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Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation

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Running title: PKC regulates platelet activation downstream of 12-lipoxygenase

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Abbreviations:

AA, arachidonic acid; 12-LOX, 12-lipoxygenase; PKC, Protein kinase C; PLA_2, phospholipase A_2 ; COX-1, cyclooxygenase-1; PAR1-AP, protease-activated receptor 1-activating peptide; PAR4-AP, protease-activated receptor 4- activating peptide; PMA, phorbol myristoyl acetate; ERK, extracellular regulated kinase; 12(S)-HpETE, (12S)-12-hydroperoxyicosa-5,8,10,14-tetraenoic acid; 12(S)-HETE, 8S-hydroxy-4,6,10-hexadecatrienoic acid; DAG, giacylglycerol

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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. Following 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 as 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 anti-platelet therapy.

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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), while other studies suggest 12-LOX activation is pro-thrombotic and is linked to calcium mobilization (Nyby et al., 1996), regulation of tissue factor activation, and thrombin generation in the 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

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activation (Harper and Poole, 2010). Further, protease-activated receptor-1 (PAR1) and PAR4 signaling in the platelet have been shown to result in Ca^{2+} mobilization and PKC-mediated aggregation and secretion (Falker et al., 2011). However, the underlying mechanism by which PKC regulates platelet activity is controversial. Kim et al (Kim et al., 2011) reported that PKC inhibition by the pan-PKC inhibitor, Ro 31-8220, potentiated epinephrine induced platelet aggregation and Unsworth et al (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 if 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.

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Materials and Methods:

Materials: 12-LOX inhibitor (NCTT-956) was synthesized at the NIH Chemical Genomics Center (Rockville, MD) and provided by David Maloney. Baicalein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK and total-ERK antibodies were purchased from Cell Signaling Technology (Boston, MA), PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). Fluo-4 AM was from Invitrogen (Eugene, OR). Human α-thrombin was purchased from Enzyme Research Labs (South Bend, IN). Convulxin was purchased from Center Chem (Norwalk, CT). Fluorescein isothiocyanate (FITC)-conjugated PAC1 antibody was purchased from BD Biosciences (San Jose, CA). C6 flow cytometer was from Accuri (Ann Arbor, MI). Aggregometer, collagen, chronolume reagent, and other aggregation supplies were purchased from Chrono-Log Corp. (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 minutes 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 minutes 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 X 10^8 platelets/ml after

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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.

GC/MS analysis of [$^2H_8$] Thromboxane synthesis in platelets: 2 ng of [$^2H_4$] TxB_2 was added to the samples as internal standards. Briefly, the sample prepurified with a C18 SepPak column. [$^2H_8$] TxB_2 was eluted with 10 ml heptane/ethyl acetate (1:1), dried, 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/electron capture negative chemical ionization mass spectrometry, using an SPB-1 column (15 meters), with a temperature gradient from 190°C to 300°C at 20°C/min. The ion corresponding to the derivatized TxB_2 was monitored by selected ion monitoring (SIM). 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 LC/APCI/MS/MS following 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 DMSO right before the recombinant enzyme was added to

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initiate the reaction. After 5 minutes of incubation, the products of the reaction were analyzed.

Platelet Aggregation: Washed platelets were adjusted to a final concentration of 3 x 10^8 platelets/ml. Where indicated, platelets were pretreated with 12-LOX inhibitors for 10 minutes or PKC inhibitor for 1minute. 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 x 10^8 platelets/ml were pretreated with 12-LOX inhibitors for 10 minutes or PKC inhibitor for 1 minute. 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 which only recognizes the active form of αIIbβ3). For these experiments, 40 µl aliquots of washed platelets adjusted to a final concentration of 2.5 x 10^7 platelets/ml were pre-treated with inhibitors for 10 minutes. After addition of 1 µl of PAC1, platelets were stimulated with agonist for 10 minutes 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 1 x 10^9 platelets/ml were stimulated with indicated agonists and lysed with 3x Laemmli buffer was then added to the samples, boiled for 5 minutes, and subjected to Western blot analysis.

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

Statistical Analysis: Comparison between experimental groups was made using appropriate statistical analyses (paired *t* test program or ANOVA with post-test analysis) using Prism software. Differences in mean values (measured as standard error of the mean) were considered significant at $p < 0.05$.

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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 (Figure 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 (Figure 1a). Values are expressed as the % 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 this drug may have a direct inhibitory effect on COX-1 or thromboxane synthase (Figure 1a). As [$^2H_8$] TxB_2 was increased in the presence of the PKC inhibitor, the level of 12-HETE formation was also measured to determine if 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 (Figure 1b). Neither 50 μM NCTT-956 nor 10 μM Ro 31-8220 inhibited $cPLA_2$ activity. 50 μM baicalein, however, 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.

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PKC regulation of platelet aggregation and dense granule secretion. Since 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, following stimulation with 20 μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen (Figure 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 if 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 (Figure 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 ATP secreted from the dense granule was decreased with full inhibition observed at 1 μM Ro- 31 8220 (Figure 3a-c).

To determine if 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 minutes (Figure 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 (Figure 4a).

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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 were 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 if 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-activating peptide (PAR4-AP) or 10 μg/ml collagen in the absence or presence of 12-LOX inhibitors (Figure 4b-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 inhibitors. While this data suggests 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 (Chari et al., 2009; Khan et al., 1993), 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 both PAR1 and PAR4 signal PLCβ resulting 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. Since there is a direct correlation between activation levels of αIIbβ3 and platelet aggregation, the role of PKC in this pathway was investigated (Figure 5). Washed platelets were stimulated with PAR1-AP, PAR4-AP, or convulxin (a snake venom known to activate the collagen GPVI receptor)

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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 (Figure 5a). To confirm that PKC could rescue agonist-mediated αIIbβ3 activation in the absence of 12-LOX activity, platelets were pre-treated with or without 25 μM NCTT-956 and stimulated with PAR1-AP, PAR4-AP, or convulxin in the presence or absence of PMA (Figures 5b-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 following 12-LOX activation in regulating agonist-mediated platelet activation. By 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). Interestingly, the effect of NCTT-956 on αIIbβ3 activation in platelets stimulated with convulxin was not rescued by PMA (Figure 5d). These results clearly indicate a very complex agonist-dependent mechanism of regulation of integrin activation in platelets.

