 6) lends strong support for PKC regulation of platelet function downstream of 12-LOX.

Several isoforms of PKC are activated by calcium in the human platelet (Grabarek and Ware, 1993; Khan et al., 1993), and previous reports have indicated that calcium may be partially regulated by 12-LOX (Nyby et al., 1996). However, this study was conducted with the less selective 12-LOX inhibitor baicalein which has been shown to inhibit a number of enzymes in addition to 12-LOX (Deschamps et al., 2006). The current study is the first to show that baicalein inhibits a number of enzymes in the bioactive lipid pathways in the platelet including $cPLA_2$, COX-1, and perhaps thromboxane synthase, whereas NCTT-956 was shown not to directly affect any of these off-target enzymes (Fig. 1). To determine whether calcium mobilization is specifically regulated by 12-LOX, platelets were treated with the highly selective 12-LOX inhibitor NCTT-956, and agonist-induced calcium mobilization was measured (Fig. 6). Calcium mobilization was significantly attenuated in the presence of NCTT-956 and baicalein, supporting the earlier reports attributing 12-LOX activation to this biochemical step (Nyby et al., 1996). Because calcium can activate PKC directly, these results give evidence for agonist-mediated activation of calcium downstream of 12-LOX activation and upstream of PKC. Of interest, calcium mobilization was not completely inhibited by NCTT-956 after platelet activation by either PAR1 or PAR4-AP, suggesting a 12-LOX-independent component of calcium signaling. In contrast, after activation by convulxin, calcium mobilization is completely abrogated by the 12-LOX-specific inhibitor. These data suggest a differential regulation of ERK phosphorylation that involves both 12-LOX-dependent and 12-LOX-independent mechanisms contingent upon the agonist used to activate the platelets.

Taken together, our results clearly show that 12-LOX plays an important role in platelet reactivity. This is the first report to show that 12-LOX activity occurs upstream of PKC and that integrin αIIbβ3 activation occurs downstream of both 12-LOX and PKC in human platelets. Of importance, this report also demonstrates the selectivity of 12-LOX sensitivity toward the PAR4 activation pathway. Finally, this study identifies PKC as an important biochemical intermediate in both 12-LOX-dependent and -independent regulation of platelet activation. Future investigations will focus on identifying the feedback mechanisms by which 12-LOX regulates platelet function, presumably through an eicosanoid-dependent pathway, resulting in PKC-dependent activation of the human platelet.

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Authorship Contributions

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Conducted experiments: Yeung, Apopa, Vesci, Boutaud, and Holinstat.

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Performed data analysis: Yeung, Apopa, Vesci, Boutaud, and Holinstat.

Wrote or contributed to the writing of the manuscript: Yeung, Apopa, Boutaud, and Holinstat.

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