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Title Page

Title: Acetylcholine inhibits platelet activation.

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Abbreviations

NO nitric oxide

CHRNA7 cholinergic receptor neuronal nicotinic alpha polypeptide 7

GPIIbIIIA glycoprotein IIb IIIa

AChR acetylcholine receptors

AChE acetylcholinesterase

TRAP thrombin receptor activating peptide 6

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PAR1 protease activated receptor 1

P2Y12 purinergic receptor P2Y

GPVI glycoprotein VI

NOS3 nitric oxide synthase isoform 3

L-NAME L-nitroarginine methyl ester

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Abstract

Platelets are key mediators of thrombosis. Many agonists of platelet activation are known, but there are fewer identified endogenous inhibitors of platelets, such as prostacyclin and nitric oxide (NO). Acetylcholinesterase inhibitors such as donepezil can cause bleeding in patients, but the underlying mechanisms are not well understood. We hypothesized that acetylcholine is an endogenous inhibitor of platelets. We measured the effect of acetylcholine or analogues of acetylcholine upon human platelet activation ex vivo. Acetylcholine and analogues of acetylcholine inhibited platelet activation, as measured by P-selectin translocation and GPIIbIIIa conformational changes. Conversely, we found that antagonists of the acetylcholine receptor such as pancuronium enhance platelet activation. Furthermore, drugs inhibiting acetylcholinesterase such as donepezil also inhibit platelet activation, suggesting that platelets release acetylcholine. We found that NO mediates acetylcholine inhibition of platelets. Our data suggest that acetylcholine is an endogenous inhibitor of platelet activation. The cholinergic system may be a novel target for anti-thrombotic therapies.

Introduction

Platelet activation is crucial for hemostasis and thrombosis (Ho-Tin-Noe et al., 2011; Joshi and Whiteheart, 2017; Stalker et al., 2014) . A variety of agonists activate platelets in vivo, including thrombin, collagen, and ADP (Boeynaems et al., 2005; Coughlin, 2005; Ghoshal and Bhattacharyya, 2014; Hechler et al., 1998; Hisada et al., 2015). An equally important aspect of platelet biology is inhibition of activation, limiting excess thrombosis which can otherwise lead to stroke or pulmonary embolism. Endogenous platelet inhibitors include factors released from endothelial cells such as nitric oxide and prostacyclin (Freedman et al., 1999; Jin et al., 2005; Moncada et al., 1977; Radomski et al., 1987b).

Studies of adverse bleeding reactions to commonly used drugs can reveal novel inhibitors of platelet function (Holly and Parise, 2011). For example, a few case reports have suggested that acetylcholinesterase inhibitors are associated with bleeding (Cholongitas et al., 2006; Gareri et al., 2005). Several clinical trials have examined the safety of donepezil, and one of these trials showed that donepezil increases the risk of bruising (Rogers et al., 1998; Tariot et al., 2001). A meta-analysis of clinical trials of acetylcholinesterase inhibitors shows that these drugs increase the risk of bruising by 1.5 fold compared to placebo, although this increased risk is not significant (Birks, 2006). These isolated clinical studies suggest that acetylcholine may be an endogenous inhibitor of platelet activation. For these reasons, we chose to examine the effect of acetylcholine signaling on platelet activation.

Prior work from other laboratories suggests that acetylcholine receptors (AChR) are involved in platelet function. Human platelets express subunits of the acetylcholine receptor (Schedel et al., 2011). Artificial agonists of AChR stimulate calcium flux across human platelet membranes (Schedel et al., 2011). Certain agonists of AChR increase human platelet activation as measured by GPIIbIIIa conformational changes and by aggregation (Schedel et al., 2011). Finally, platelets from mice lacking AChR subunit *Chrna7* have increased activation when

stimulated by ADP (Kooijman et al., 2015). These important experimental studies suggest that acetylcholine signaling plays a role in inhibiting platelets both in vitro and in vivo.