12-LOX regulation of PKC and p-ERK. Previous work suggests that PKC activation is not upstream of 12-LOX since PMA does not result in formation of 12-HETE in human platelets (Holinstat et al., 2011). To determine if 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 (Figure 6a). Stimulation with PAR1-AP, PAR4-AP, CVX, or PMA resulted in a significant increase in phosphorylation of a number of PKC substrates.. As pleckstrin is a well established phosphorylated substrate for active PKC, the pleckstrin band was

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determined by immunoblot in the platelet samples to be approximately 47kDa (Figure 6a right panel and supplemental figure 1). The phospho-(Ser) PKC band corresponding to pleckstrin was blocked following treatment with the pan-PKC inhibitor, Ro- 31-8220 (Figures 6a-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 other PKC substrates, was significantly decreased following stimulation with PAR4-AP and CVX (middle and right panels) compared to control (no inhibitor). PAR1-AP-induced pleckstrin phosphorylation however, was not inhibited in the presence of NCTT-956 (left panel). Equal 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 (Figure 6a-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 (Figure 6c). ERK phosphorylation was measured since 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 a 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. Interestingly, PAR1-AP-mediated ERK phosphorylation was not significantly affected by treatment with NCTT-956. To determine if ERK phosphorylation was regulated downstream of 12-LOX solely through the PAR-4 pathway, platelets were

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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). As calcium mobilization also plays a role in regulation of eicosanoid production and platelet activation, calcium levels were monitored following stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin in the absence or presence of 12-LOX inhibitors (Figure 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 following stimulation with thrombin, PARs, or convulxin (Figure 7a). Inhibition with baicalein more severely attenuated platelet mobilization compared to NCTT-956 which may be due to the higher level of selectivity toward 12-LOX exhibited with NCTT-956 relative to baicalein (Figure 7b) (Kenyon et al., 2011).

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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, while 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 stereo-specific 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 (Ding et al., 2001; Szekeres et al., 2000). Inhibition of 12-LOX in tumor cells was shown to induce apoptosis and was blocked by either over expression of 12-LOX or addition of 12-HETE (Chen et al., 2008; Szekeres et al., 2000). In platelets however, the underlying signaling mechanisms regulating 12-LOX-mediated platelet reactivity have not been well characterized. Our data demonstrates that inhibition of 12-LOX significantly attenuates agonist-mediated platelet aggregation. This is in line with the pro-thrombotic actions attributed to production of 12(S)-HETE in the platelet (Thomas et al., 2010). Furthermore, our data suggests that 12-LOX-mediated regulation of platelet activity may be regulated, at least in part, through activation of PKC.

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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). While 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. Similarly, 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, as 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 PMA activation of platelet aggregation did not result in calcium mobilization or formation of 12-HETE (Holinstat et al., 2011), this data is suggestive of a signaling cascade in which PKC is downstream of 12-LOX activation. While 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 to published work investigating regulation through PKC and 12-LOX (Chari et al., 2009; Konopatskaya and Poole, 2010; Liu et al., 1995; McPhail et al., 1984; Nadal-Wollbold et al., 2002; Seth et al., 2001; Shearman et al., 1989; Szekeres et al., 2000; Yacoub et al., 2006) 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.

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Future investigations will focus on elucidating the mechanisms by which PKC regulates platelet reactivity and stability following 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 (Raso 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 following activation of platelets by PAR4-AP. Interestingly, 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 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 JNK (Bugaud et al., 1999; Kramer et al., 1995; Samiei et al., 1993; 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 pertusis toxin suggesting the potential involvement of a G protein coupled receptor (Szekeres et al., 2000). ERK has also been shown to be regulated following PKA activation (Borsch-Haubold et al., 1996; Nadal-Wollbold et al., 2002). Our data supports a role for 12-LOX

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regulation of ERK in human platelets as well, since 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 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 (Figure 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, while NCTT-956 was shown not to directly affect any of these off-target enzymes (Figure 1). To determine if 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 (Figure 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). Since calcium can activate PKC directly, these results give evidence for agonist-mediated activation of calcium downstream of 12-LOX activation

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and upstream of PKC. Interestingly, calcium mobilization was not completely inhibited by NCTT-956 following platelet activation by either PAR1 or PAR4-AP, suggesting a 12-LOX-independent component of calcium signaling. By 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. Importantly, this report also demonstrates the selectivity of 12-LOX sensitivity towards 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

Participated in research design: Yeung, Apopa, Boutaud, and Holinstat

Contributed new reagents or analytic tools: Kenyon, Rai, Jadhav, Simeonov, Holman, and Maloney

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

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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Footnotes

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Figure Legends:

Figure 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 control. 50 μM baicalein induced an inhibition of [$^2H_8$] TxB_2 compared to control levels and [$^2H_8$] TxB_2 formation in the presence of baicalein was significantly inhibited compared to [$^2H_8$] 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.

Figure 2: PKC regulation of platelet aggregation. Platelets were pre-treated with increasing concentrations of pan-PKC inhibitor, Ro 31-8220 from 0 to 10 μM for 1 minute followed by stimulation with 20μM PAR1-AP, 200 μM PAR4-AP, or 10 μg/ml collagen and platelets aggregation measured. Pre-treatment with increasing concentration of Ro 31-8220 attenuated agonist-mediated platelet aggregation in a dose dependent manner. The bars represent mean±SEM (*P<0.05; **P<0.001) for aggregation, n=3.

Figure 3: **PKC is an important determinant of platelet ATP secretion.** Washed platelets were treated with or without increasing concentrations of pan-PKC inhibitor, Ro 31-8220 for 1 minute and platelet ATP secretion was measured following stimulation with (a) 20 μM PAR1-AP, (b) 200 μM PAR4-AP, or (c) 10 μg/ml collagen. The right panel graph represents normalized ATP secretion (n=3).

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Figure 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 minutes. 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 measured for 12 minutes. 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 measured. (***P<0.001) for aggregation, n=3.

Figure 5: Role of PKC in PAR-mediated integrin αIIbβ3 activation. a) Washed platelets were pre-incubated with PAC1 antibody and treated with 2.5 μM or 10 μM pan-PKC inhibitor, Ro 31-8220, for 1minute. αIIbβ3 activation by flow cytometry was measured following stimulation with 5 μM PAR1-AP, 25 μM PAR4-AP, or 0.1 μg/ml convulxin for 10 minutes. The histograms shown are representative of three different experiments. b) Washed platelets were pre-incubated with PAC1 antibody and treated with the 25 μM NCTT-956 for 10 minutes followed by stimulation with PAR1-AP, c) PAR4-AP, or d) convulxin in the presence or absence of 1 μM PMA. αIIbβ3 activation was measured by flow cytometry with FITC-PAC1. The bars represent mean±SEM for fluorescence, n=3. *,P<0.05.

Figure 6: 12-LOX regulates agonist-mediated regulation of pleckstrin and ERK phosphorylation. a) Washed Platelets were treated with or without NCTT-956 or Ro

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31-8220 for 15 minutes 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 minute 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 unstimulated condition for each lane in 6a (N=3). *, $P<0.05$. c) Washed Platelets were treated with 25 μM NCTT-956 for 10 minutes 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 minutes 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 is presented on the left. Total ERK antibody was used as a loading control. The bar graphs on the right indicate the mean phosphorylation (± SEM) of each condition as a percentage of maximal phosphorylation with agonist alone. Conditions were compared using a paired t-test (N=4). *, $P<0.05$; **, $P<0.01$.

