BIOCHEMICAL PHARMACOLOGY 77 (2009) 835–844

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RhoA downstream of G_q and $G_{12/13}$ pathways regulates protease-activated receptor-mediated dense granule release in platelets

Jianguo Jin [a,c], *Yingying Mao* [a], *Dafydd Thomas* [b], *Soochong Kim* [a,c], *James L. Daniel* [b,c], *Satya P. Kunapuli* [a,b,c,*]

[a] *Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA*
[b] *Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, USA*
[c] *The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA*

ARTICLE INFO

Article history:
Received 21 October 2008
Accepted 14 November 2008

Keywords:
Platelet secretion
Dense granule
$G_{12/13}$
ADP
RhoA

ABSTRACT

Platelet secretion is an important physiological event in hemostasis. The protease-activated receptors, PAR 1 and PAR 4, and the thromboxane receptor activate the $G_{12/13}$ pathways, in addition to the G_q pathways. Here, we investigated the contribution of $G_{12/13}$ pathways to platelet dense granule release. 2MeSADP, which does not activate $G_{12/13}$ pathways, does not cause dense granule release in aspirin-treated platelets. However, supplementing 2MeSADP with YFLLRNP (60 μM), as selective activator of $G_{12/13}$ pathways, resulted in dense granule release. Similarly, supplementing PLC activation with $G_{12/13}$ stimulation also leads to dense granule release. These results demonstrate that supplemental signaling from $G_{12/13}$ is required for G_q-mediated dense granule release and that ADP fails to cause dense granule release because the platelet P2Y receptors, although activate PLC, do not activate $G_{12/13}$ pathways. When RhoA, downstream signaling molecule in $G_{12/13}$ pathways, is blocked, PAR-mediated dense granule release is inhibited. Furthermore, ADP activated RhoA downstream of G_q and upstream of PLC. Finally, RhoA regulated PKCδ T505 phosphorylation, suggesting that RhoA pathways contribute to platelet secretion through PKCδ activation. We conclude that $G_{12/13}$ pathways, through RhoA, regulate dense granule release and fibrinogen receptor activation in platelets.

1. Introduction

Platelets are an important part of the hemostatic mechanism that are activated following vascular injury [1,2]. Numerous agonists, such as thrombin and ADP, cause platelet activation through stimulation of G protein pathways [3–8]. Upon activation, platelets secrete their granule contents that help amplify platelet responses to many of the physiological agonists [9]. Human platelets contain two types of storage granules, α-granules and dense granules. Substances released from the α-granules supplement thrombin generation at the site of vascular injury [9,10]. ADP is the most important constituent of the dense granules that is essential for recruiting platelets to the site of vascular injury [11,12].

Platelets express a number of heterotrimeric G proteins, including G_q, G_{12}, G_{13}, and G_i [6,13,14]. Protease-activated

* *Corresponding author at*: Department of Physiology, Temple University, Department of Physiology, Rm. 224, OMS, 3420 N. Broad Street, Philadelphia, PA 19140, USA. Tel.: +1 215 707 4615; fax: +1 215 707 4003.
E-mail address: spk@temple.edu (S.P. Kunapuli).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.11.017

receptors (PARs) and thromboxane receptors couple to G_q as well as $G_{12/13}$ [11,12], whereas ADP activates G_q and G_i pathways through the $P2Y_1$ and $P2Y_{12}$ receptors, respectively [15]. Downstream of these G protein-coupled receptor stimulation, G_q is known to be important for platelet secretion [16]. In platelets deficient in $G\alpha_q$ or phospholipase C-β_2 (PLCβ_2), stimulation with thrombin and thromboxane A_2 (TxA_2) results in markedly decreased platelet secretion [16–18]. Similarly, collagen fails to cause dense granule secretion in PLCγ_2-deficient platelets [19]. Activation of PLC leads to generation of inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) [18,20] and platelet dense granule secretion is dependent on the IP_3-induced rise in intracellular calcium and DAG-induced activation of protein kinase C (PKC) [21,22].

