

**Protease activated receptors differentially regulate human platelet activation through a
phosphatidic acid-dependent pathway**

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Running Title: PA regulation of PAR1 versus PAR4 in platelets

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Abbreviations:

1. Protease Activated Receptor-1 (PAR1)
2. PAR1-activating peptide (PAR1-AP)
3. Protease Activated Receptor-3 (PAR3)
4. Protease Activated Receptor-4 (PAR4)
5. PAR4-activating peptide (PAR4-AP)
6. Diacylglycerol (DAG)
7. Phosphatidylcholine (PC)
8. Phosphatidic Acid (PA)
9. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG)
10. Phorbol 12-myristate 13-acetate (PMA)
11. lipid phosphate phosphatase-1 (LPP-1)
12. Guanine nucleotide exchange factor (GEF)
13. Phospholipase C (PLC)
14. Phospholipase D (PLD)
15. Beta Adrenergic Receptor (β -AR)
16. G Protein Coupled Receptor (GPCR)

Abstract:

Pathological conditions such as coronary artery disease are clinically controlled via therapeutic regulation of platelet activity. Thrombin, through PAR1 and PAR4, plays a central role in regulation of human platelet function as it is known to be the most potent activator of human platelets. Currently, direct thrombin inhibitors used to block platelet activation result in unwanted side effects of excessive bleeding. An alternative therapeutic strategy would be to inhibit PAR-mediated intra-cellular platelet signaling pathways. To elucidate the best target, we are studying differences between the two platelet thrombin receptors, PAR1 and PAR4, in mediating thrombin's action. In this study we show that platelet activation by PAR1-activating peptide (PAR1-AP) requires a phospholipase D (PLD)-mediated phosphatidic acid (PA) signaling pathway. We show that this PAR1-specific PA-mediated effect is not regulated through differential granule secretion following PAR-induced platelet activation. Perturbation of this signaling pathway via inhibition of lipid phosphate phosphatase-1 (LPP-1) by propranolol or inhibition of the phosphatidylcholine (PC)-derived phosphatidic acid (PA) formation by PLD with a primary alcohol significantly attenuated platelet activation by PAR1-AP. Platelet activation by thrombin or PAR4-AP was insensitive to these inhibitors. Further, activation of Rap1 was significantly attenuated by these inhibitors following stimulation by PAR1-AP, but not thrombin or PAR4-AP. Since PA metabolites such as diacylglycerol play an important role in intracellular signaling, identifying crucial differences in PA-regulation of PAR-induced platelet activation may lead to a greater understanding of the role of PAR1 versus PAR4 in progression of thrombosis.

Platelets are regulated through numerous agonist-induced signaling pathways, the most potent of which is thrombin (Davey and Luscher, 1967; Jamieson, 1997). Human platelets express two functional thrombin receptors, protease activated receptor-1 and -4 (PAR1 and PAR4) (Coughlin, 2005; Kahn et al., 1998), whereas mouse platelets express PAR3 and PAR4. Previous work from our lab has indicated that PAR1 and PAR4 mediate platelet activation through distinct signaling pathways (Holinstat et al., 2006). It is still unclear how differential signaling through the thrombin receptors is regulated and what potential physiological consequences result from selective activation of PAR1 and PAR4. Several intermediates within this signaling scheme such as PKC α and the small GTPase Rap1 have been identified to be of some importance to regulating platelet function (Chrzanowska-Wodnicka et al., 2005; Franke et al., 2000). Genetic studies in the mouse have confirmed the role of Rap1b in platelet aggregation and subsequent clot formation (Chrzanowska-Wodnicka et al., 2005). How Rap1 is involved in the initial and irreversible phases of platelet aggregation (Franke et al., 2000), as well as whether Rap1 is differentially regulated in human platelets by PAR1 and PAR4, remains elusive.

In mouse platelets, the guanine nucleotide exchange factor (GEF) CalDAG-GEF1 is thought to regulate the initiation of Rap1 activation (Crittenden et al., 2004). Upon activation by the direct binding of either Ca²⁺ or diacylglycerol (DAG), CalDAG-GEF1 catalyzes the exchange of GTP for GDP on Rap1 (Dupuy et al., 2001; Kawasaki et al., 1998). The manner by which Rap1 is activated may be an important determinant of its downstream signaling effects in platelets. In human platelets, PI phospholipase C (PLC) and calcium mobilization may not be required for Rap1 activation and platelet aggregation (Woulfe et al., 2002). We sought to determine if another mechanism for producing DAG such as the metabolism of PLD-derived phosphatidic acid (PA) plays a role in PAR-mediated Rap1 activation and platelet aggregation.

The role of PA-mediated DAG production in platelets is poorly understood. One mechanism of PA production downstream of thrombin is activation of enzymes such as phospholipase D (PLD) (Ha and Exton, 1993a). Studies using primary alcohols that inhibit the pathway from PC to PA to DAG, as well as propranolol, an inhibitor of lipid phosphate phosphatase-1 (LPP-1) which blocks conversion of PA to DAG together indicate that PA-derived DAG is an important component of thrombin signaling in platelets (Haslam and Coorssen, 1993; Natarajan and Garcia, 1993). How this pathway functions in PAR1 and PAR4 signaling has not been reported. We investigated whether PAR1 and PAR4 require PLD and PA-derived metabolites such as DAG to activate human platelets. Using primary alcohol and propranolol to inhibit the formation of PA-derived metabolites, we found strong evidence that PAR1-AP (but not thrombin or PAR4-AP) signaling in platelet activation requires the PLD pathway. Specifically, our data show this pathway is necessary for PAR1 to maintain Rap1 activation and form stable platelet aggregates. This is the first report of how PAR-induced activation of human platelets may involve production of PA-derived metabolites through unique signaling correlates differentially regulated by PAR1 and PAR4.

Materials and Methods:

Materials: Human α -thrombin (2700 NIH units/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). Activating peptides for PAR1 (PAR1-AP; SFLLRN) and PAR4 (PAR4-AP; AYPGKF) were purchased from GL Biochem (Shanghai, China). 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), Phorbol 12-myristate 13-acetate (PMA), Propranolol, nadolol, 1-butanol and tert-Butanol was purchased from Sigma-Aldrich (Saint Louis, MO). Anti-Rap1 antibody was purchased from Santa Cruz Biotechnology (SanDiego, CA). Blocking buffer and

anti-rabbit IRDYE 800 antibody were purchased from LI-COR Biosciences (Lincoln, NE). Fluorescein isothiocyanate (FITC) conjugated anti-PAC1, phycoerythrin (PE) conjugated anti-P-selectin and FITC conjugated anti-CD63 antibodies were purchased from BD Pharmingen (San Jose, CA). Ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), o-Phthalaldehyde (OPT) and chloroform were purchased from Fisher Scientific. Aggregometers, Chrono-Lume reagent, and other aggregation supplies were purchased from Chrono-Log Co. (Havertown, PA).

Human Platelets: Human platelets were obtained from healthy volunteers from within the Vanderbilt University community. These studies were approved by the Vanderbilt University Institutional Review Board. Informed consent was obtained from all individuals prior to platelet donation. Blood was centrifuged at 170 X g for 15 minutes at room temperature. Platelet rich plasma (PRP) 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 800 X g for 10 minutes at room temperature. Platelets were resuspended in Tyrodes buffer (12 mM NaHCO₃, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM glucose, 10 mM HEPES), and the final platelet concentration was adjusted as indicated after counting using a Coulter counter. Reported results are the data obtained using platelets from at least three different subjects. Unless otherwise noted, all experiments were conducted with 10 nM thrombin, 20 μM PAR1-AP or 200 μM PAR4-AP. Inhibitors were used at a concentration of 100 μM propranolol, 100 μM nadolol, 0.4% 1-butanol and 0.4% tert-butanol unless otherwise specified.

Platelet Aggregation: Washed platelets were adjusted to a final concentration of 3 x 10⁸ platelets/ml. Where indicated platelets were pre-treated with propranolol or nadolol for 10

minutes or 1-butanol or tert-butanol for 3 minutes. The aggregation response to thrombin, PAR1-AP, or PAR4-AP, was measured using an aggregometer with stirring at 1,000 rpm at 37 °C.

Measurement of RAP1 Activity: Rap1 activity was measured using GST-RalGDS-Rap1-binding domain (RalGDS-RBD) that specifically interacts with activated Rap1 as described elsewhere (Holinstat et al., 2006). Briefly, 250 μ l of 3×10^8 platelets/ml in Tyrodes buffer were pre-treated with propranolol or nadolol for 10 minutes or 1-butanol or tert-butanol for 3 minutes. Following stimulation of platelets with thrombin, PAR1-AP, or PAR4-AP, for the time indicated, platelets were lysed with an equal volume of 2x lysis buffer (100 mM Tris-HCl pH 7.4, 2% Triton X-100, 150 mM NaCl, 2% IGEPAL, 1% sodium deoxycholate, .05% SDS, 2 mM Na_3VO_4 , 2 mM PMSF, 2 μ g/ml Leupeptin, 2 μ g/ml Aprotinin) and centrifuged at $10,621 \times g$ for 7 minutes. RalGDS-RBD was prepared by coupling 0.3 ml of GST-Sepharose 4B with 5 ml bacterial lysates containing RalGDS-RBD construct (van Triest et al., 2001) for 1.5 hours at 4°C. The sepharose was then washed twice with lysis buffer and 30 μ l/tube was aliquoted into each 1.5 ml centrifuge tube. Platelet lysate was incubated with the sepharose coupled RalGDS-RBD for 1 hour at 4°C. The sepharose was washed twice in lysis buffer, boiled in 1x Laemmli buffer for 10 minutes, and separated by SDS-PAGE. Activated Rap1 was detected by immunoblotting with the anti-Rap1 antibody. In parallel experiments using whole platelet lysate, Rap1 expression was analyzed to confirm equal protein loading for each sample.