Gaps remain in our collective knowledge pertaining to the effect of acetylcholine upon platelets. The effect of acetylcholine on platelets stimulated with endogenous agonists other than ADP is not yet completely known. The effect of acetylcholine on platelet degranulation is not fully understood. The effect of endogenous acetylcholine signaling on hemostasis and thrombosis is not well defined. The expression of genes involved in acetylcholine signaling in human platelets is not fully described. And the mechanisms through which clinical drugs targeting acetylcholine affect bleeding in humans has not yet been explored. Determining the role that acetylcholine signaling plays in inhibition of platelet function may help clinicians avoid the toxicity of drugs that target the parasympathetic nervous system, and may help us uncover new pathways which inhibit platelet function.

Materials and Methods

Human Platelet Collection

Human blood collection was performed as previously described using protocols approved by the Institutional Review Board at the University of Rochester Medical Center (IRB Protocol RSRB00028659) (Cameron et al., 2015). Normal healthy blood donors were recruited. Subjects were excluded if they had used aspirin or any nonsteroidal anti-inflammatory agent within 10 days before the blood draw. Blood was collected by venipuncture into sodium citrate anticoagulant tubes. Whole blood was centrifuged at 180 × *g* for 15 min to isolate the top layer of platelet-rich plasma (PRP). PRP was diluted 1:20 in room temperature Tyrode's Buffer (134 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.34 mM Na2HPO4, 20 mM HEPES, pH 7.0, 5 mM glucose, 0.35% bovine serum albumin) and dispensed in 100 µL volumes for treatment with various drugs.

Platelet Drug Treatment

Human platelets were suspended in Tyrode's buffer and placed into microcentrifuge tubes. Drugs were added and the platelets were incubated for 15 min at room temperature. To some samples, L-nitroarginine methyl ester (L-NAME) was added first and incubated for 15 min, then carbachol (Sigma Aldrich) or acetylcholine (Sigma Aldrich) for 15 min, and then TRAP (Tocris Bioscience) or thrombin (Cayman Chemical) for 15 min. Platelets were first treated for 15 minutes with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and trifluoperazine (TFP) (Sigma Aldritch) for some experiments. For experiments involving cholinesterase inhibition, platelets were pre-treated with donepezil for 15 minutes prior to stimulation. For experiments with nAChRα7-selective agonist PNU-282987, platelets were pretreated for 15 minutes with PNU prior to stimulation. For calcium flux experiments with Fura-2 AM, platelet rich plasma was loaded with Fura-2 AM at 5uM for 1 hour at 37 degrees Celsius,

and then further prepared as above to yield platelets loaded with Fura-2. HEK293 cells were also loaded as a positive control. Cells were analyzed on a Flexstation 3 (Molecular Devices) for the 340/380 Fura-2 AM ratio.

Detection of platelet activation by flow cytometry

Phycoerytherin-labeled antibody to CD62P (P-selectin) (Bectin Dickinson) at a dilution of 1:100 was added to platelets following stimulation or drug treatment for 30 min. Platelets were then fixed in 1% formalin. Surface P-selectin was measured by flow cytometry (LSRII, Becton Dickinson). To detect conformational changes in GPIIbIIIa, FITC-fibrinogen (Abcam) was added for 30 minutes, and platelets were analyzed by flow cytometry. We have previously used these techniques to measure platelet activation (Zhu et al., 2014)

Quantification of cGMP levels by ELISA

Platelets were treated and stimulated as described above. The reactions were stopped and cells lysed by the addition of HCl to a final concentration of 0.1 M. Samples were cleared by centrifugation (14,000 rpm) for 20 minutes. Samples were then analyzed for cGMP content using a commercially available ELISA (Cayman Chemical).

Statistical analyses.

Data were analyzed by two-tailed Student's t-test for comparison of two groups, and by Bonferroni corrected two-way ANOVA to compare means of three or more groups. Statistical significance was defined as $P < 0.05$.

Study approval.