It is known that stronger platelet agonists like thrombin, protease-activated receptor-1 (PAR1) activating peptide SFLLRN, protease-activated receptor-4 (PAR4) activating peptide AYPGKF and thromboxane A_2 analogue (U46619) cause dense granule secretion. However, weaker agonists like ADP fail to cause dense granule secretion when thromboxane generation is blocked [4]. ADP, through activation of the $P2Y_1$ receptor, stimulates G_q and PLCβ_2, and causes increases in intracellular calcium and PKC activation [15,16,23–25], but fails to cause dense granule secretion [4]. Our investigation focuses on why some agonists cause dense granule release while others do not even though both agonists activate PLC. Specifically, what are the signaling differences between ADP and U46619 or thrombin that account for the lack of dense granule release by ADP? It is only recently that we began to clearly understand the signaling events downstream of agonist receptors. In G_q-deficient mouse platelets, U46619 or thrombin, but not ADP, causes shape change through activation of $G_{12/13}$ and RhoA-mediated p160ROCK pathways [26,27]. Hence it is now clear that receptors for thrombin and thromboxane A_2 couple to $G_{12/13}$, in addition to G_q. Could the inability of ADP to couple to $G_{12/13}$ explain its inability to cause dense granule release? Offermanns et al. have shown that $G\alpha_{13}$ deficient mice have a severe defect in primary hemostasis and complete protection against arterial thrombosis in vivo [16,27]. In addition these mouse platelets have defective agonist-induced dense granule release [27]. Thus, we investigated the role of $G_{12/13}$ pathways in agonist-induced dense granule release using complementary approaches.

In this study, we demonstrate that ADP causes dense granule release in aspirin-treated platelets when supplemented with selective activation of $G_{12/13}$ pathways. In addition, we show that $G_{12/13}$ pathways contribute to dense granule release partially through RhoA pathways. We also provide evidence for the PLC-independent activation of RhoA pathways downstream of G_q stimulation. Here, we suggest that at least one reason ADP fails to cause dense granule secretion is its inability to activate $G_{12/13}$ signaling pathways.

2. Materials and methods

2.1. Materials

Apyrase (Type VII), fibrinogen (Type 1), bovine serum albumin (fraction V), 2MeSADP were obtained from Sigma (St. Louis, MO). Chrono-lume reagent was purchased from Chrono-Log Corp. (Havertown, PA). AYPGKF and YFLLRNP were obtained from New England Peptide (Gardner, MA). YM254890 was a gift from Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki, Japan). m-3M3FBS was purchased from Calbiochem, Inc. (San Diego, CA). Exoenzyme C3 transferase was obtained from Cytoskeleton Inc. (Denver, CO). PKC δ isoform selective antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). All the other reagents were of reagent grade and bought from Sigma (St. Louis, MO).

2.2. Isolation of human platelets

Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230 × *g* for 20 min at room temperature to obtain platelet-rich plasma (PRP). PRP was incubated with 1 mM acetylsalicylic acid for 30 min at 37 °C. The PRP was then centrifuged for 10 min at 980 × *g* at room temperature to pellet the platelets. Platelets were resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 10 mM Hepes, pH 7.4, 0.2% bovine serum albumin) containing 0.01 U/ml apyrase. Cells were counted using the Coulter Z1 Particle Counter and concentration of cells was adjusted to 2×10^8 platelets/ml. All experiments using washed platelets were performed in the absence of extracellular calcium unless otherwise mentioned.

2.3. ADP-ribosylation of RhoA by exoenzyme C3 transferase

Platelet-rich plasma was obtained from ACD buffed blood by centrifugation at 230 × *g* for 20 min at ambient temperature. Platelets, free of reticulocytes and other contaminants, were isolated from plasma by centrifugation at 980 × *g* for 10 min and re-suspended in Tyrode's buffer with 0.01 U/ml apyrase, 1 mM acetylsalicylic acid and with or without 20 μg/ml exoenzyme C3 transferase (2×10^8 cells/ml), and incubated at 37 °C for 4 h. Finally, the platelet count was adjusted to 1.5×10^8 cells/ml.

2.4. Measurement of platelet secretion

Platelet secretion was determined by measuring the release of ATP using the lumichrome reagent. The activation of platelets was performed in a lumi-aggregometer at 37 °C with stirring at 900 rpm and the secretion was measured and expressed as nmoles of ATP released/10^8 platelets. In experiments where inhibitors were used, the platelet sample was incubated with the inhibitors for 10 min at 37 °C prior to the addition of agonists. The secretion was subsequently measured as described above.

2.5. Aggregometry

Aggregation of 0.5 ml washed platelets was analyzed using a P.I.C.A. lumi-aggregometer (Chrono-log Corp., Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37 °C. Agonists were

added simultaneously for platelet stimulation, each sample was allowed to aggregate for at least 3 min. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

2.6. RhoA GST-pulldown studies

Synthesis of GST-Rhotekin-RBD was performed according to Ren and Schwartz [28] with some minor modifications. Protein expression was then induced by 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) (Research Products international, Prospect, IL) treatment for 3 h at 30 °C.