Dense-granule Secretion: ATP and serotonin release were used to detect dense granule secretion. For ATP studies, 270 μ l aliquots of washed platelets adjusted to a final concentration of 2.3×10^8 platelets/ml were pre-treated with propranolol or nadolol for 10 minutes. Following addition of 30 μ l Chrono-lume reagent, ATP release in response to thrombin, PAR1-AP, or PAR4-AP,

was measured using a Lumi-aggregometer at 37 °C with stirring at 1,000 rpm. For serotonin studies, 500 μ l aliquots of washed platelets adjusted to a final concentration of 1.5×10^8 platelets/ml were pre-treated with propranolol for 10 minutes. While stirring at 1,000 rpm, samples were stimulated for 5 minutes at 37 °C with thrombin, PAR1-AP or PAR4-AP. Reactions were terminated by addition of 50 μ l of 0.5M EDTA and placing the samples on ice. Serotonin was extracted from a 300 μ l aliquot of the sample using 60 μ l of 6.0 M TCA. Following centrifugation at 12,000 x rpm on an Eppendorf 5417C centrifuge for 2 minutes at room temperature, a 250 μ l aliquot of the TCA extract was transferred to a tube containing 1 ml of 0.05% OPT. Samples were boiled for 10 minutes, cooled on ice, and then washed twice with chloroform. Serotonin release was measured using a Varian fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 475 nm.

Flow cytometry: Flow cytometry was used to measure the secretion of α and lysosomal granules and the activation of glycoprotein (GP) IIbIIIa. Specifically, P-selectin expression was used to detect α granule secretion, CD63 expression was used to detect lysosomal (and dense) granule secretion, and PAC1 binding was used to selectively detect the conformational activation of GPIIbIIIa (Shattil et al., 1987). For these experiments, 50 μ l aliquots of washed platelets adjusted to a final concentration of 2×10^7 platelets/ml were pre-treated with propranolol or nadolol for 10 minutes. Following addition of 20 μ l of the indicated antibody, platelets were stimulated with thrombin, PAR1-AP, or PAR4-AP for 15 minutes and then diluted to a final volume of 500 μ l using Tyrodes buffer. The fluorescence intensity of 10,000 platelets was immediately measured using a flow cytometer.

Statistical Analysis: Comparison between experimental groups was made using a paired t-test program using Prism software. Differences in mean values were considered significant at $p < 0.05$.

Results:

LPP-1 regulates PAR1-AP stimulated platelet aggregation and Rap1 activation: DAG has been reported to play a role in thrombin stimulated platelet aggregation (Franke et al., 2000; Werner and Hannun, 1991). We sought to determine the dependence of PAR1 and PAR4 on LPP-1-mediated DAG production. Platelets were pre-treated with either propranolol, a β -AR antagonist known to inhibit LPP-1 (Fukami and Takenawa, 1992; Kanaho et al., 1991; Kim et al., 2004), or nadolol, a chemically similar β -AR antagonist without effects on LPP-1 signaling. We first measured the dose-dependent effects of propranolol and nadolol on aggregation stimulated by thrombin, PAR1-AP and PAR4-AP (Figure 1A) to determine if LPP-1 or inhibition of the β -AR plays a role in PAR signaling. Nadolol had no effect on platelet aggregation at any concentration tested, indicating that the effects of propranolol are not through the β -AR. Propranolol, however, differentially inhibited PAR stimulated aggregation. Propranolol inhibited PAR1-AP-mediated aggregation at all concentrations (EC_{50} for thrombin, PAR1-AP and PAR4-AP are 290 μ M, 250 μ M and 52 μ M, respectively following propranolol pre-treatment). Thus, there is a differential effect of propranolol on signaling through PAR1. To confirm that pre-treatment with nadolol or propranolol did not alter platelet function, we measured platelet aggregation following pre-treatment with each drug for 10 minutes in the absence of agonist stimulation. No noticeable change in platelet aggregation was observed at any concentration of nadolol or propranolol tested (supplemental data). To confirm that the observed inhibition of PAR1-AP-, but not PAR4-AP- or thrombin-induced platelet aggregation was not due to a lower level of platelet aggregation by

PAR1-AP versus PAR4-AP, platelets were treated with or without 100 μ M propranolol followed by stimulation with increasing concentrations of thrombin, PAR1-AP and PAR4-AP (Figure 1B). As shown in our previous work (Holinstat et al., 2006), we confirm here that 20 μ M PAR1-AP has an equivalent effect on platelet aggregation to 200 μ M PAR4-AP (both are well above the EC_{100} for platelet aggregation) and all concentrations of PAR1-AP are attenuated by propranolol whereas there was no effect on thrombin- or PAR4-AP-induced platelet aggregation at any recorded concentration. Hereafter, thrombin, PAR1-AP and PAR4-AP will be used at the concentrations tested in figure 1B (10 nM thrombin, 20 μ M PAR1-AP and 200 μ M PAR4-AP). In PAR1-AP-stimulated aggregation, propranolol treatment (100 μ M) decreased both maximal aggregation ($88.7 \pm 2.96\%$ for PAR1-AP alone and $37.25 \pm 2.94\%$ for combination) and platelet aggregate stability (decreased to $17.6 \pm 10.2\%$ after 6 minutes). This propranolol-dependent effect on platelet aggregation was not observed in the thrombin or PAR4-AP conditions. (Figure 2A-D).

The small GTPase Rap1 is known to be involved in GPCR as well as integrin-mediated platelet activation (Woulfe et al., 2002). The temporal response of Rap1 activity was measured to determine its relationship to LPP-1 (Figure 3). Treatment of platelets with either PAR1-AP or PAR4-AP alone stimulates a sustained activation of Rap1 for at least 15 minutes (Figure 3A and C). Consistent with the aggregation data, propranolol differentially altered the temporal profile of Rap1 activity. Whereas sustained activation of Rap1 by PAR4-AP was not affected by propranolol (Figure 3C and D), PAR1-AP stimulated Rap1 was activated more transiently in platelets in the presence of propranolol, compared to control, returning to the basal level within 10 minutes (Figure 3A and B).

DAG formation via PLD-mediated PA production is disrupted by primary alcohols (e.g., 1-butanol), but not tertiary alcohols (e.g., tert-butanol) (Kiss, 1996). Evidence has been previously presented indicating thrombin can signal through this pathway in platelets (Rubin, 1988) and therefore it is likely that PARs would also have the potential to signal DAG formation through the metabolism of PC. To verify that the effects of propranolol are due to inhibiting the LPP-1-regulated PA metabolism to DAG, we measured the effects of 1-butanol and tert-butanol on aggregation (Figure 4) and Rap1 activation (Figure 5). Consistent with the effects of propranolol, 1-butanol selectively impaired PAR1-AP stimulation of platelets. 1-Butanol attenuated PAR1-AP stimulated platelet aggregation and caused the aggregate to become unstable within minutes (Figure 4C and D), but had no effect on aggregation stimulated by thrombin or PAR4-AP (Figure 4A, B, and D). Likewise, Rap1 activation by PAR1-AP became transient in the presence of 1-butanol (Figure 5A), but there was no effect on the sustained Rap1 activation by PAR4-AP (Figure 5B). Importantly, tert-butanol had no effect on aggregation (Figure 4) or Rap1 activation (Figure 5). Thus, Figures 1-5 indicate a PAR1-specific signaling pathway that appears to be requisite for the second phase of irreversible platelet activation. More importantly, PAR4-AP and thrombin (which activates both PAR1 and PAR4) are insensitive to perturbation of this pathway.

PA metabolites regulate GPIIbIIIa activation: Stability of the platelet plug is partly determined by the activation of integrins on the surface of platelets, and we hypothesized that, by blocking LPP-1, propranolol may interfere with integrin activation. In particular, glycoprotein $\alpha_{IIb}\beta_3$ (GPIIbIIIa) undergoes a conformational change upon activation thereby increasing its affinity for fibrinogen and von Willebrand factor. The antibody PAC1 specifically interacts with the conformationally activated form of GPIIbIIIa and its binding can be measured by flow

cytometry. Independent of the mode of activation with thrombin, PAR1-AP or PAR4-AP, propranolol treatment of platelets caused an inhibition of active GPIIbIIIa as compared to the control (no pre-treatment) or nadolol treated conditions (Figure 6).

PA-mediated signaling selectively regulates lysosomal and α -granule secretion: Both PAR1 and PAR4 induce secretion of dense, α and lysosomal granules from human platelets (Rendu and Brohard-Bohn, 2001). We wanted to determine if the observed effects of propranolol and 1-butanol were due to a differential PAR-specific effect on granule secretion. Platelets were pre-treated with either propranolol or nadolol followed by stimulation with thrombin, PAR1-AP or PAR4-AP and FACS analysis of P-selectin was performed. Treatment with propranolol, but not nadolol, inhibited surface expression of P-selectin (Figure 7A) indicating that propranolol inhibits α granule secretion irrespective of the mechanism by which platelets were activated. Surface expression of CD63 was then measured in platelets stimulated with PAR1-AP or PAR4-AP following treatment with or without propranolol or nadolol (Figure 7B). Similar to P-selectin, CD63 expression was significantly inhibited following propranolol, but not nadolol, indicating that secretion of lysosomal granules are also inhibited in a propranolol-dependent manner.

To determine the effect of propranolol on dense granule secretion, serotonin and ATP (constituents of the dense granule) were measured in the platelet buffer following treatment with propranolol or nadolol and stimulation with thrombin, PAR1-AP or PAR4-AP (Figure 8). ATP release was measured from washed platelets treated with or without propranolol using a lumi-aggregometer (Figure 8A). No significant differences were observed in ATP release under any of the conditions tested. Serotonin secretion from washed platelets was also measured under the same conditions as a confirmation that dense granule secretion is not affected by treatment with

propranolol (Figure 8B). Again, propranolol did not significantly inhibit serotonin secretion following thrombin, PAR1-AP, or PAR4-AP stimulation.