Figure 7: 12-LOX inhibition attenuates calcium mobilization in human platelets. Calcium mobilization was measured in re-calcified washed platelets in the presence or absence of 12-LOX inhibitors following 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 minutes were incubated with NCTT-956 for an additional 10 minutes followed by

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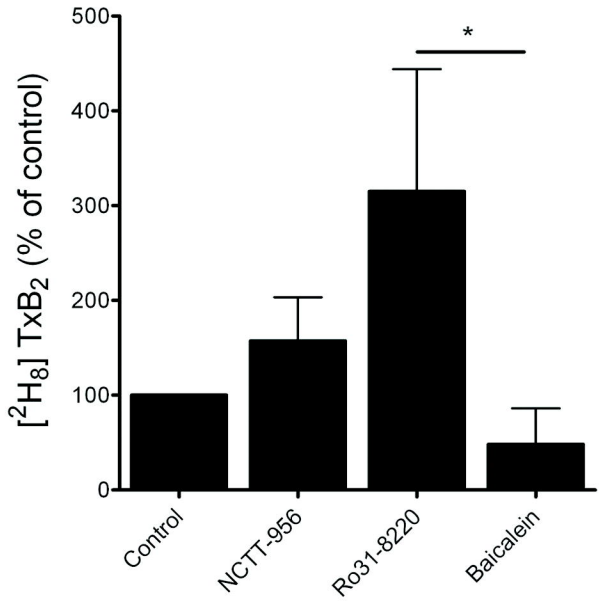
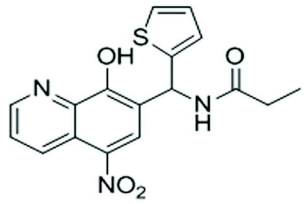
stimulation with thrombin, PAR1-AP, PAR4-AP, or convulxin and calcium mobilization was monitored for 8 minutes post-stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 minutes. The bar graphs on the right indicate the maximal 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 minutes post-stimulation. Representative curves on the left show the fold change in free calcium relative to the unstimulated condition over 8 minutes. The bar graphs on the right indicate the maximal increase in calcium mobilization (N=3). Composite bar graphs are calculated as mean ± SEM; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

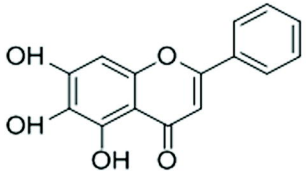
Figure 1:

a





NCTT-956



BAICALEIN

b

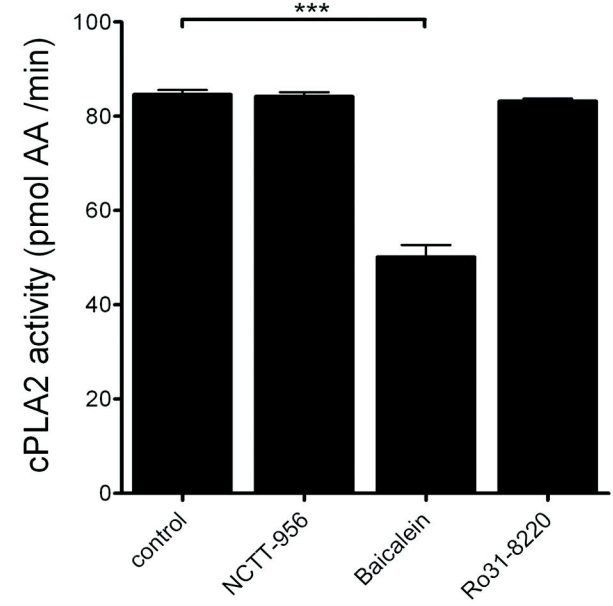


Figure 2:

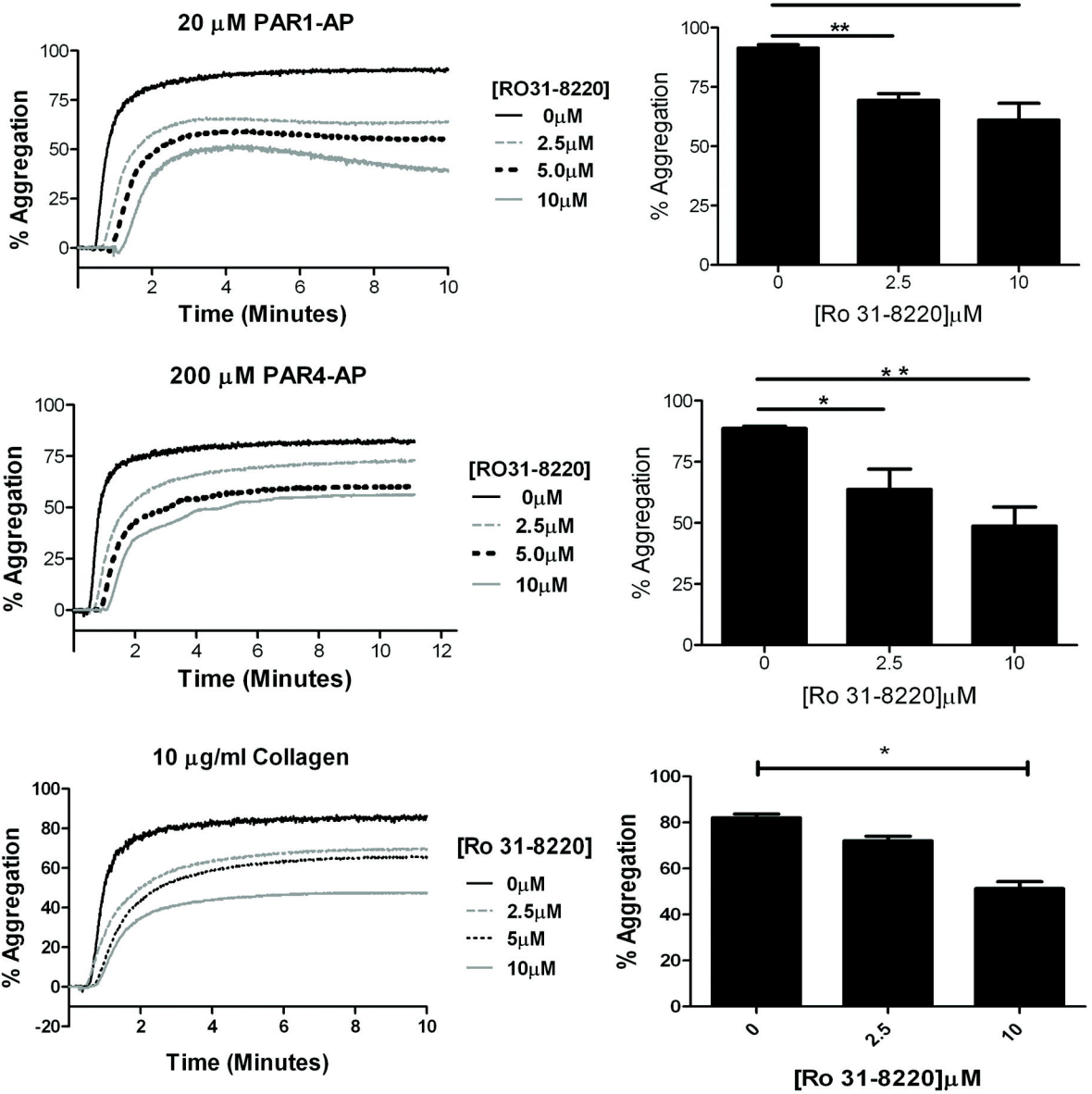
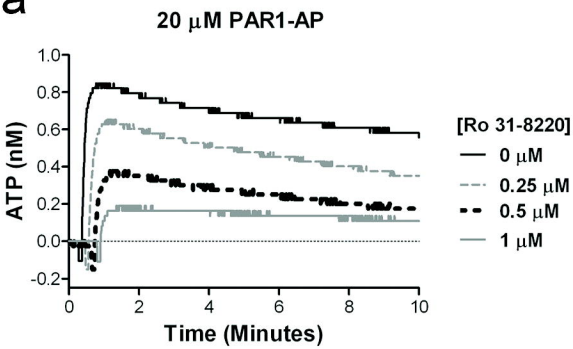
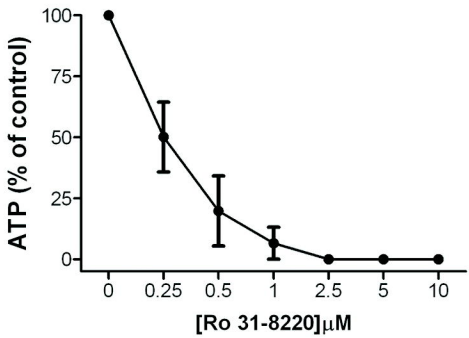