For RhoA activation assays, platelets were resuspended in HEPES buffered Tyrodes at 3×10^8 cells/ml containing 0.4 units/ml apyrase. One ml of platelets per sample was then treated with 1 min preincubation with GR144053 at 37 °C to prevent platelet aggregation. Platelets were then lysed with lysis buffer (100 mM Tris, pH 7.5, 2% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 M NaCl, 20 mM $MgCl_2$, 20 μg/ml leupeptin, 2 mM PMSF), snap frozen, thawed on ice and spun at 13,000 × *g* for 10 min at 4 °C. Lysates were then treated as described by Ren and Schwartz [28], separated via SDS/Poly Acrylamide Gel Electrophoresis (PAGE, 15% acrylamide), blotted onto Immobilon-P (Millipore, Billerica, MA), blocked with 5% non-fat milk/0.5% Tween in Tris buffered saline and active RhoA was detected using anti-Rho clone 55 monoclonal antibody (Millipore, Billerica, MA), appropriate HRP-conjugated secondary antibody (Santa Cruz, CA), and Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA).

2.7. Western blot analysis

Platelets were stimulated with agonists in the presence or absence of inhibitors for the appropriate time, and the reaction was stopped by the addition of 3× SDS Laemmlli's buffer. Platelet samples were boiled for 10 min and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked by incubation in Tris-buffered saline-Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin (BSA) for 30 min at room temperature, and membranes were incubated overnight at 4 °C with the primary antibody (1:1000 dilution in TBST with 2% BSA) with gentle agitation. After three washes for 5 min each with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5000 dilution in TBST with 2% BSA) for 1 h at room temperature. After additional washing steps, membranes were then incubated with CDP-Star chemiluminescent substrate (Tropix, Bedford, MA) for 10 min at room temperature and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH, Japan).

3. Results

3.1. Effect of $G_{12/13}$ activation on 2 MeSADP-induced platelet dense granule release

As a difference in signaling between ADP and thrombin in platelets is the activation of $G_{12/13}$ pathways, we investigated the effect of selective activation of $G_{12/13}$ pathways on ADP-induced dense granule release. YFLLRNP, a partial PAR1 agonist, only activates $G_{12/13}$ pathway at low concentrations [29,30]. We stimulated washed and aspirin-treated human platelets with ADP and/or YFLLRNP and evaluated platelet functional responses. 2MeSADP activating G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$, but not $G_{12/13}$ pathway, caused platelet aggregation, but could not cause dense granule release (Fig. 1). YFLLRNP (60 μM), which selectively activates only $G_{12/13}$ pathways, caused platelet shape change, but not aggregation or dense granule release (Fig. 1). However, 100 nM 2MeSADP together with 60 μM YFLLRNP-induced dense granule release in platelets (Fig. 1B). In addition, 60 μM YFLLRNP potentiated 2MeSADP-induced platelet aggregation (Fig. 1A). These data indicate that $G_{12/13}$ pathways contribute to dense granule release and aggregation in platelets.

3.2. Effect of selective activation of phospholipase C and $G_{12/13}$ pathways on platelet dense granule release

PAR stimulation in platelets leads to stimulation of G_q and $G_{12/13}$ pathways. Downstream of G_q, PLC is activated resulting in