PKC-dependent platelet activation is sensitive to propranolol: PKCs are known to be involved in platelet secretion (Haslam and Coorsen, 1993; Ma et al., 2005). Based on the results in figures 7 and 8, it is possible that PKC regulation of platelet function is upstream of PLD and PA-derived DAG production (resulting in both Rap1 activation and aggregation) (Henage et al., 2006). DAG can be activated downstream of PKC through regulation of enzymes such as PLD (Chen and Exton, 2004; Hu and Exton, 2003). To test this possibility, we used two DAG analogs known to act as direct activators of PKC, OAG and PMA. As shown in Figure 9A-B, OAG and PMA both induce aggregation, and this was blocked by propranolol. Pre-treatment with 100 μ M nadolol had negligible effects on PMA or OAG-mediated platelet aggregation (data not shown). Additionally, propranolol was able to fully inhibit OAG and PMA-induced Rap1 activation at 1 and 5 minutes following stimulation, indicating Rap1 activation downstream of these agonists (and possibly PKC) is dependent on LPP-1.

Discussion:

The mechanisms by which PARs signal platelet activation have not been well defined. Although there is evidence that PAR1 and PAR4 signaling pathways are not redundant (Holinstat et al., 2006; Kahn et al., 1998; Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005), delineating the specific signaling correlates downstream of each receptor has proven to be problematic. Some work in the field is beginning to elucidate the signaling differences inherent in PAR1- versus PAR4-mediated activation human platelets (Holinstat et al., 2006; Ma et al., 2005). In this study, we show that platelet aggregation by PAR1-AP, but not thrombin or PAR4-AP, is partially

dependent on signaling through PLD and PA metabolites. Inhibition of this pathway attenuated PAR1-AP-induced platelet aggregation. Additionally, the sustained activation of Rap1 by PAR1-AP may require a PA-derived DAG signal since Rap1 activation becomes transient when either PC-derived PA production or LPP-1-mediated metabolism of PA to DAG is blocked. Differences in PAR signaling are highlighted by the observation that inhibition of the PA-mediated pathway has negligible effects on either thrombin or PAR4-AP-mediated platelet aggregation and Rap1 activation. To further elucidate the signaling pathways responsible for PAR1 versus PAR4 regulation of DAG and subsequent platelet activation, our lab is currently investigating potential differences in DAG species formed following PAR1-AP or PAR4-AP stimulation in human platelets. Finally, we present evidence suggesting that PKC activation of PLD may play a central role in this signaling cascade (Figure 9) since propranolol fully inhibits platelet aggregation and Rap1 activation induced by the PKC activators OAG and PMA. Thus, these data provide compelling evidence that PAR1 and PAR4 signal through distinct pathways, which differentially regulate platelet activity through a PAR1-required PA-derived signaling pathway.

Thrombin receptors are known to signal to several lipases including PLC and PLD (McKenzie et al., 1992). Work in the field indicates that although PLC activation is important in integrin-mediated Rap1 activation (Bernardi et al., 2006), its role in thrombin-induced platelet aggregation and Rap1 activation is less well understood. Thrombin-mediated PLD activation in platelets has also been observed, although the specific role of PLD in platelet activation is unknown (Haslam and Coorssen, 1993; Martinson et al., 1995). The importance of a potential PA-derived DAG signaling pathway has been indicated in numerous cell types (Exton, 2002). For this reason, studies focusing on the identification of which phospholipases are involved in

PAR-mediated platelet activation are necessary. To investigate the PA signaling pathway, we used 1-butanol to inhibit PC metabolism to PA and DAG and propranolol to block the LPP-1 dependent metabolism of PA to DAG (Pappu and Hauser, 1983; Walter et al., 1996). Propranolol attenuated platelet activation specifically through PAR1. The platelet aggregates that did form were unstable and disaggregated within 5 minutes of stimulation (Figure 1). This effect was limited to PAR1-AP, supporting our hypothesis that LPP-1 metabolism of PA to DAG is crucial for PAR1 to induce irreversible platelet aggregation. Interestingly, propranolol had no effect on the secretion of dense granules, yet it blocked the secretion of α and lysosomal granules as well as the activation of GPIIb/IIIa. These findings suggest that α and lysosomal granule secretion are not required for PAR-dependent platelet activation, particularly since thrombin and PAR4-AP were able to form stable platelet aggregates in the absence of P-selectin or CD63 expression. Though PLD metabolism of PC to PA and DAG influence secretion, effects on secretion are unable to explain the differential effects of PAR1 versus PAR4 signaling. Others have indicated that ADP released from the dense granule is important for augmenting a second phase of platelet activation (Falker et al., 2004; Hechler et al., 2005). Our data are consistent with the hypothesis that PAR4 signals in cooperation with ADP, and PAR1 signaling does not require ADP (Holinstat et al., 2006).

PKC α , which is known to be activated downstream of thrombin in human platelets, is involved in cytoskeletal rearrangement, vesicle secretion, and PLD activation (Brass, 2003; Reed et al., 1999). As shown in Figure 9, platelet aggregation and Rap1 activation by two DAG analogs known to directly activate the PKC pathway (Cardoso et al., 1997; Ha and Exton, 1993b) were inhibited by propranolol, suggesting that PKC may be interposed between PAR1 and PA production. PKC's potent activation of PLD is well known, in synergy with small GTPases such

as Rho and Arf, and Ca^{2+} (Henage et al., 2006; Preininger et al., 2006). Ongoing studies focusing on the identification of DAG species produced downstream of PAR1 and PAR4 signaling will enable us to further elucidate the mechanisms by which each receptor triggers platelet activation.

Rap1 is known to be integral in platelet signaling, having been shown to be activated by receptors for thrombin, ADP, integrins, and others (Bernardi et al., 2006; Bos, 2005; Holinstat et al., 2006). In platelets, Rap1 is shown to be activated by CalDAG-GEF1 (Crittenden et al., 2004). Since CalDAG-GEF1 may be activated by direct binding of calcium or DAG, or perhaps, by some other undetermined mechanism, we investigated how PAR1 and PAR4 differentially regulate Rap1 activation. Whereas chelation of $[\text{Ca}^{2+}]_i$ has no effect on Rap1 activation by PAR1-AP or PAR4-AP (Holinstat et al., 2006), inhibition of the PA pathway selectively attenuates Rap1 activation by PAR1-AP and renders it transient (Figures 3 and 5). Since PKC can regulate PLD and subsequently the formation of DAG, PKC could regulate Rap1 activation through CalDAG-GEF-1. Additionally, the instability of PAR1-AP stimulated platelet aggregates associated with inhibiting PA and DAG formation is temporally paralleled by a transient Rap1 activation, suggesting that prolonged Rap1 activation is important for forming stable platelet aggregates. Taken together, these findings reveal the possibility that PAR1 requires PKC and PA-derived DAG to cause a sustained Rap1 activation that is necessary for stable platelet aggregation. This hypothesis is consistent with an earlier finding that PKC functions in the second phase of Rap1 mediated platelet activation (Franke et al., 2000). Similar to that study, the initial activation of Rap1 by PAR1-AP is not affected by either 1-butanol or propranolol (or in the case of that study, initial Rap1 activity was independent of PKC inhibition, whereas prolonged Rap1 activation was dependent on PKC activity), indicating that multiple mechanisms for activating Rap1 exist in the human platelet. The connection of PAR1 to Rap1

through PKC and PA-derived DAG likely represents a PAR1-specific mechanism for sustained activation of Rap1.

Activation of the human platelet is known to involve a complex signaling scheme initiated by activation of receptors for thrombin, ADP, or integrins (Macfarlane et al., 2001; Offermanns, 2000; Siess, 1989). Clinically, therapeutic approaches to thrombosis involve inhibiting thrombin with direct thrombin inhibitors (DTI), and blocking ADP as well as integrin receptors on the platelet surface. Our data indicate that it is possible to modulate the signaling through one thrombin receptor without disrupting the signaling through the other receptor. Hence, attenuation of Rap1 activity through inhibition of a specific PA-derived DAG pathway, for example, may be more desirable than inhibiting thrombin (which activates both PAR1 and PAR4 signaling in the platelet) and lead to a lower risk of bleeding relative to what is currently observed following administration of DTIs.

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Footnotes:

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Figure Legends

Figure 1: Propranolol regulation of PAR-mediated aggregation. Washed human platelets were treated with A) increasing concentrations of propranolol or nadolol (inset) for 10 minutes followed by stimulation with 10 nM thrombin, 20 μ M PAR1-AP, or 200 μ M PAR4-AP for 10 minutes and the maximal aggregation was recorded. B) Platelets were pre-treated with or without 100 μ M propranolol for 10 minutes followed by stimulation with increasing concentrations of each agonist. At all concentrations tested (thrombin-2, 5, and 10 nM; PAR1-AP-5, 10, and 20 μ M; PAR4-AP-100, 150, and 200 μ M, respectively) PAR1-AP was the only agonist significantly affected by propranolol. Each point on the curve is representative of the average of at least 3 independent experiments \pm S.E.M. *, $P < 0.05$; ***, $P < 0.0001$.

Figure 2: Propranol effects on PAR-regulated primary and secondary platelet aggregation. Washed human platelets were treated with or without 100 μ M propranolol or 100 μ M nadolol for 10 minutes followed by stimulation with A) 10 nM thrombin, B) 20 μ M PAR1-AP or C) 200 μ M PAR4-AP. D) Maximal aggregation was measured in response to agonist stimulation. Each point on the curve is representative of the average of at least 3 independent experiments \pm S.E.M. *, $P < 0.05$.

Figure 3: Propranolol regulation of Rap1 activity. A) Washed platelets were pre-treated with or without 100 μ M propranolol followed by stimulation for various times with 20 μ M PAR1-AP. Active Rap1 (Rap1-GTP) was measured using a Rap1 activation assay (N=3). B) Change in PAR1-AP-mediated Rap1 activation following propranolol treatment (*; $P < 0.05$; N=3). C) Washed platelets were pre-treated with or without 100 μ M propranolol followed by stimulation for various times with 200 μ M PAR4-AP (N=3). D) Change in PAR4-AP-mediated Rap1 activation following propranolol treatment (*; $P < 0.05$; N=3).

Figure 4: 1-Butanol inhibits PAR1-mediated aggregation. Washed human platelets were pre-treated with or without 0.4% 1-Butanol or 0.4% tert-butanol for 4 minutes followed by stimulation with A) 10 nM thrombin, B) 200 μ M PAR4-AP or C) 20 μ M PAR1-AP. D) Maximal aggregation was measured in

response to agonist stimulation. Each point on the curve is representative of the average of at least 3 independent experiments \pm S.E.M.