Human blood collection was performed using protocols approved by the Institutional Review Board at the University of Rochester Medical Center.

Results

Acetylcholine receptors regulate platelet activation

Since patients taking acetylcholine inhibitors have an increased risk of bleeding, we hypothesized that increased acetylcholine signaling directly inhibits platelet activation. To test this hypothesis, we first analyzed the effect of carbachol, an analog of acetylcholine, on platelet activation. We treated human platelets with increasing concentrations of carbachol, and then stimulated the platelets with the thrombin receptor agonist thrombin receptor activating peptide 6 (TRAP). Carbachol inhibits human platelets activation in a dose dependent manner (Figure 1A). We next explored the effect of acetylcholine on platelet activation. Acetylcholine inhibits TRAP activation of human platelets in a dose responsive manner by over 25% of maximal stimulation (Figure 1B), and acetylcholine inhibits platelet activation over a range of TRAP doses (Figure 1C).

We tested the effect of acetylcholine signaling upon platelets stimulated with different agonists, including: TRAP, which activates the thrombin receptor PAR1; ADP, which activates the ADP receptor P2Y12; U44619 which activates the thromboxane receptor TP; and convulxin, which activates the collagen receptor GPVI. Carbachol inhibits platelet activation by other agonists (Figure 1D-F).

The above data show that acetylcholine inhibits alpha-granule release. Next we tested the effect of acetylcholine signaling on other aspects of platelet activation, namely dense granule secretion and GPIIbIIIa conformational changes. We found that the acetylcholine analogue carbachol decreases dense granule exocytosis measured by release of ATP (Figure 1G) and inhibits GPIIbIIIa activation measured by FITC-fibrinogen binding (Figure 1H). Furthermore, endogenous acetylcholine has the same effect (as shown when the acetylcholine esterase inhibitor pyridostigmine is added) (Figure 1G).

We also tested the effect of the nicotinic receptor agonist PNU-282987 upon platelet activation. We found that PNU inhibits thrombin induced platelet exposure of P-selectin (Fig. 1I) and GPIIbIIIA activation (Fig. 1J).

Taken together, these data suggest that stimulation of the acetylcholine receptor inhibits platelet activation as measured by 3 separate functions: alpha-granule release, dense granule release, and GPIIbIIIa activation.

Endogenous acetylcholine inhibits platelet activation

While acetylcholine signaling inhibits platelet activation, the potential source of acetylcholine in vivo remains unclear. We hypothesized that platelets release acetylcholine which inhibits platelet activation in an autocrine or paracrine manner. We treated platelets with the acetylcholinesterase inhibitor pyridostigmine bromide prior to activation. We observed that inhibition of acetylcholinesterase (AChE) decreases platelet activation (Figure 2A). This is consistent with the idea that pyridostigmine bromide inhibits acetylcholinesterase, increasing the amount of acetylcholine released by platelets which is available to signal through the acetylcholine receptor. We then confirmed that pancuronium bromide, which antagonizes the acetylcholine receptor, enhances platelet activation (Figure 2B). We tested the effect of these compounds on platelet GPIIbIIIa activation using FITC-fibrinogen, and observed that agonism of acetylcholine receptors inhibits, and antagonism of acetylcholine receptors enhances binding (Figure 2C).

Patients who take donepezil may have an increased risk of bleeding (Cholongitas et al., 2006; Rogers et al., 1998; Tariot et al., 2001). Since donepezil is an acetylcholinesterase inhibitor, we hypothesized that donepezil inhibits platelet activation. To test this hypothesis, we treated platelets with donepezil hydrochloride and then stimulated them with TRAP. Donepezil inhibits platelet activation (Figure 2D). These data are consistent with the hypothesis that endogenous acetylcholine released from platelets inhibits platelet activation.

Collectively, these data suggest platelets can release acetylcholine which limits activation, and endogenous acetylcholinesterase blunts the extent of endogenous acetylcholine signaling.