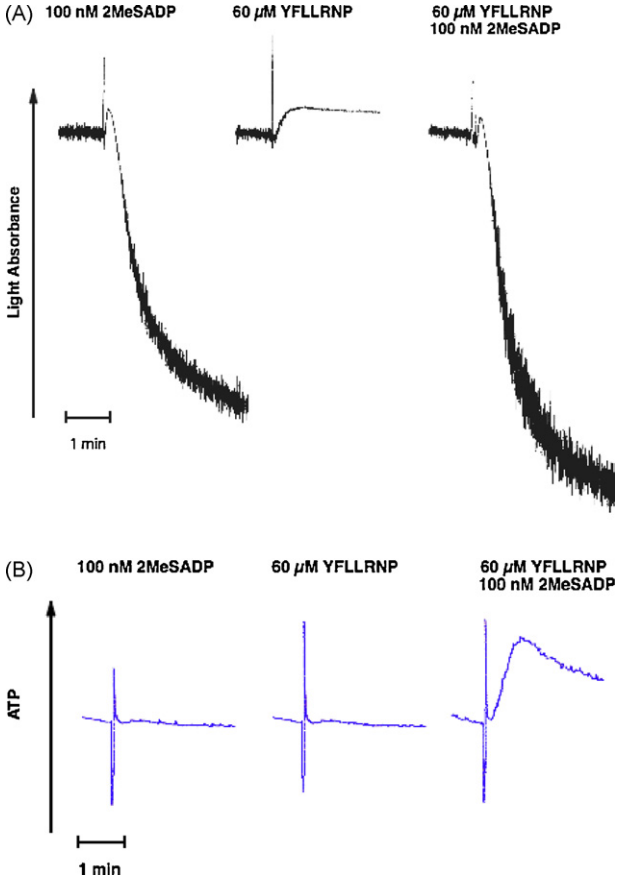

Fig. 1 – Effect of $G_{12/13}$ activation on 2MeSADP-induced platelet dense granule release in human platelets. Aspirin-treated and washed human platelets were stimulated with 100 nM 2MeSADP, 60 μM YFLLRNP or 60 μM YFLLRNP + 100 nM 2MeSADP at 37 °C while stirring. Platelet aggregation (A) and secretion (B) were measured in a Lumi-aggregometer. The tracings are representative of data from at least three independent experiments.

increased intracellular calcium and activation of protein kinase C isoforms, both of which are reported to be important for dense granule release. Phospholipase C can be directly activated by m-3M3FBS [31,32]. We evaluated the effect of selective $G_{12/13}$ signaling and selective phospholipase C activation on dense granule release using two strategies. In both approaches, G_q signaling is completely abolished. First, we used a G_q inhibitor YM254890 [33,34], to completely block G_q pathways. Under these conditions, activation of human platelets with AYPGKF results in selective activation of $G_{12/13}$ pathways. We have established the conditions and demonstrated the specificity of the YM254890 in our recent publication [35]. As shown in Fig. 2, in the YM254890-treated human platelets, AYPGKF caused shape change (Fig. 2A), but not aggregation (Fig. 2A) or dense granule release (Fig. 2B). Selective activation of PLC using m-3M3FBS (80 μM) resulted in a small shape change (Fig. 2A), but not aggregation (Fig. 2A)

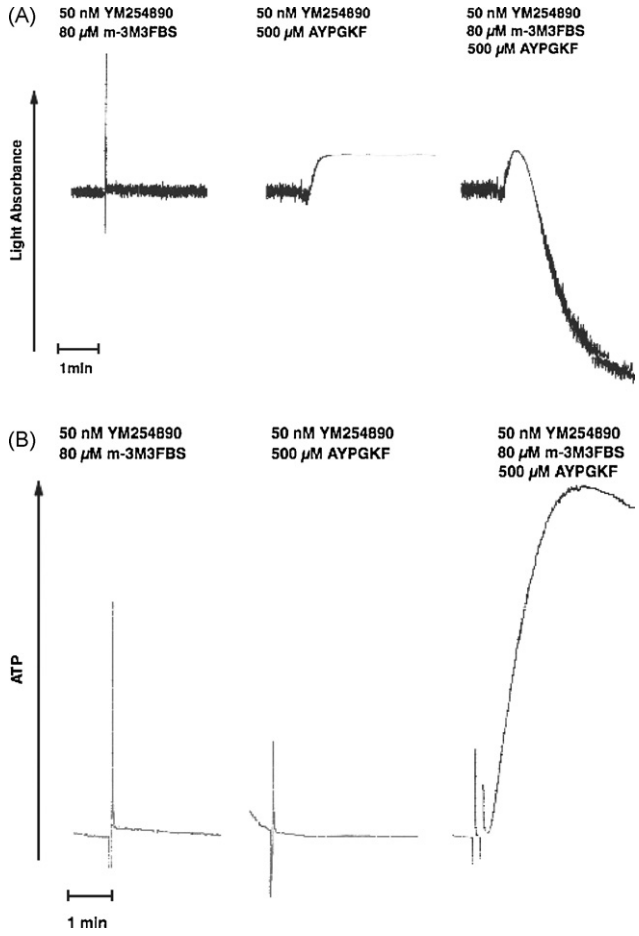

Fig. 2 – Effect of selective activation of phospholipase C and $G_{12/13}$ pathways on platelet dense granule release in human platelets. Aspirin-treated and washed human platelets were stimulated with 80 μM m-3MFBS, 500 μM AYPGKF or 80 μM m-3MFBS + 500 μM AYPGKF at 37 °C while stirring. Platelet samples were incubated with 50 nM YM254890 (G_q protein inhibitor) at 37 °C for 5 min before the addition of agonists. Platelet aggregation (A) and secretion (B) were measured in a Lumi-aggregometer. The tracings are representative of data from at least three independent experiments.

or dense granule release (Fig. 2B). However, combined stimulation of $G_{12/13}$ pathways and PLC resulted in platelet aggregation (Fig. 2A) and dense granule release (Fig. 2B), under conditions of G_q blockade. These results indicate that $G_{12/13}$ pathways contribute to both platelet aggregation and dense granule release.