Figure 5: 1-Butanol attenuates Rap1 activation. Washed platelets were pre-treated with or without 0.4% 1-Butanol or 0.4% tert-butanol for 4 minutes. A) Following pre-treatment, platelets were stimulated with 20 μ M PAR1-AP for various times (0 to 20 minutes). Top panel indicates Rap1 activation without pre-treatment, middle panel shows Rap1 activation following pre-treatment with 1-Butanol and bottom panel shows Rap1 activation after pre-treatment with tert-butanol. Active Rap1 (Rap1-GTP) was measured using a Rap1 activation assay (N=3). Bar graph indicates the fold change in Rap1 activation compared to the unstimulated condition (Average \pm S.E.M.). B) Following pre-treatment, platelets were stimulated with 200 μ M PAR4-AP for various times (0 to 20 minutes). Top panel indicates Rap1 activation without pre-treatment, middle panel shows Rap1 activation following pre-treatment with 1-Butanol and bottom panel shows Rap1 activation after pre-treatment with tert-butanol. Active Rap1 (Rap1-GTP) was measured using a Rap1 activation assay (N=3). Bar graph indicates the fold change in Rap1 activation compared to the unstimulated condition (Average \pm S.E.M.).

Figure 6: Propranolol inhibits PAR-mediated activation of GPIIbIIIa. GPIIbIIIa activation was measured through FACS analysis using FITC conjugated anti-PAC1, which only recognizes the active form of GPIIbIIIa (N=3). Platelets were pre-treated with or without 100 μ M nadolol or 100 μ M propranolol followed by stimulation with either 10 nM thrombin, 20 μ M PAR1-AP or 200 μ M PAR4-AP.

Figure 7: Propranolol inhibits PAR-mediated α and lysosomal-granule secretion. Platelets were pre-treated with or without 100 μ M nadolol or 100 μ M propranolol followed by stimulation with either 10 nM thrombin, 20 μ M PAR1-AP or 200 μ M PAR4-AP. A) P-selectin surface expression was measured through FACS analysis using PE conjugated anti-P-selectin (N=3). B) CD63 surface expression was measured through FACS analysis using FITC conjugated anti-CD63 (*; $P < 0.05$; N=3).

Figure 8: Propranolol does not inhibit PAR-mediated dense-granule secretion. A) Washed platelets were pre-treated with or without nadolol or propranolol followed by stimulation with either 10 nM thrombin, PAR1-AP or PAR4-AP. ATP secretion was quantitatively measured with a lumi-aggregometer (N=3). B) Washed platelets were pre-treated with or without propranolol followed by stimulation with either thrombin, PAR1-AP or PAR4-AP. Serotonin secretion was fluorometrically measured with OPT following stimulation (N=3).

Figure 9: Propranolol regulates PKC-mediated platelet activation. A-B) Washed human platelets were pre-treated with or without 100 μ M propranolol for 10 minutes followed by stimulation with A) 60 μ M OAG or B) 30 nM PMA. C) Maximal platelet aggregation was measured in response to agonist stimulation. ***; $P < 0.0001$; each condition is representative of the average of at least 3 independent experiments \pm S.E.M. D) Washed platelets were pre-treated with or without 100 μ M propranolol followed by stimulation for 1 or 5 minutes with OAG or PMA. Active Rap1 (Rap1-GTP) was measured using a Rap1 activation assay (N=3).