20 μM PAR1-AP
% Aggregation
Time (Minutes)
[RO31-8220]
0μM
2.5μM
5.0μM
10μM
*
**
% Aggregation
[Ro 31-8220]μM
200 μM PAR4-AP
% Aggregation
Time (Minutes)
[RO31-8220]
0μM
2.5μM
5.0μM
10μM
* *
*
% Aggregation
[Ro 31-8220]μM
10 μg/ml Collagen
% Aggregation
Time (Minutes)
[Ro 31-8220]
0μM
2.5μM
5μM
10μM
*
% Aggregation
[Ro 31-8220]μM

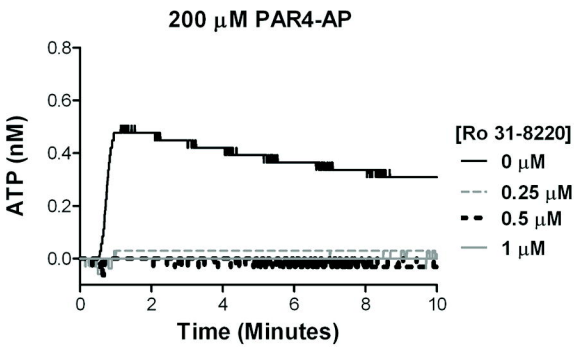
Figure 3:

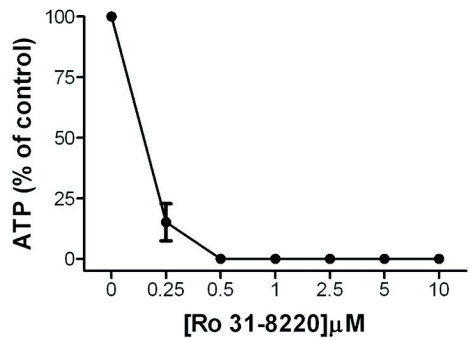
a


20 μM PAR1-AP
ATP (nM)
Time (Minutes)
[Ro 31-8220]
0 μM
0.25 μM
0.5 μM
1 μM

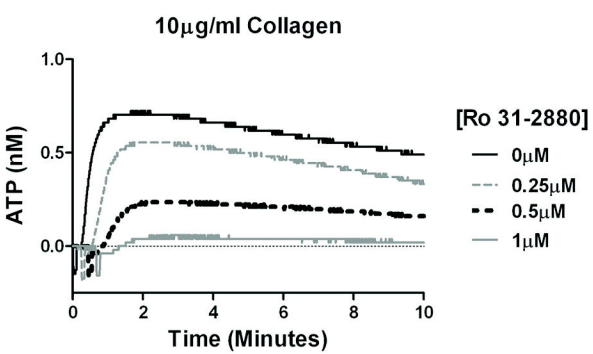

ATP (% of control)
[Ro 31-8220]μM

b


200 μM PAR4-AP
ATP (nM)
Time (Minutes)
[Ro 31-8220]
0 μM
0.25 μM
0.5 μM
1 μM


ATP (% of control)
[Ro 31-8220]μM

c


10μg/ml Collagen
ATP (nM)
Time (Minutes)
[Ro 31-2880]
0μM
0.25μM
0.5μM
1μM

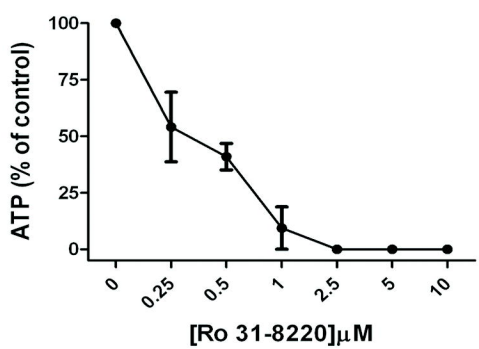
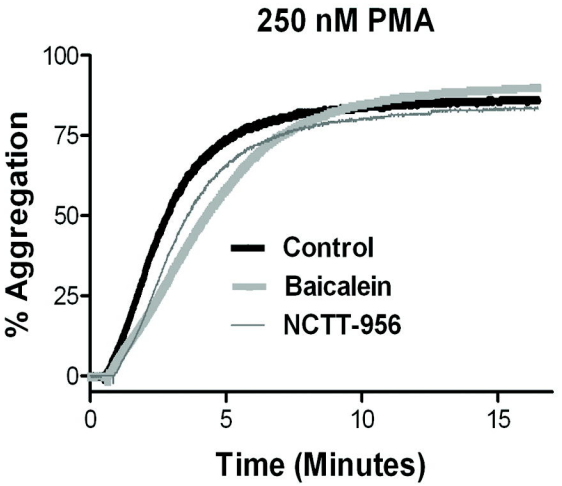
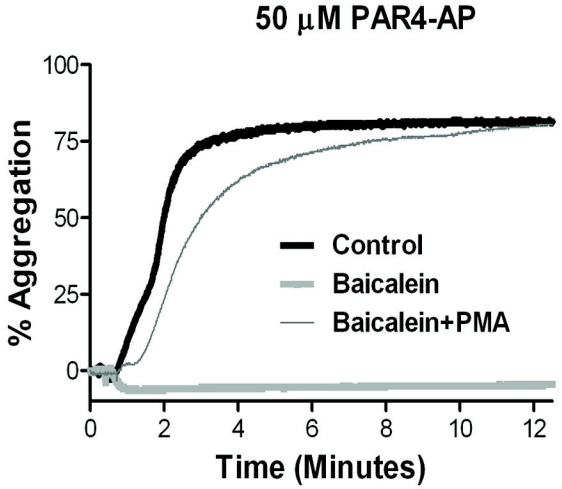