In the second approach we used platelets from mice lacking $G\alpha_q$. Thrombin fails to cause platelet aggregation, dense granule release, or intracellular calcium mobilization in these platelets [16]. As shown in Fig. 3A, stimulation of platelets from $G\alpha_q$ null mice with AYPGKF resulted in shape change but not aggregation or dense granule release. Similarly, selective PLC activation with 80 μM m-3M3FBS caused only shape change in these platelets (Fig. 3A). However, stimulation of $G\alpha_q$ null mouse platelets with AYPGKF and m-3M3FBS resulted in platelet aggregation and dense granule release (Fig. 3A and B), indicating that $G_{12/13}$ pathways contribute to platelet aggregation and dense granule release.

In both approaches, we limited the concentration of m-3M3FBS to 80 μM as at this concentration we observed only shape change without aggregation or dense granule release. However, when we used higher concentrations of m-3M3FBS, platelet aggregation and dense granule release were observed (data not shown).

Our recent studies and other's have shown that co-stimulation of $G_{12/13}$ and G_i or G_z pathways leads to a small raise in intracellular calcium and aggregation [36,37]. Hence, it is possible that co-stimulation of $G_{12/13}$ and G_i or G_z pathways may be sufficient to cause dense granule release. We stimulated platelets from $G\alpha_q$ null mice with 2MeSADP and AYPGKF together. In these platelets 2MeSADP activates only G_i pathways and AYPGKF activates only $G_{12/13}$ pathways [38]. Although selective stimulation of G_i or $G_{12/13}$ with 2MeSADP or AYPGKF, respectively, in these murine platelets failed to cause any aggregation, combined stimulation of G_i and $G_{12/13}$ pathways resulted in platelet aggregation (Fig. 3C). As shown in Fig. 3D, selective activation of $G_{12/13}$ and G_i pathways did not result in dense granule secretion indicating the importance of G_q pathways in this event.

3.3. *Role of RhoA in dense granule secretion*

As stimulation of $G_{12/13}$ pathways is known to cause activation of RhoA [26,39], we evaluated the role of RhoA using pharmacological inhibitor, exoenzyme C3 transferase [40]. When platelets were pretreated with exoenzyme C3 transferase for 4 h, we evaluated the effectiveness of RhoA inhibition using shape change as a read out. Since it is known that RhoA activation occurs downstream of $G_{12/13}$ pathways and that this signaling molecule contributes to shape change in the absence of G_q pathways, we blocked G_q pathways with YM254890 and evaluated the effect of C3 exoenzyme [41] treatment on AYPGKF-induced platelet shape change. When G_q pathways are blocked, AYPGKF caused platelet shape change through $G_{12/13}$-RhoA pathways in control platelets (Fig. 4A). As seen in Fig. 4A, exoenzyme C3 transferase treatment completely blocked this RhoA-mediated platelet shape change in these platelets, indicating that exoenzyme C3 transferase completely inhibited RhoA under our experimental conditions. When platelets were pretreated with exoenzyme C3 transferase,

PAR-mediated platelet aggregation was inhibited compared to control platelets (Fig. 4B). We also observed that the AYPGKF-induced dense granule release reaction was inhibited by exoenzyme C3 transferase treatment (Fig. 4C). Similar inhibition by exoenzyme C3 transferase was seen for thrombin, SFLLRN-, or AYPGKF-induced dense granule release (Fig. 4D). These data indicate that RhoA plays an important role in platelet dense granule release.