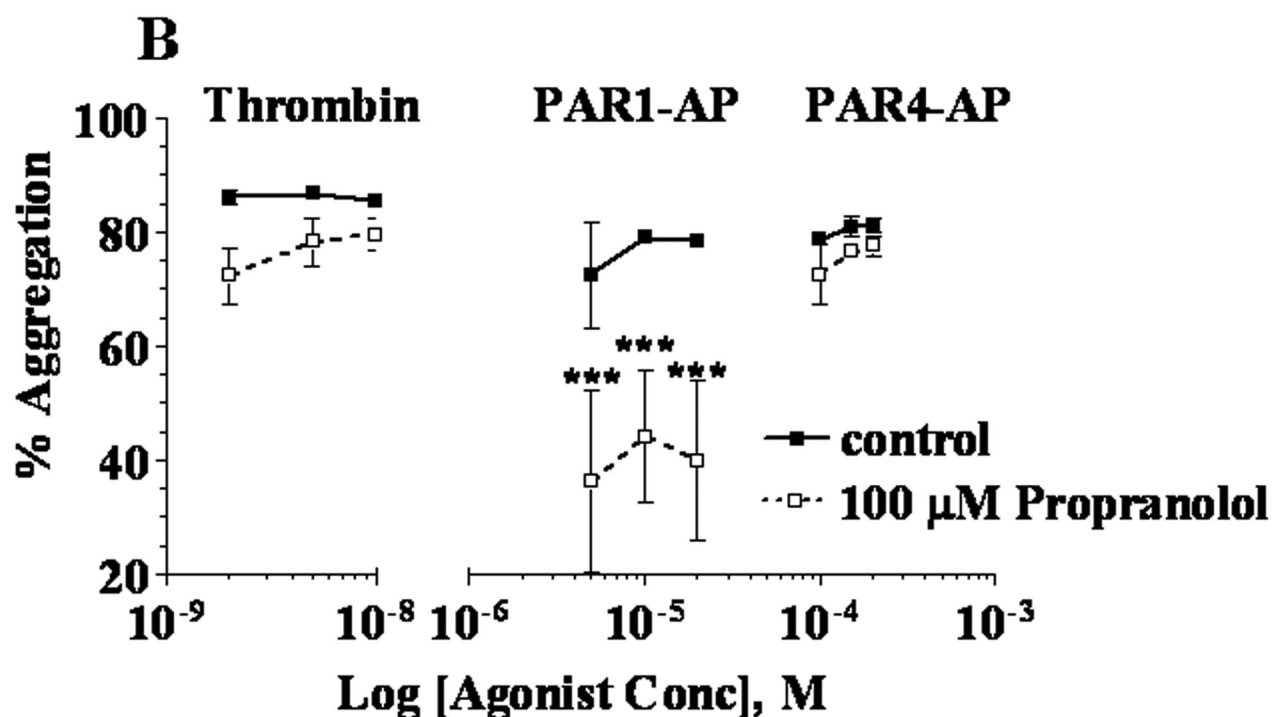
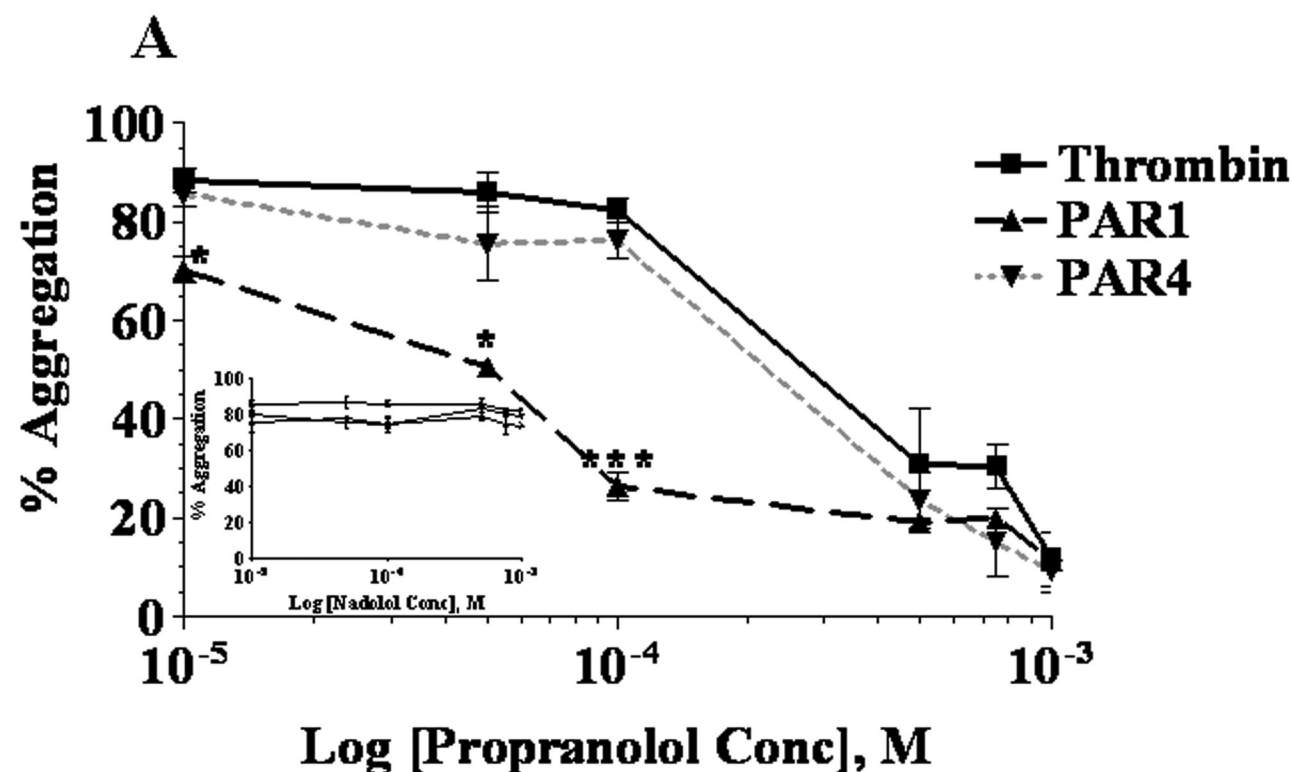
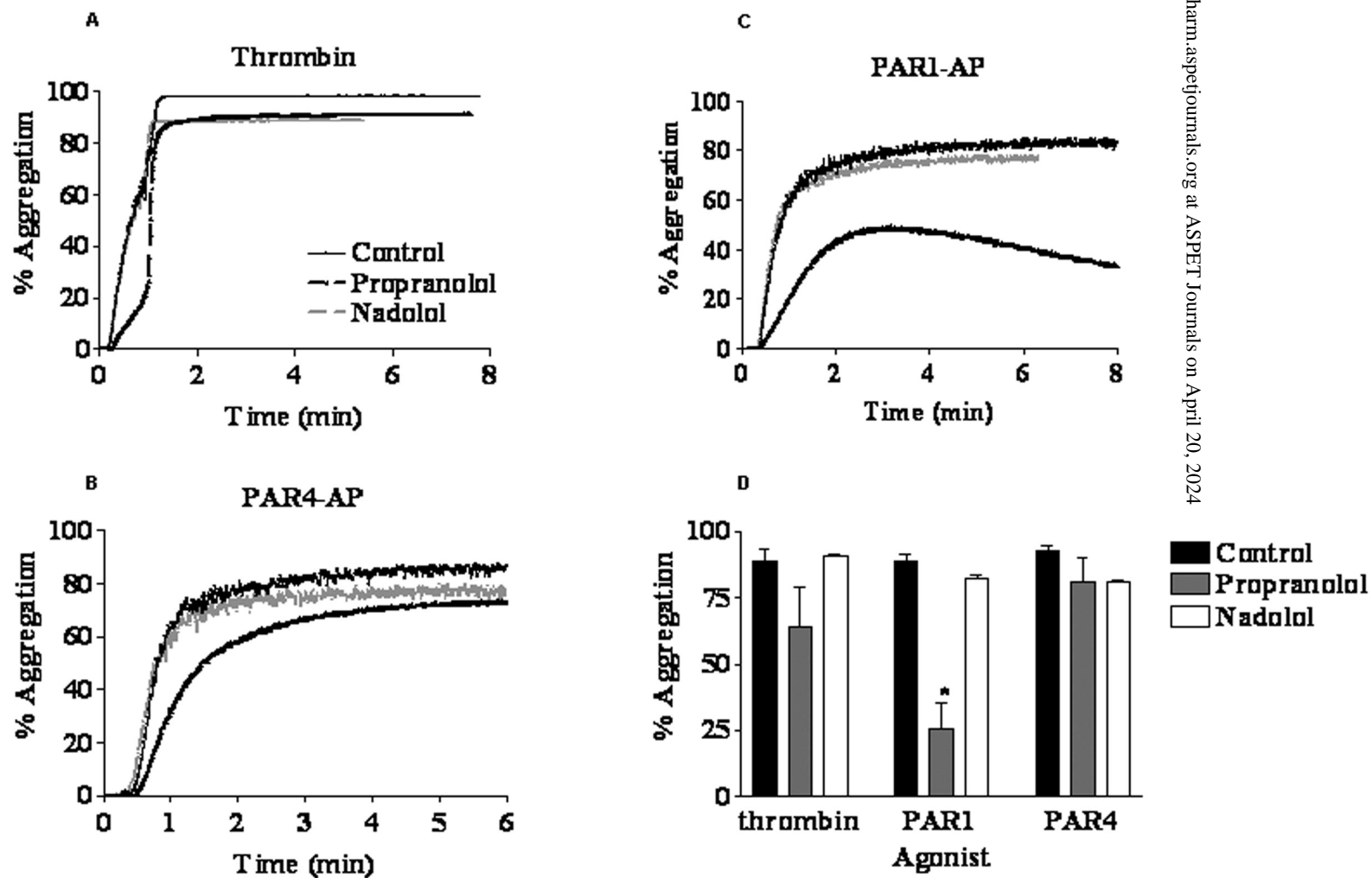
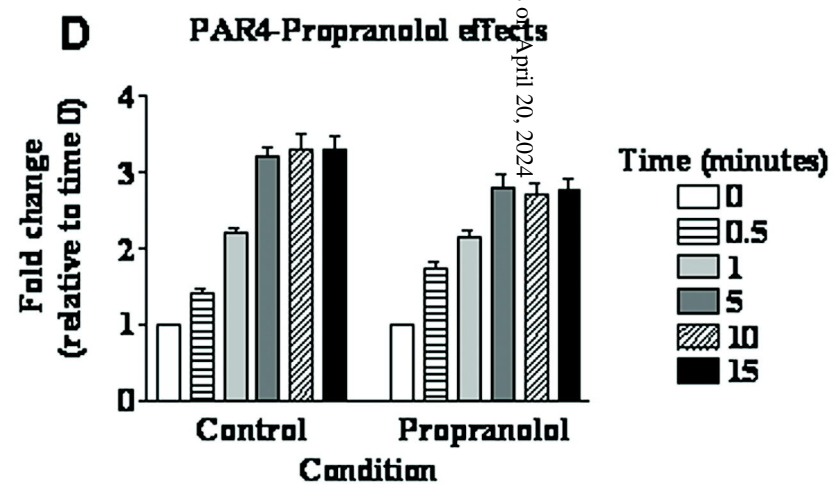
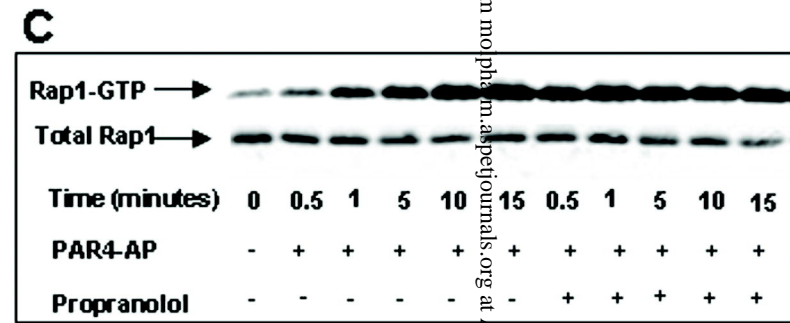
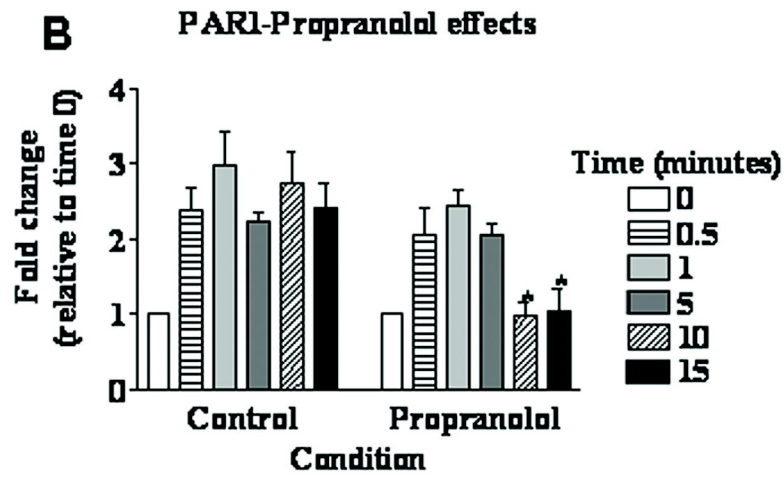
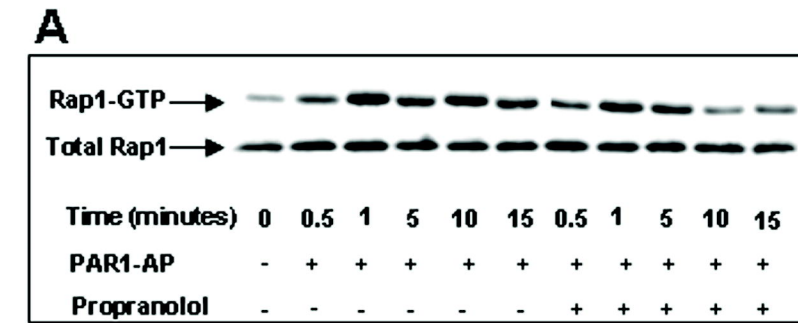
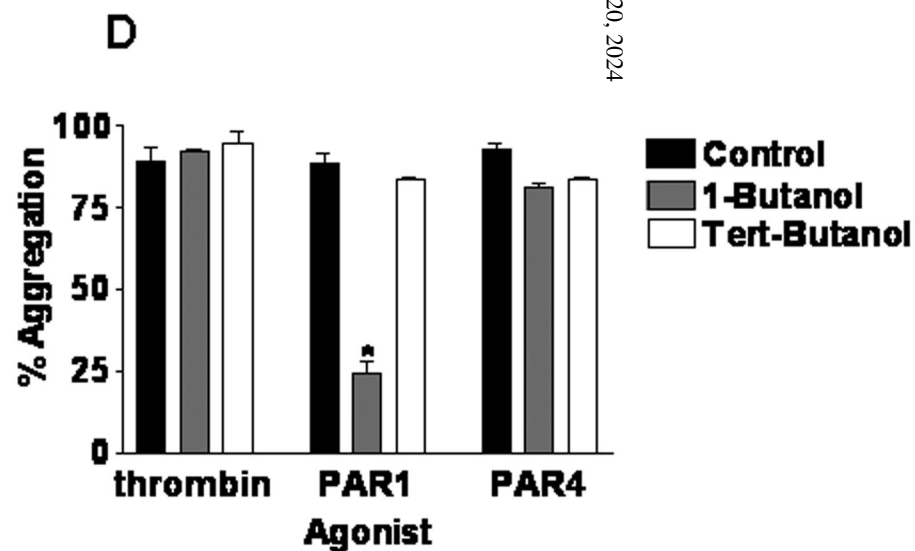
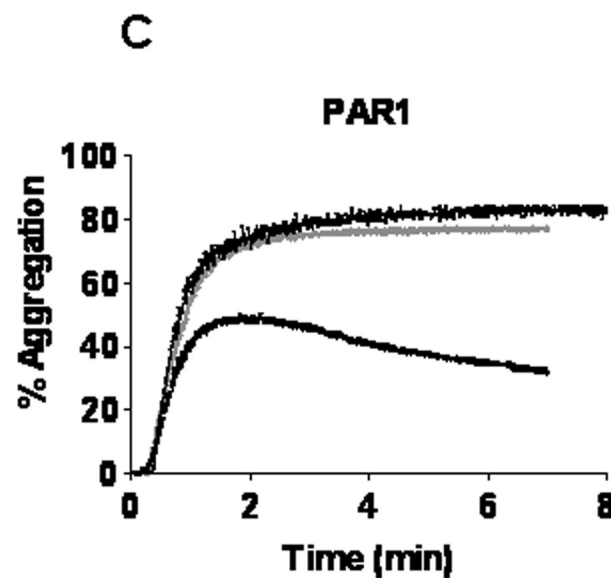
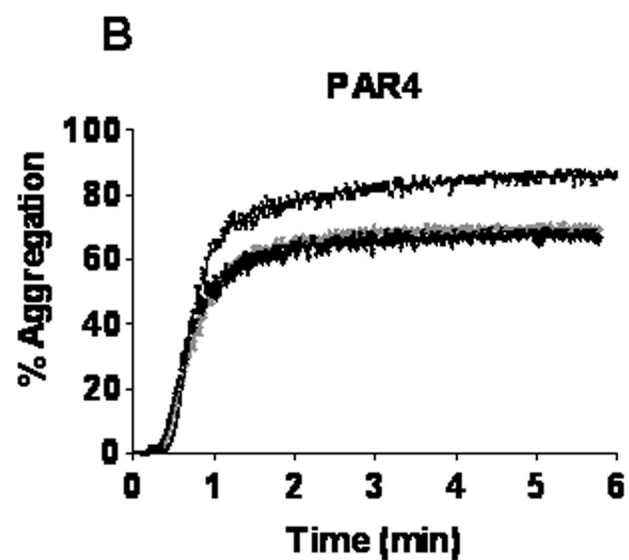
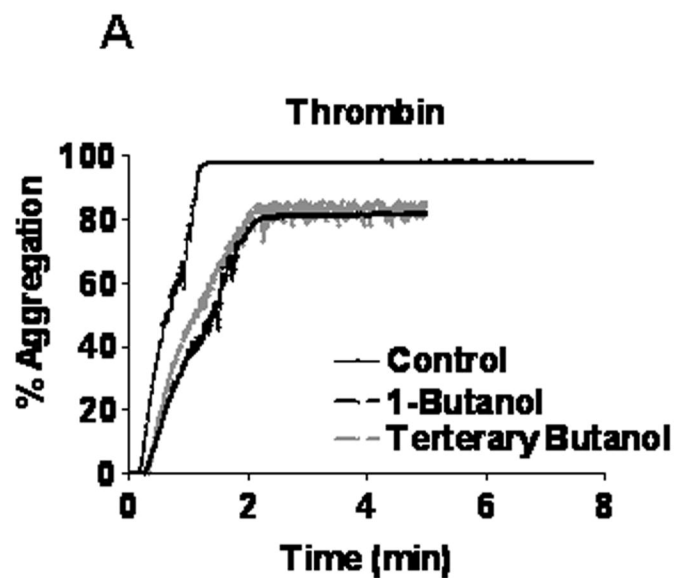