Nitric oxide mediates acetylcholine inhibition of platelet activation

We next explored the mechanism through which acetylcholine signaling inhibits platelet activation. Acetylcholine receptors increase the synthesis of nitric oxide in endothelial cells (Zuccolo et al., 2017). Platelets express NOS3 (Sase and Michel, 1995). We proposed that nitric oxide mediates acetylcholine inhibition of platelets. In order to test our idea, we treated human platelets with an inhibitor of nitric oxide synthase, L-nitroarginine methyl ester (L-NAME), and then treated with carbachol and stimulated with TRAP. We observed that carbachol inhibits platelets, but NOS inhibition blocks the effects of carbachol (Figure 3A). To confirm that acetylcholine signaling triggers NO synthesis in platelets, we measured carbachol stimulation of cGMP, a messenger downstream of NO. Carbachol increases cGMP levels in human platelets, and the effect of carbachol is blocked by the NOS inhibitor L-NAME (Figure 3B). The inhibitory effect of NO was further tested with a range of L-NAME doses. We found that L-NAME inhibits the effects of acetylcholine on platelets in a dose-dependent manner (Figure 3C). Since calcium signaling can regulate NOS activation, we explored a calcium signaling pathway in platelets. First, carbachol increases intracellular calcium levels in platelets (Figure 3D). Second, the calcium chelator BAPTA blocks the ability of carbachol to inhibit platelets (Figure 3E). Finally, calmodulin is important for acetylcholine inhibition of platelet activation (Figure 3F). Taken together, our data suggest that NO mediates acetylcholine inhibition of platelets via a calcium-calmodulin dependent mechanism.

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Discussion

The major finding of our study is that acetylcholine inhibits platelet activation. Acetylcholine signals through the acetylcholine receptor, increasing NO levels, and inhibiting platelet activation. Acetylcholine inhibits activation of platelets from humans by over 15%. Taken together, our results suggest that acetylcholine receptor activation is a potential endogenous inhibitory pathway which prevents platelet activation.

Two types of acetylcholine receptors have been described: muscarinic acetylcholine receptors which are G-protein coupled receptors, and nicotinic acetylcholine receptors are ligand gated ion channels (Beker et al., 2003; Itier and Bertrand, 2001). Nicotinic acetylcholine receptors are composed of 5 subunits in different combinations, including alpha, beta, delta, epsilon, and gamma subunits (Mishina et al., 1986; Morales-Perez et al., 2016; Unwin, 2005). The precise nature of the acetylcholine receptor in human platelets is not yet defined. Further research is needed to identify the subtypes of acetylcholine receptor and their various functions on platelets.

We show that NO mediates acetylcholine inhibition of platelets. Others have demonstrated that platelets express NOS3 and synthesize NO (Radomski et al., 1990a; b; Sase and Michel, 1995). Prior work has shown that NO inhibits platelet adhesion, activation, and aggregation (Freedman et al., 1999; Gkaliagkousi et al., 2007; Radomski et al., 1987a; b; c). For example, we showed that NO inhibits platelet exocytosis (Matsushita et al., 2003). Others have shown that activators of NO can inhibit platelet function (Doni et al., 1991; Liu et al., 2015). Our work extends these prior studies and shows that calcium-calmodulin signaling and NOS activity mediate acetylcholine inhibition of platelet activation. Our work also suggests that diseases or drugs which change nitric oxide production may affect platelet activation.

Acetylcholine inhibits activation of platelets by multiple agonists (Figure 1). Although both PAR1 and P2Y12 are GPCR, they signal through different intracellular messenger pathways (Boeynaems et al., 2005; Jin et al., 1998; Ramachandran et al., 2017; Sanchez Centellas et al.,

2017). Convulxin signals through GPIV (Marlas et al., 1983; Niedergang et al., 2000). While these pathways ultimately converge to stimulate platelet activation as measured by conformational changes in GPIIbIIIa, the prior signaling events are different, and might be differentially susceptible to NO. There are clinical drugs which take advantage of pathway specificity for platelet activation. For example, ticagrelor inhibits platelet activation by inhibiting ADP signaling through the P2Y12 receptor, but not other receptors(Goel, 2013; Patel et al., 2013; von Kugelgen, 2017).