ATP (% of control)
[Ro 31-8220]μM

Figure 4:

a


250 nM PMA
% Aggregation
100
75
50
25
0
0
5
10
15
Time (Minutes)
Control
Baicalein
NCTT-956

b


50 μM PAR4-AP
% Aggregation
100
75
50
25
0
0
2
4
6
8
10
12
Time (Minutes)
Control
Baicalein
Baicalein+PMA

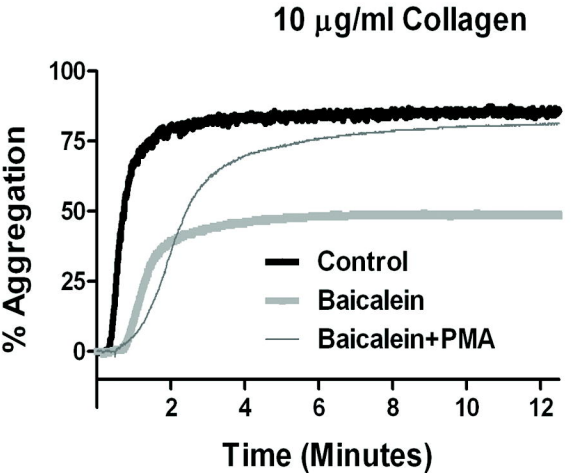
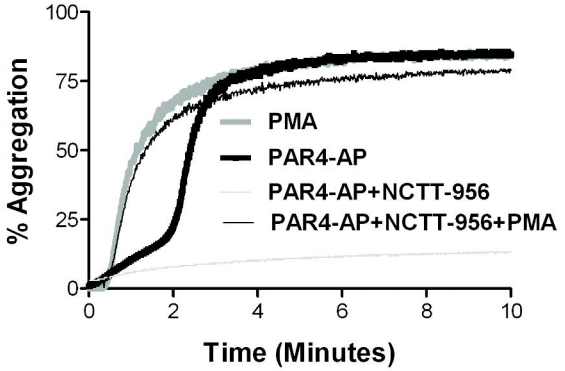
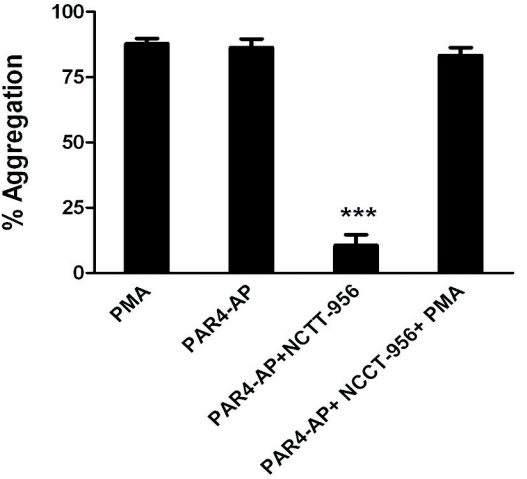
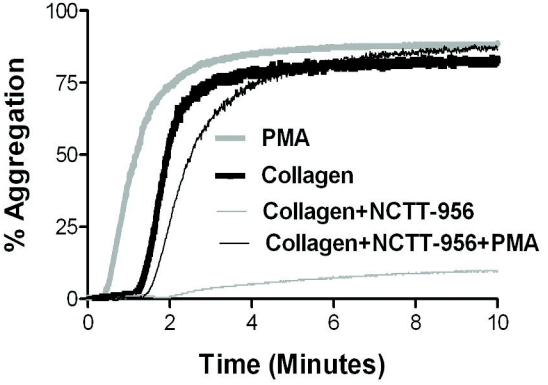

10 μg/ml Collagen
% Aggregation
100
75
50
25
0
2
4
6
8
10
12
Time (Minutes)
Control
Baicalein
Baicalein+PMA

Figure 4 continued:







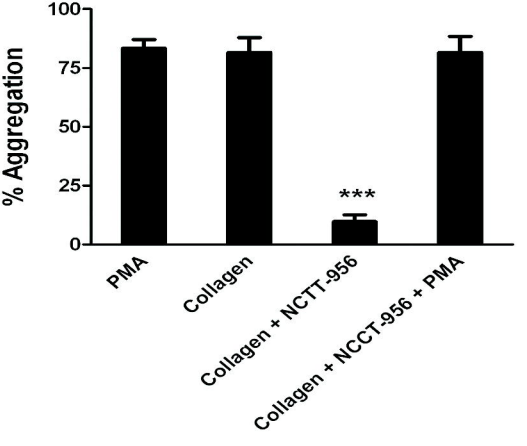


Figure 5:

a

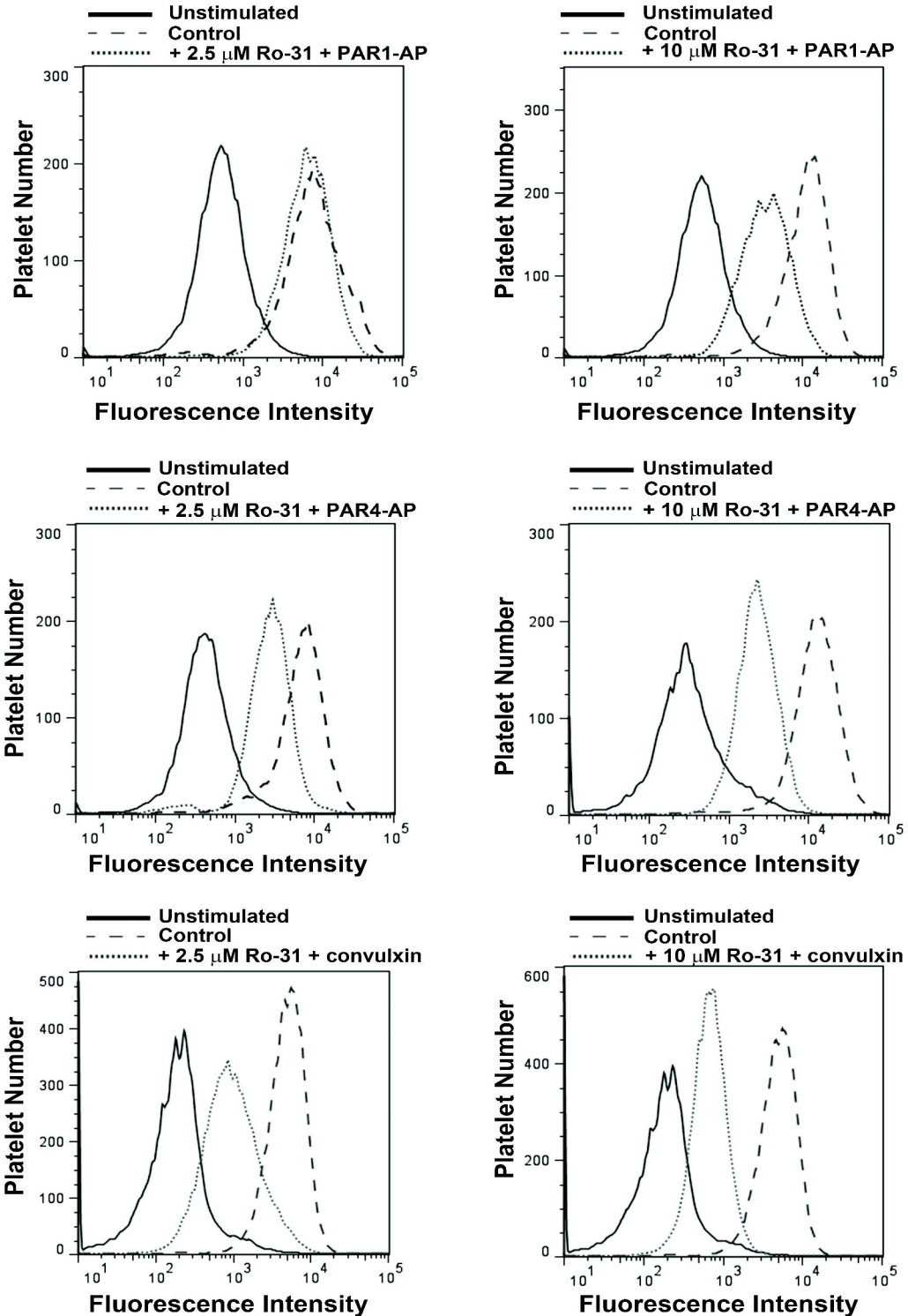

Unstimulated
Control
+ 2.5 μM Ro-31 + PAR1-AP
Platelet Number
Fluorescence Intensity
Unstimulated
Control
+ 10 μM Ro-31 + PAR1-AP
Platelet Number
Fluorescence Intensity
Unstimulated
Control
+ 2.5 μM Ro-31 + PAR4-AP
Platelet Number
Fluorescence Intensity
Unstimulated
Control
+ 10 μM Ro-31 + PAR4-AP
Platelet Number
Fluorescence Intensity
Unstimulated
Control
+ 2.5 μM Ro-31 + convulxin
Platelet Number
Fluorescence Intensity
Unstimulated
Control
+ 10 μM Ro-31 + convulxin
Platelet Number
Fluorescence Intensity

Figure 5 continued:

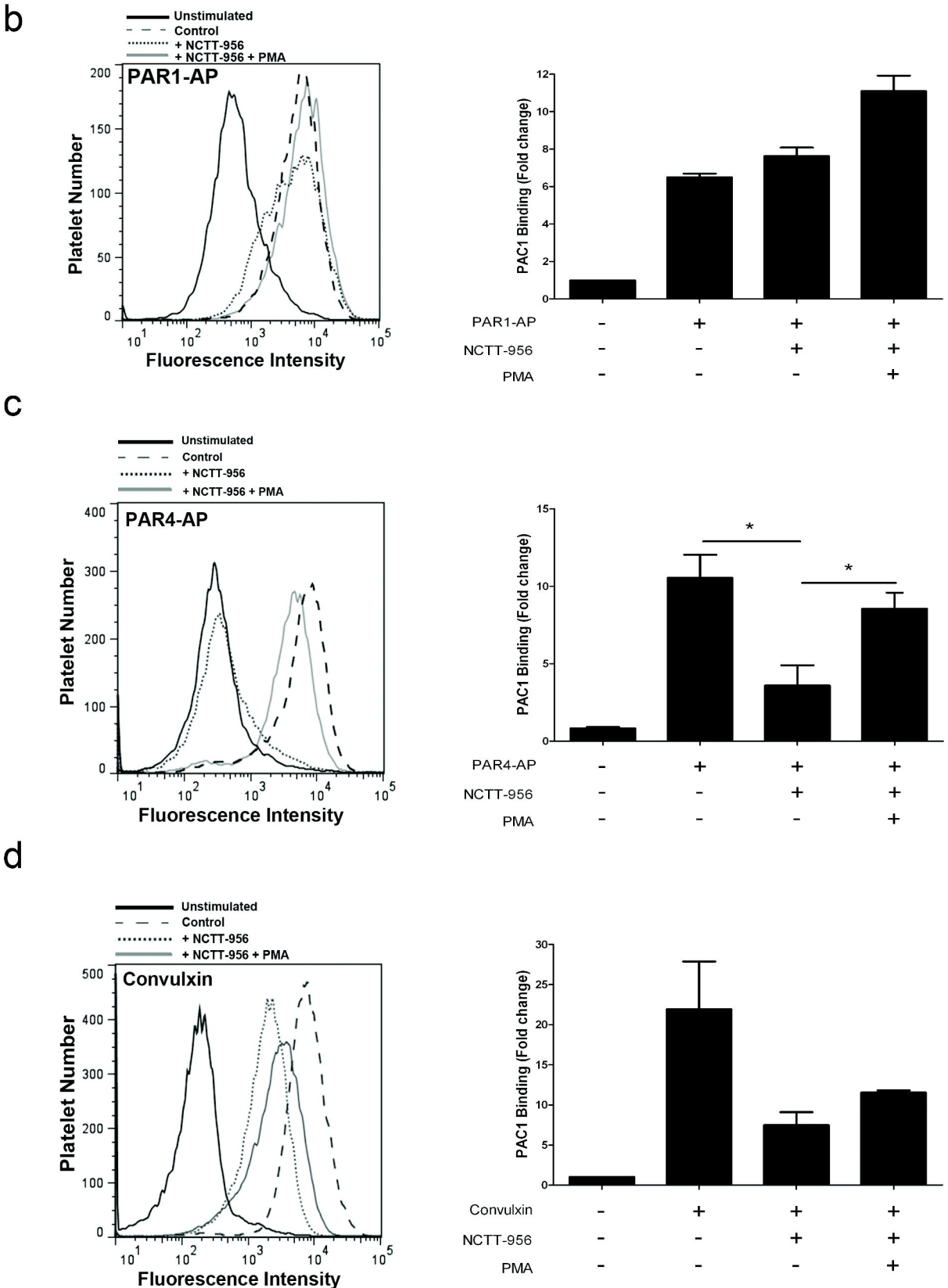


Figure 6:

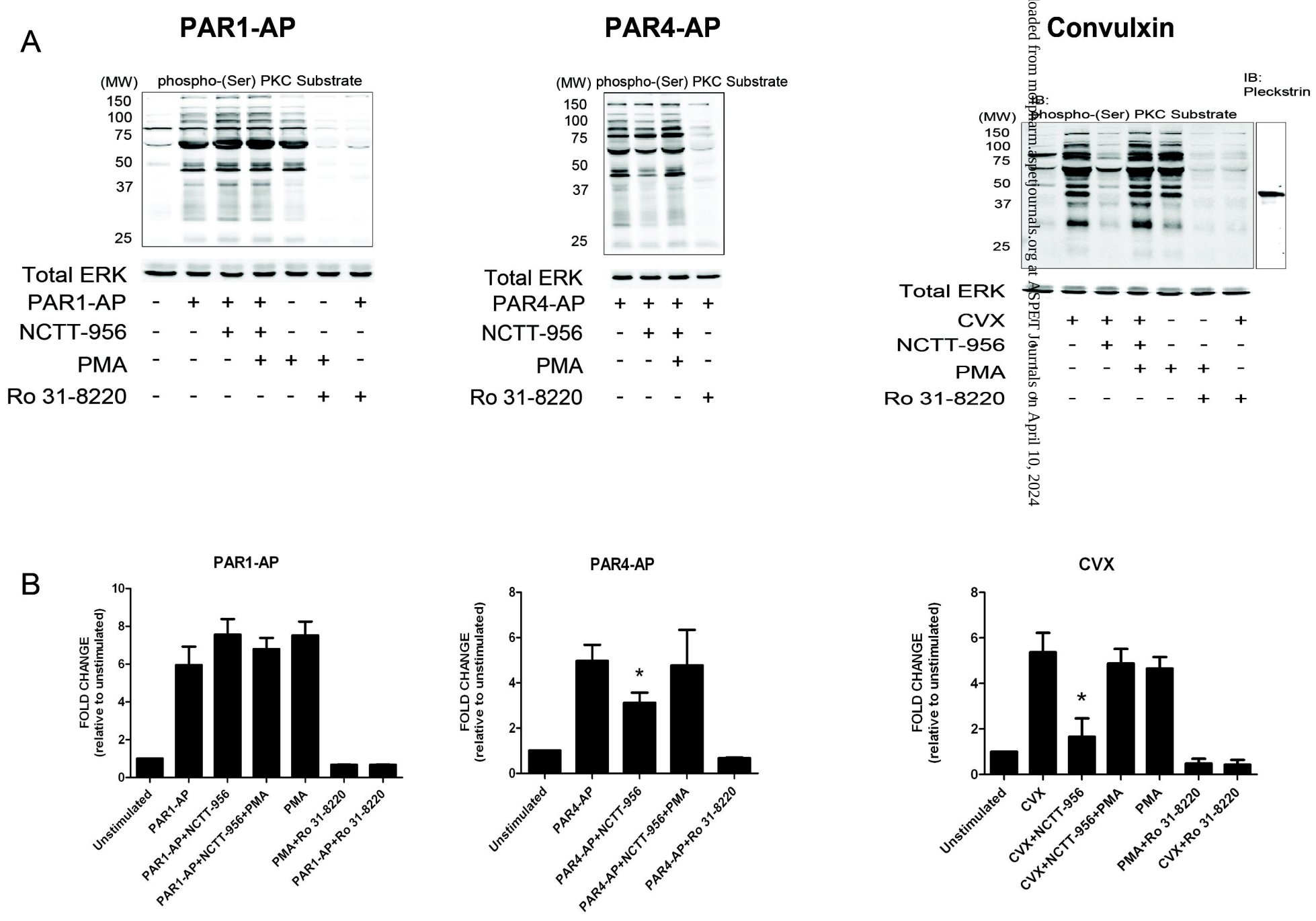
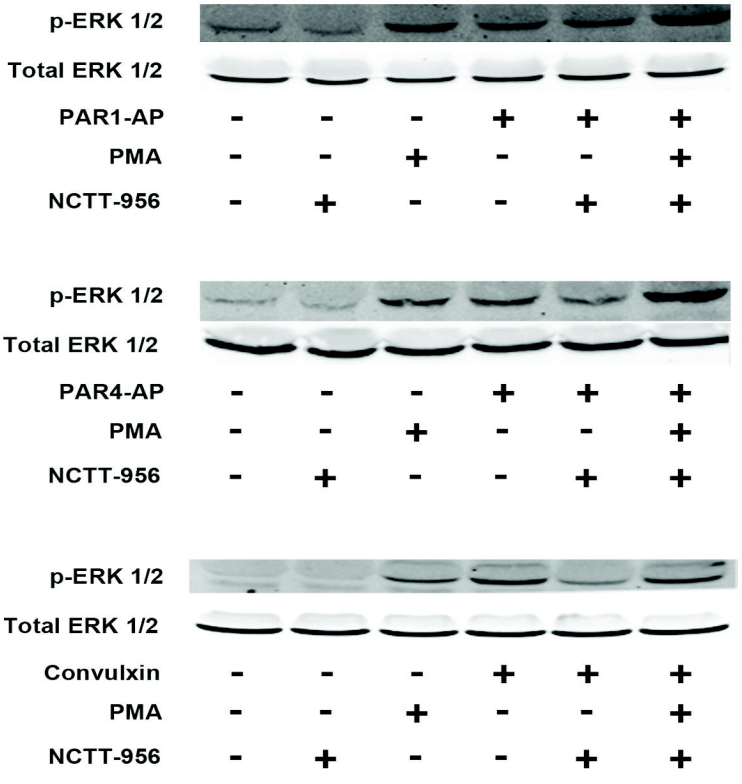
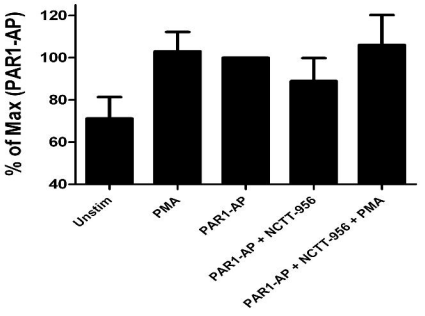
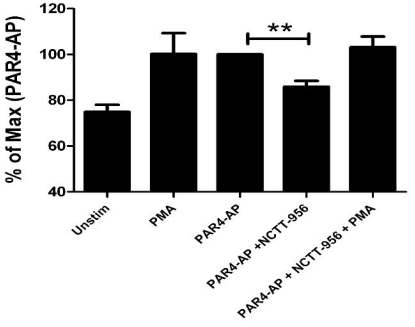