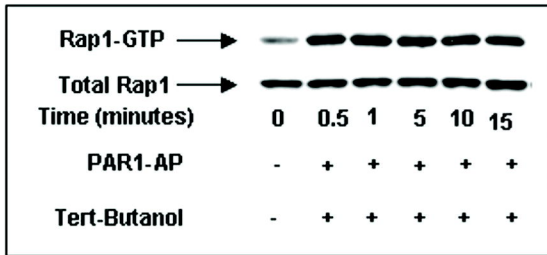
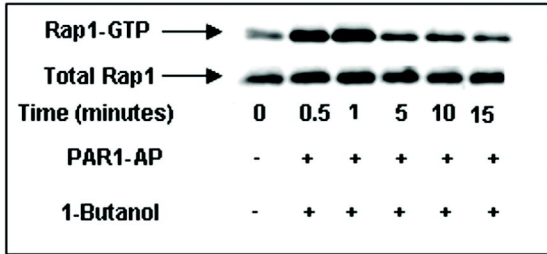
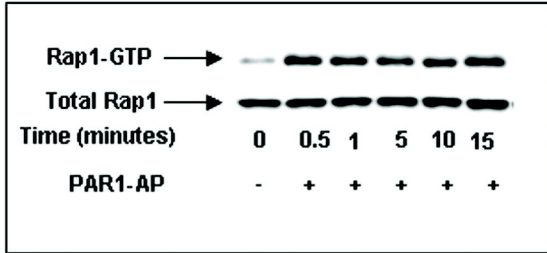
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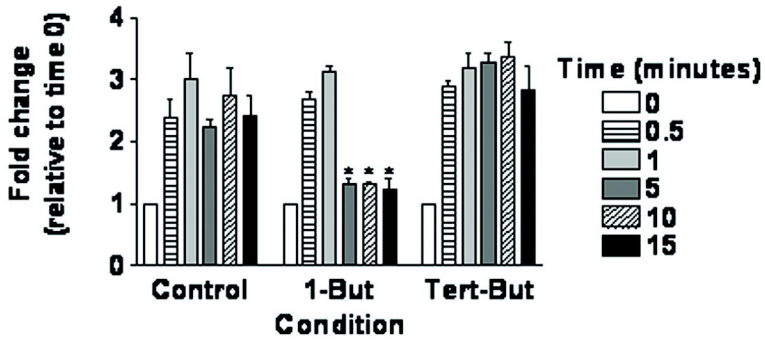




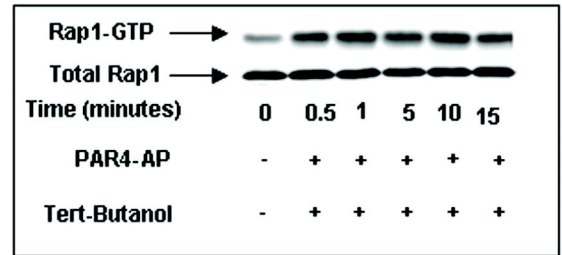
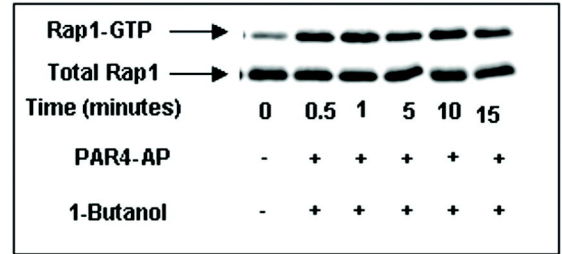
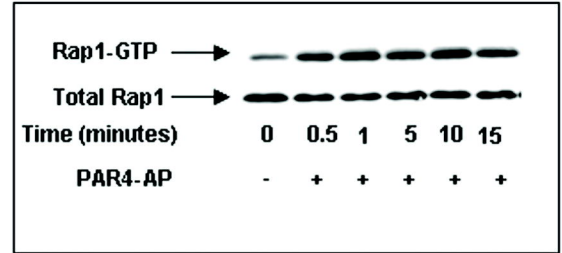
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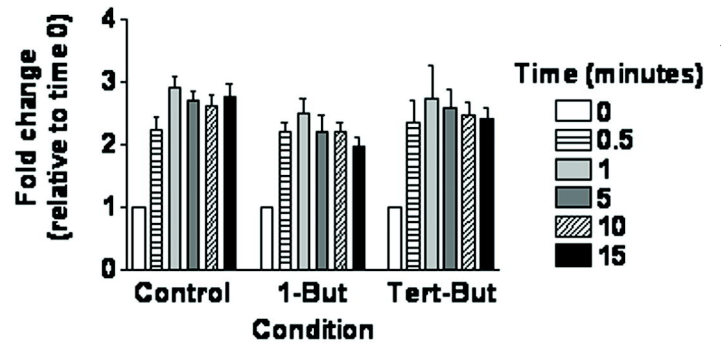
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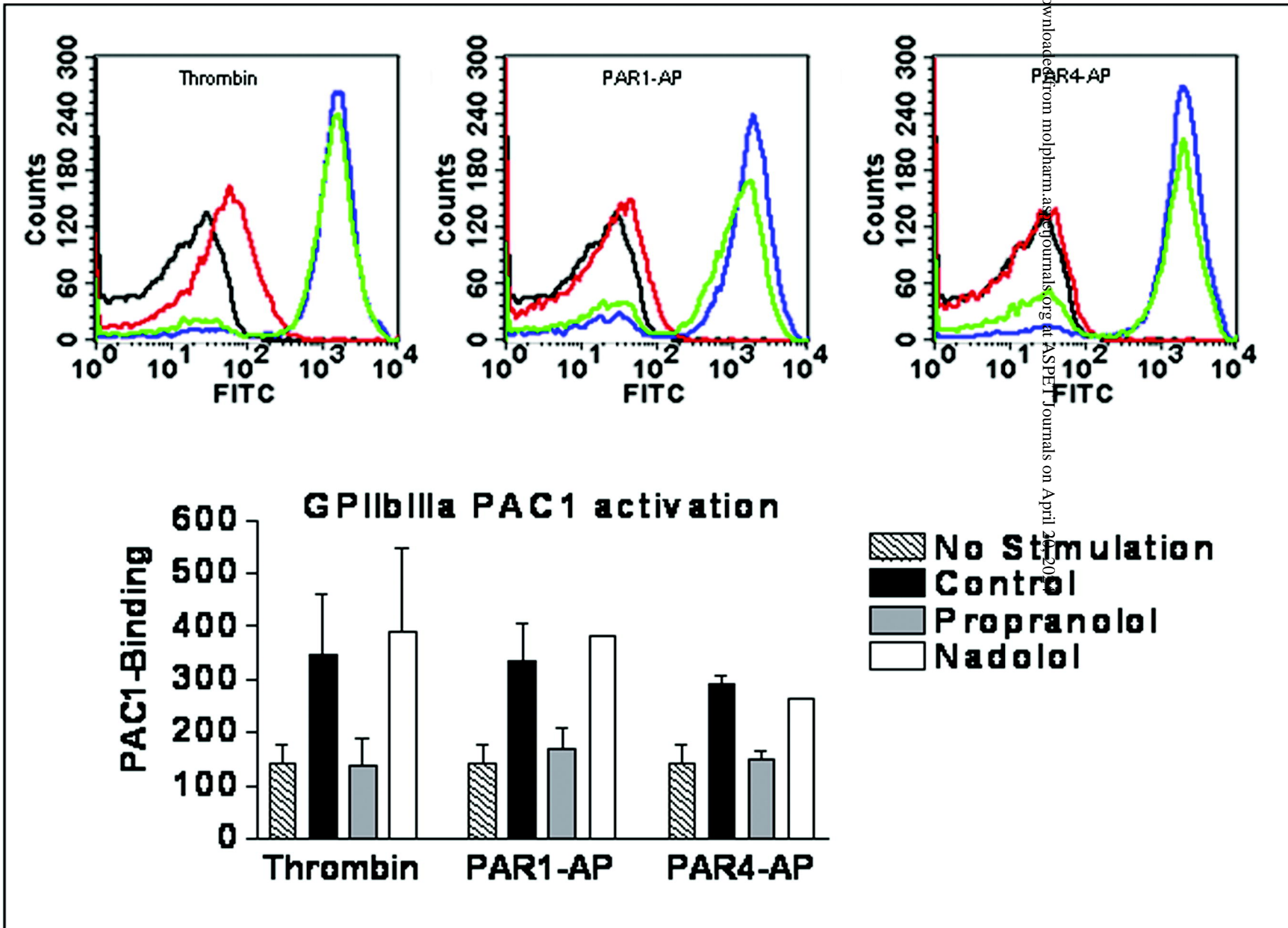