We found that acetylcholine inhibits platelet activation in vitro by about 15% (Figure 1B). Carbachol, an analog of acetylcholine, has a much stronger effect upon platelet activation, inhibiting P-selectin translocation by over 90% (Figure 1A and 5A). This is likely due to poor hydrolysis of carbachol by acetylcholinesterase or butyrylcholinesterase. Thus exogenous agonists like carbachol have a powerful effect upon platelet activation, but endogenous agonists such as acetylcholine have a more modest inhibitory effect on platelet activation. This suggests a role for endogenous acetylcholine as a novel mechanism to limit aberrant platelet activation.

Our work extends prior research on cholinergic signaling in platelets. Others have shown that agonists of AChR increase human platelet activation ex vivo as measured by GPIIbIIIa conformational changes and by aggregation induced by ADP (Schedel et al., 2011). We show that acetylcholine itself inhibits platelet degranulation (Figure 1B), and PNU-282987 inhibits P-selectin externalization and also inhibits GPIIBIIIA activation (Fig 3G-H). This confirms our hypothesis that acetylcholine signaling inhibits PAR-1 induced platelet activation. The difference between our work and Schedel et al can potentially be explained by the choice of agonist. Supporting both our studies and hypothesis, others have shown that platelets from mice lacking *Chrna7* have increased aggregation when stimulated by ADP ex vivo (Kooijman et al., 2015).

Our study has several limitations which suggest future studies. We have not yet defined the composition of the acetylcholine receptor on platelets, and we have not identified the role of all acetylcholine subunits in mediating platelet inhibition. Another limitation is that we have

indirect evidence that platelets store acetylcholine in their granules, since acetylcholinesterase inhibitors boost platelet inhibition, but we have not directly measured acetylcholine inside platelet granules.

Our studies have pharmacological relevance to humans. We show that donepezil inhibits platelet activation ex vivo at a concentration between 5 – 50 uM (Figure 2D). This matches the concentration of donepezil of 47 uM in serum of humans taking donepezil as a treatment for Alzheimer's Disease (Hefner et al., 2015). Reports in the literature suggest that drugs targeting the acetylcholine signaling pathway have modest effects on hemostasis; for example, donepezil increase bruising by about 2% more than placebo (Birks, 2006). Another recent trial shows a benefit of acetylcholinesterase inhibitors for reducing the incidence of acute coronary syndrome in patients with dementia by 17% (Wu et al., 2015). Dementia patients frequently have co-morbidities such as diabetes with elevated risk of thrombosis, so giving these patients more refined and targeted AChE inhibitors may be clinically useful. Our data support our proposal that drugs that target acetylcholinesterase can promote bleeding in humans, and may explain why donepezil is associated with hemostatic abnormalities in humans.

Our study also has therapeutic implications for the management of thrombosis. Our data suggest that drugs targeting acetylcholine receptor subunits might inhibit thrombosis. Furthermore, our data suggest that drugs increasing acetylcholine signaling will increase the risk of bleeding and bruising in patients.

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Footnotes

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The data presented in this manuscript are available as part of a pre-print paper.