Figure 6c:


p-ERK 1/2
Total ERK 1/2
PAR1-AP - - - + + +
PMA - - + - - +
NCTT-956 - + - - + +
p-ERK 1/2
Total ERK 1/2
PAR4-AP - - - + + +
PMA - - + - - +
NCTT-956 - + - - + +
p-ERK 1/2
Total ERK 1/2
Convulxin - - - + + +
PMA - - + - - +
NCTT-956 - + - - + +


% of Max (PAR1-AP)
120
100
80
60
40
Unstim
PMA
PAR1-AP
PAR1-AP + NCTT-956
PAR1-AP + NCTT-956 + PMA


% of Max (PAR4-AP)
120
100
80
60
40
**
Unstim
PMA
PAR4-AP
PAR4-AP +NCTT-956
PAR4-AP + NCTT-956 + PMA

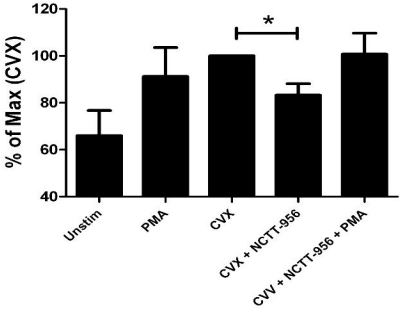
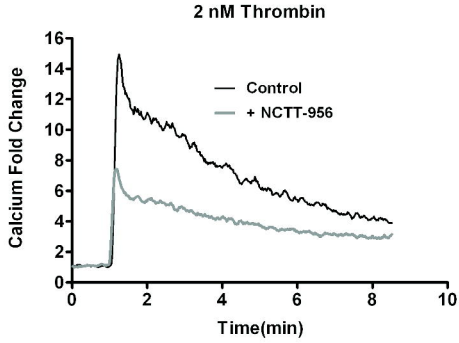
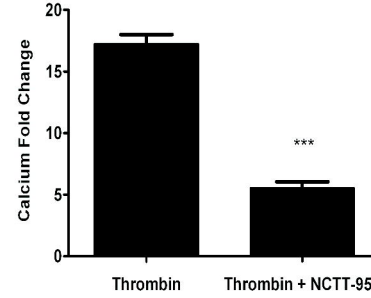
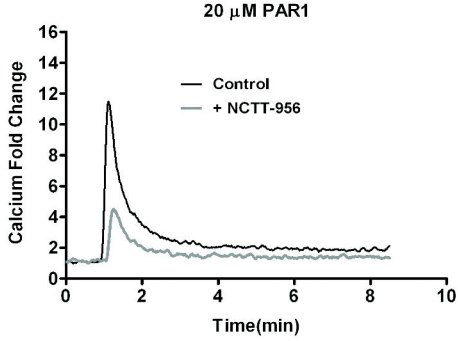

% of Max (CVX)
120
100
80
60
40
*
Unstim
PMA
CVX
CVX + NCTT-956
CVV + NCTT-956 + PMA

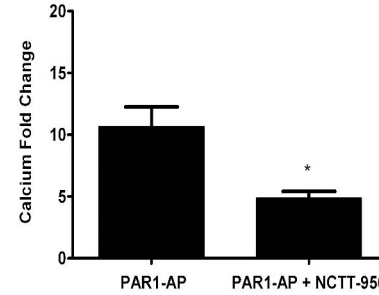
Figure 7:

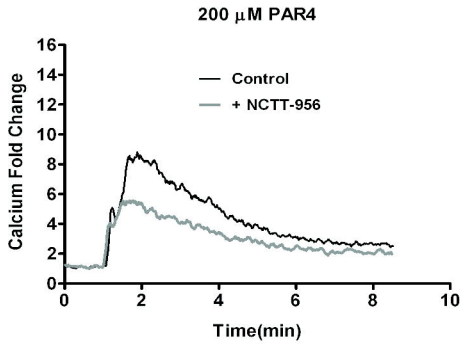
a

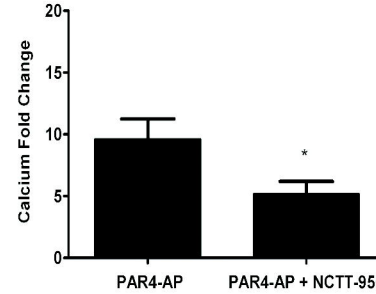

2 nM Thrombin
Control
+ NCTT-956
Calcium Fold Change
Time(min)

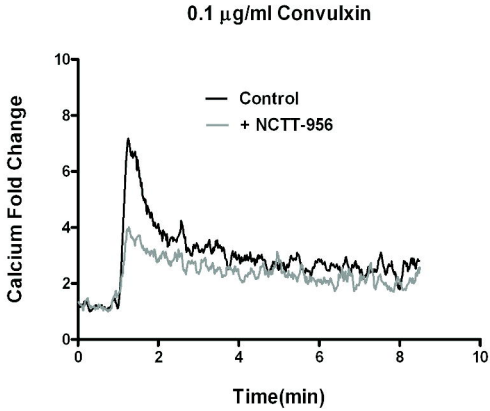

Calcium Fold Change
***
Thrombin
Thrombin + NCTT-956


20 μM PAR1
Control
+ NCTT-956
Calcium Fold Change
Time(min)


Calcium Fold Change
*
PAR1-AP
PAR1-AP + NCTT-956


200 μM PAR4
Control
+ NCTT-956
Calcium Fold Change
Time(min)


Calcium Fold Change
*
PAR4-AP
PAR4-AP + NCTT-956


0.1 μg/ml Convulxin
Control
+ NCTT-956
Calcium Fold Change
Time(min)

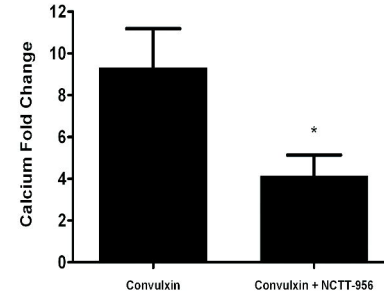

Calcium Fold Change
*
Convulxin
Convulxin + NCTT-956

Figure 7 continued:

b