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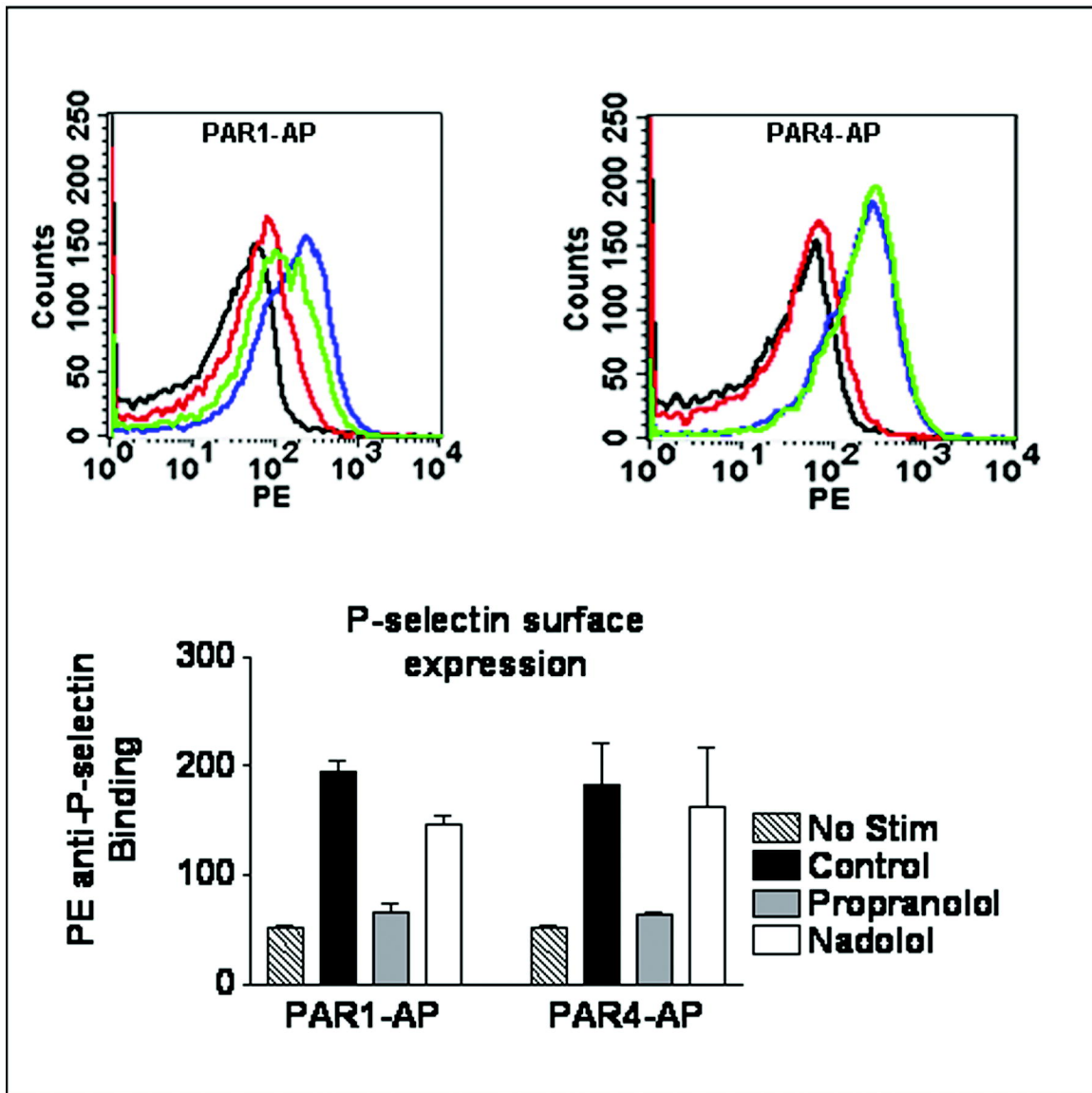
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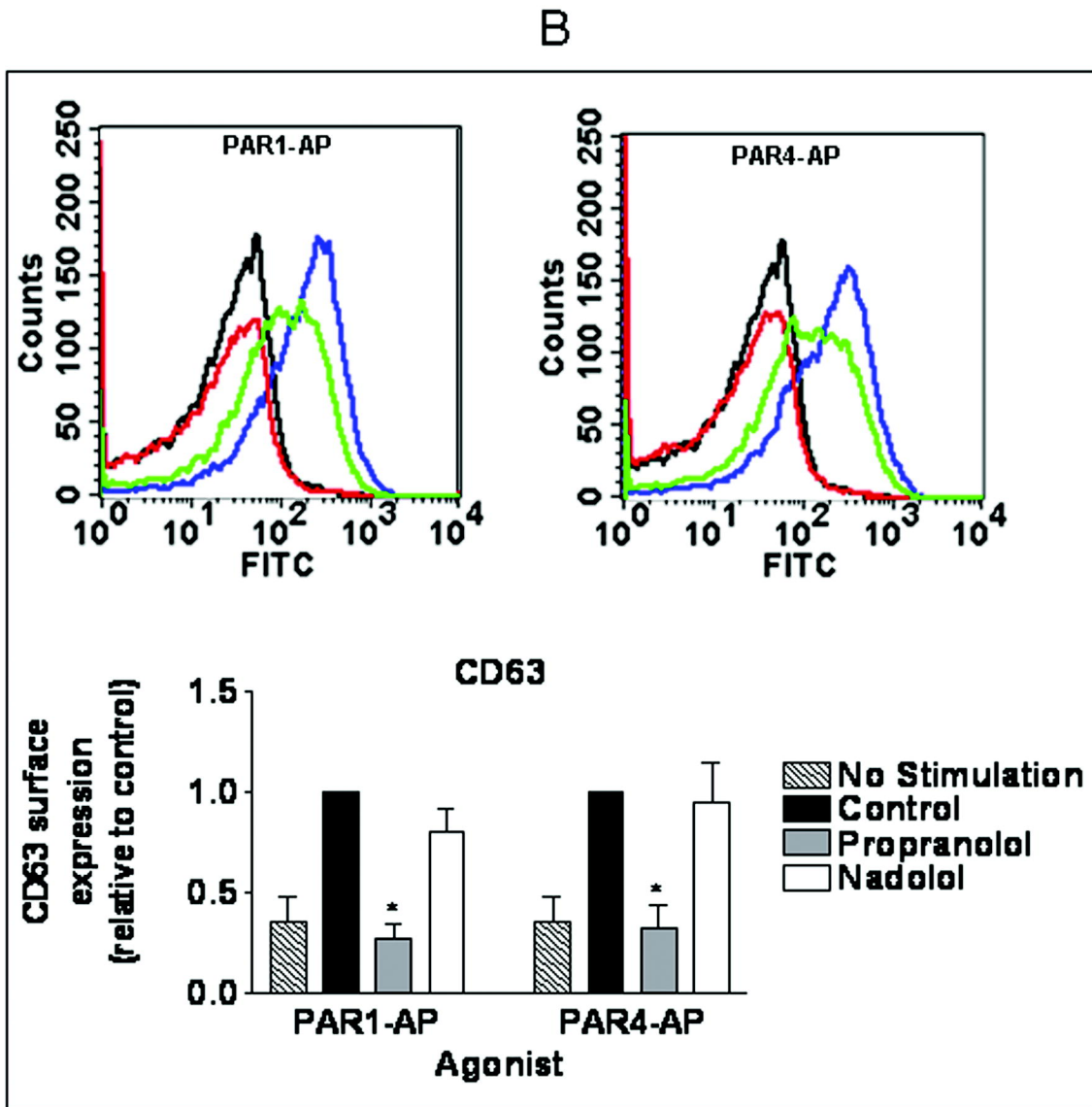