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Legends for Figures

Figure 1. Acetylcholine receptors regulate platelet activation. (A) Carbachol inhibits platelet activation. Human platelets were isolated and treated with PBS or carbachol, stimulated with PBS or 10 uM TRAP, and analyzed for surface expression of P-selectin using flow cytometry. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + carbachol.) (B) Acetylcholine inhibits platelet activation. Human platelets were treated with PBS or ACh, stimulated with PBS or 10 uM TRAP and analyzed as above. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + ACh.) (C) Carbachol inhibits platelet activation over a range of TRAP doses. Platelets were stimulated with varying concentrations of TRAP and analyzed for surface expression of P-selectin as above. (N=4 ± S.D. *P < 0.05 for the indicated concentration of TRAP vs. TRAP + carbachol. (D) Carbachol inhibits platelet activation by ADP (E) Carbachol inhibits platelet activation by U46619 (F) Carbachol inhibits platelet activation by convulxin. For (D-G), Isolated human platelets were treated with PBS or 10 nM carbachol, then stimulated with various agonists, and analyzed via flow cytometry. (N=4 ± S.D. *P < 0.05 for agonist vs. agonist + carbachol.) (G) Carbachol inhibits platelet dense granule release. Platelets were isolated and treated with 10 nM carbachol, 100 uM pyridostigmine bromide or 100 nM pancuronium bromide, and then stimulated with PBS or TRAP and analyzed for surface expression of P-selectin. . (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP and indicated compound.) (H) Carbachol inhibits GPIIbIIIa activation as measured by FITC-fibrinogen binding to platelets. Platelets were isolated and treated with 10 nM carbachol, and then stimulated with the indicated concentrations of TRAP and analyzed for surface expression of P-selectin. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + carbachol.). (I) Treatment with the nAChRα7-selective agonist PNU-282987 inhibits P-selectin exposure. Platelets were treated with PNU-282987 at the indicated concentrations, then stimulated with TRAP6 and analyzed for surface expression of p-selectin. *P < 0.05 for TRAP6 + vehicle vs TRAP6 + indicated concentration of PNU. (J) PNU inhibits GPIIbIIIa activation. Platelets were treated with PNU-282987 at the indicated concentrations, then stimulated with TRAP6 and analyzed for

activation of GPIIbIIIa as above. *P < 0.05 for TRAP6 + vehicle vs TRAP6 + indicated concentration of PNU.

Figure 2. Endogenous acetylcholine inhibits platelet activation. (A) Pyridostigmine inhibition of AChE permits endogenous acetylcholine inhibition of activation of human platelets. Isolated human platelets were treated with 100 uM pyridostigmine, or 100 uM pyridostigmine and 100 uM ACh, stimulated with 10 uM TRAP and then analyzed for P-selectin using flow cytometry. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + pyridostigmine/ACh.) (B) Pancuronium antagonism of acetylcholine receptor blocks endogenous acetylcholine inhibition of human platelets. Isolated human platelets were treated with pancuronium, and then stimulated with 10 uM TRAP and analyzed for P-selectin using flow cytometry. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + pancuronium.) (C) Endogenous ACh inhibits GPIIbIIIa conformational changes. Platelets were isolated and treated with 10 nM carbachol, 100 uM pyridostigmine or 100 nM pancuronium bromide and analyzed for FITC-fibrinogen binding to measure GPIIbIIIa activation. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + indicated compound.) (D) Donepezil inhibition of AChE permits endogenous acetylcholine inhibition of activation of human platelets. Isolated human platelets were treated with donepezil hydrochloride, then stimulated with 10 uM TRAP and analyzed for P-selectin using flow cytometry. (N=4 ± S.D. *P < 0.05 for TRAP vs. TRAP + donepezil.)

Figure 3. Nitric oxide mediates Ach inhibition of platelet activation. (A) NOS mediates carbachol inhibition of platelet activation. Isolated human platelets were treated with PBS, carbachol, L-NAME or L-NAME + carbachol, stimulated with 10 uM TRAP, and then analyzed for P-selectin using flow cytometry. (N = 4 ± S.D. *P < 0.05 for TRAP + carbachol vs. TRAP + carbachol + L-NAME.) (B) NOS mediates carbachol induced production of cGMP. Isolated human platelets were treated as above, and cGMP content was measured using a commercial

kit. (N = 4 ± S.D. *P < 0.05 for TRAP-6 + carbachol vs. TRAP + carbachol + L-NAME.) (C) L-NAME reversal of carbachol mediated platelet inhibition is dose dependent. Platelets were isolated as above and treated with 10 nM carbachol, 1 mM, 0.1 mM or 0.01 mM L-NAME and then stimulated with TRAP and analyzed for surface expression of P-selectin. . (N = 4 ± S.D. *P < 0.05 for TRAP + carbachol vs. TRAP + carbachol + indicated concentration of L-NAME.) (D) Carbachol elevates intracellular calcium. Platelets or HEK293 cells were loaded with Fura-2 AM, treated with carbachol and analyzed for calcium flux. (E) Calcium mediates the inhibitory effect of carbachol. Isolated human platelets were treated with BAPTA, then carbachol and then stimulated with TRAP and analyzed for surface expression of p-selectin. (N=4) *P < 0.05 for carbachol + TRAP vs carbachol + TRAP + BAPTA). (F) Calmodulin activity is required for the inhibitory effect of carbachol. Platelets were treated with TFP, then carbachol and then stimulated with TRAP and analyzed for surface expression of p-selectin. *P < 0.05 for TRAP + carbachol vs. TRAP + carbachol + TFP).

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Figures:

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Figure 1.