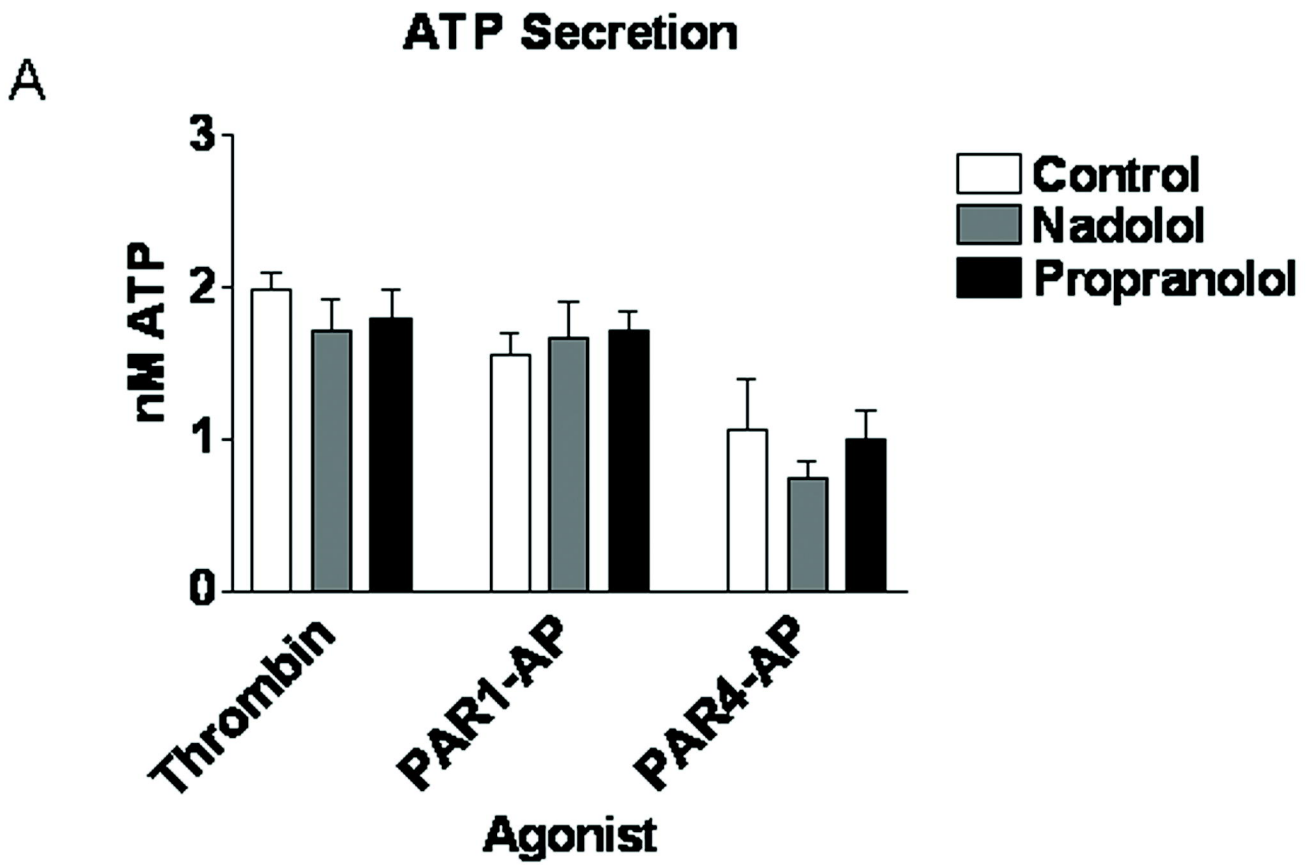


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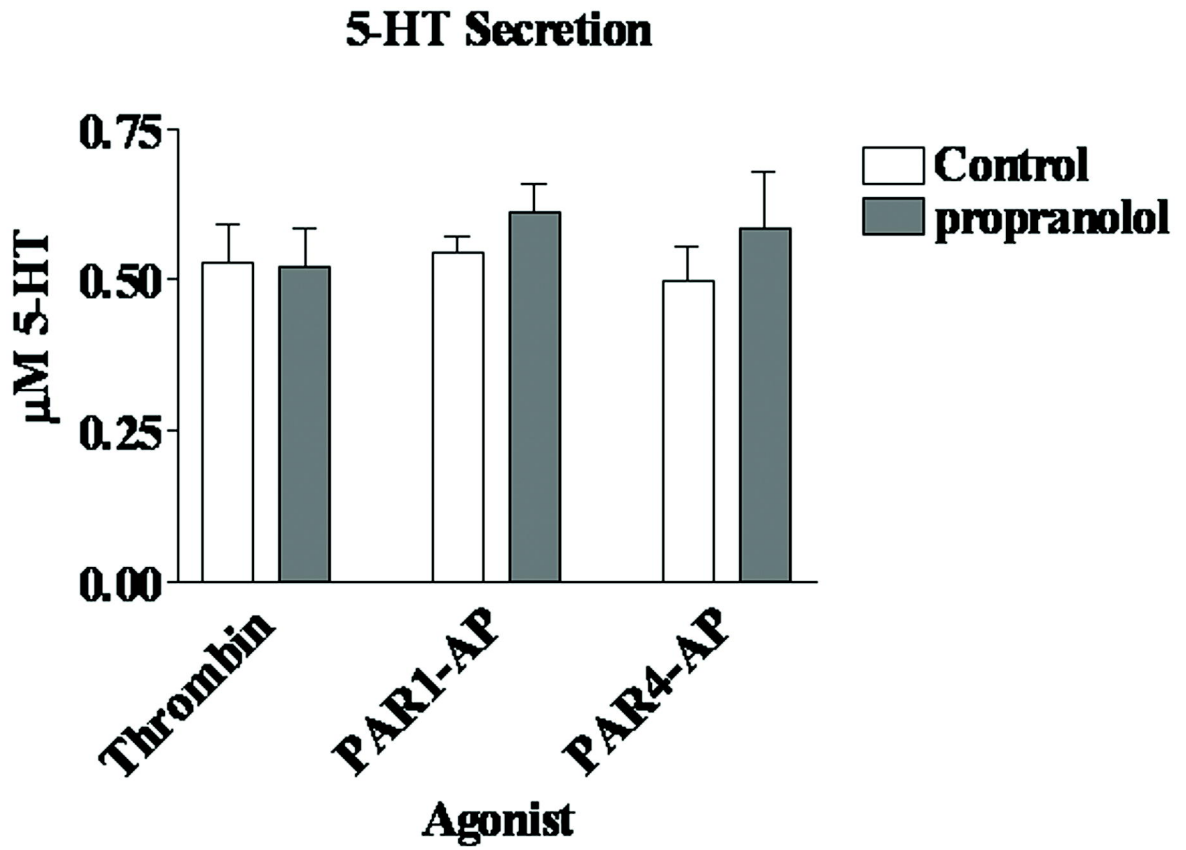
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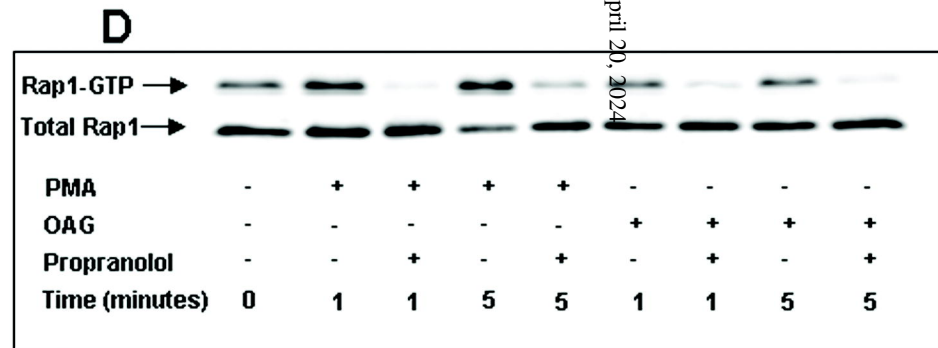
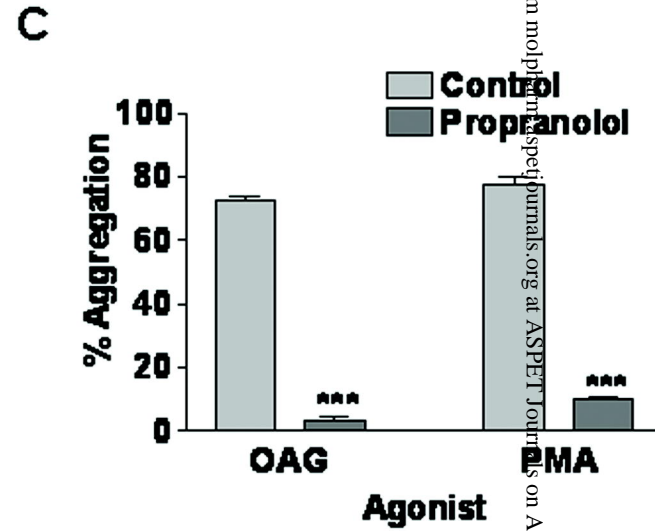
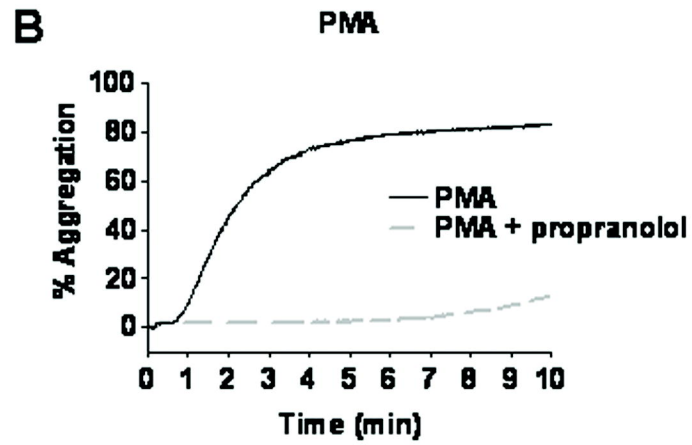
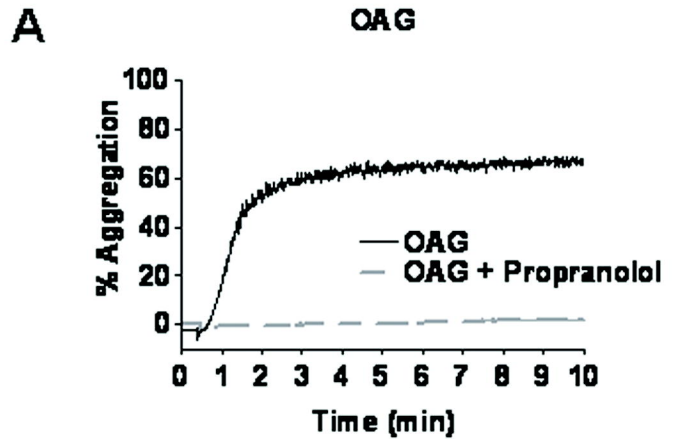






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