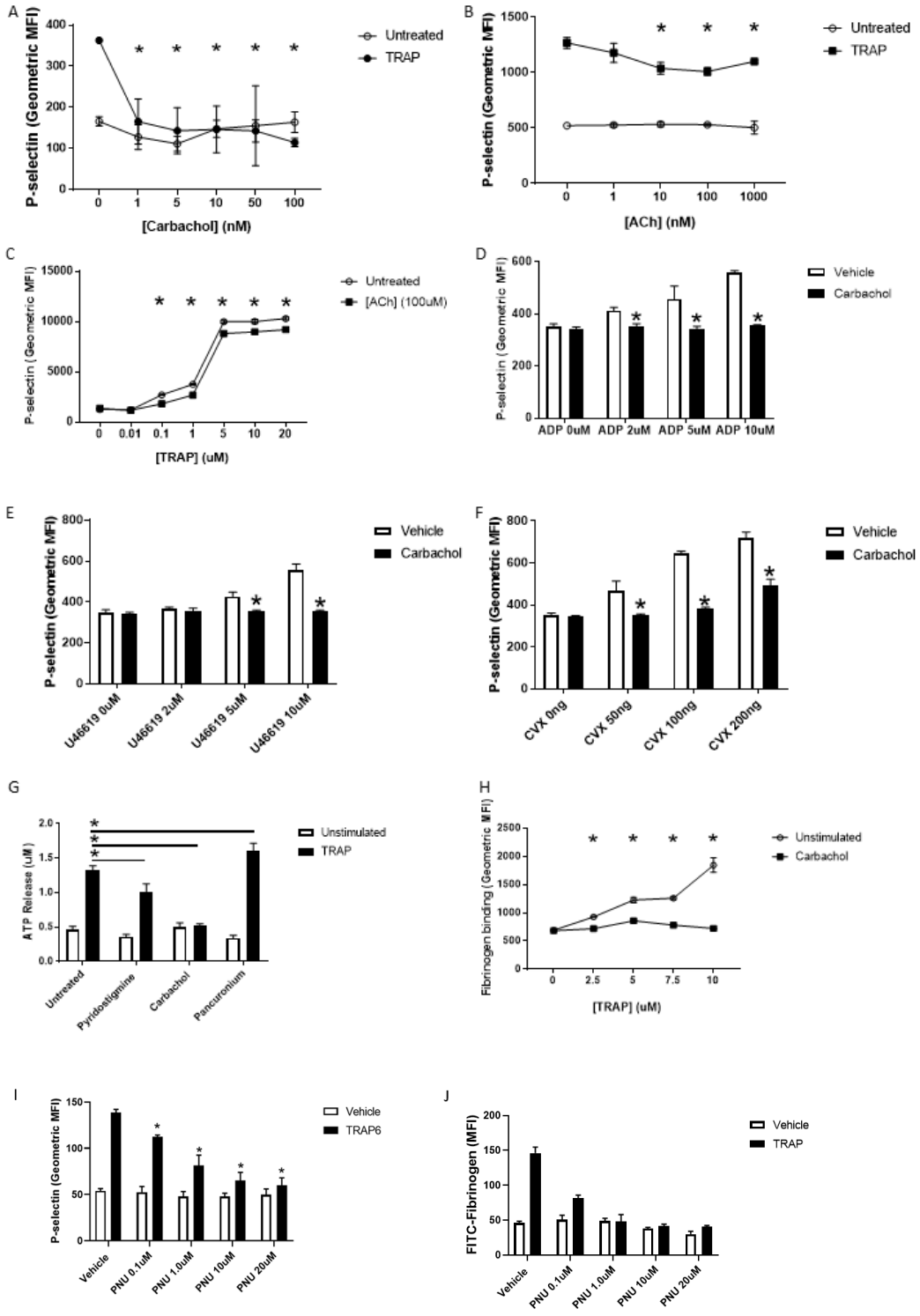

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Figure 2.

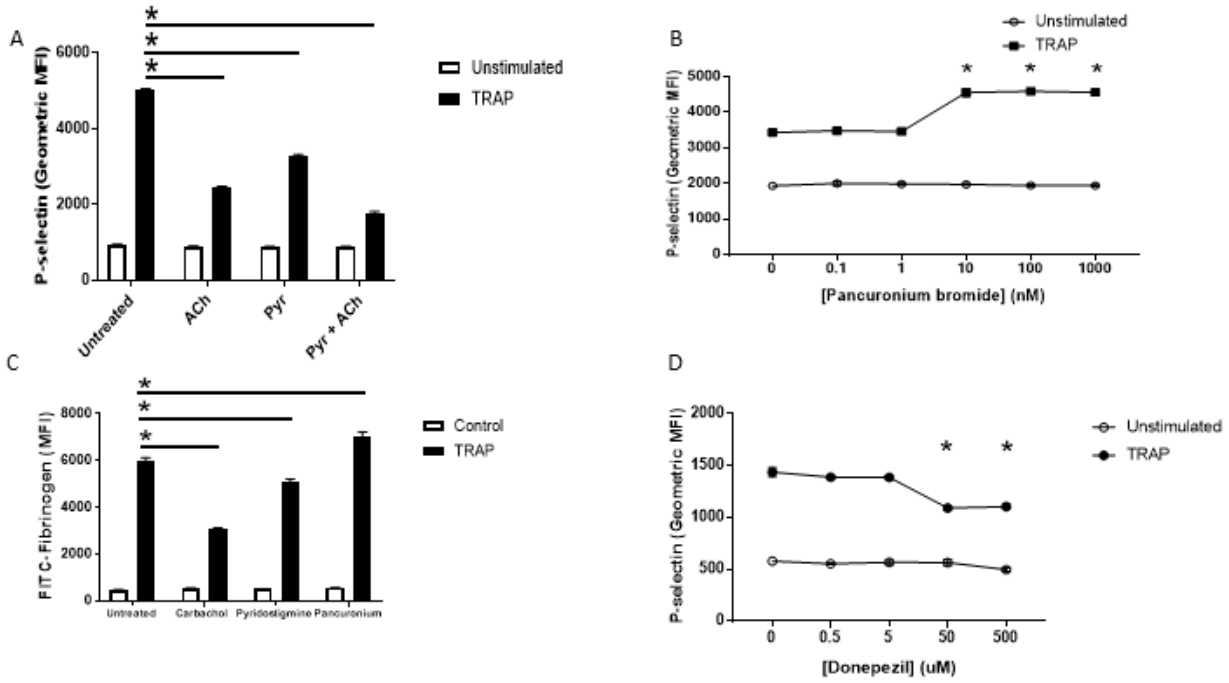

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Figure 3.