3.4. *RhoA stimulation downstream of G_q pathways in platelets*

Although it has been known that $G_{12/13}$ and G_q pathways independently activate RhoA [26,39,42], the role of PLC downstream of G_q pathways in the activation of RhoA has not been addressed. Similarly, we have shown that ADP, through the P2Y1 receptor, causes calcium-independent shape change through p160ROCK pathways using pharmacological inhibitors [43]. However, the mechanism of RhoA activation downstream of the P2Y1 receptor is not established. Hence, we investigated whether ADP causes RhoA activation and the initial signaling steps contributing to its activation in platelets. As shown in Fig. 5A, RhoA was activated upon stimulation of platelets with 2MeSADP and this activation was inhibited by a G_q inhibitor YM-254890, suggesting that RhoA activation downstream of the P2Y1 receptor requires G_q stimulation. As shown in Fig. 5B, PLC inhibitor U73122 did not affect 2-MeSADP-induced RhoA activation indicating that

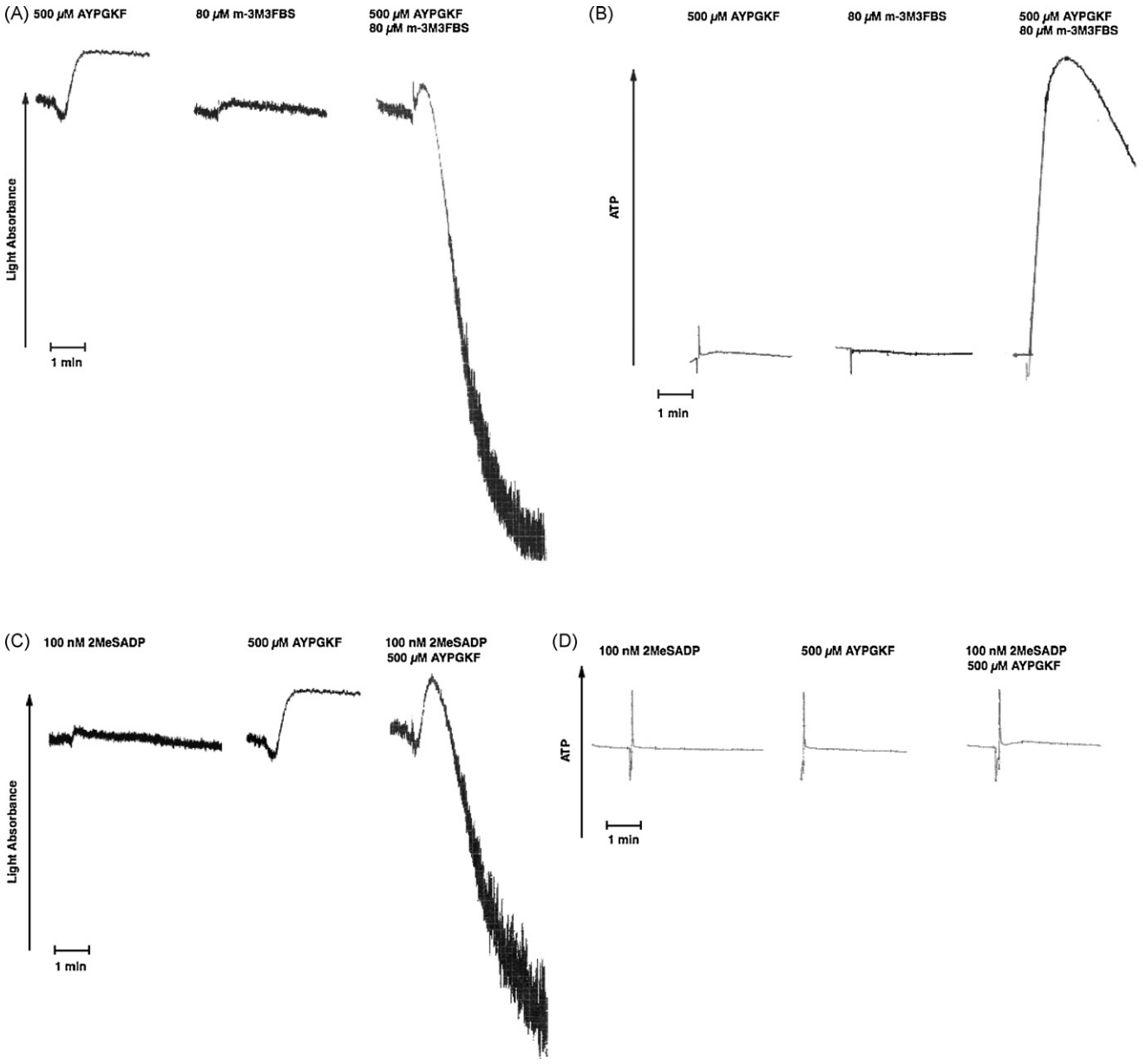

Fig. 3 – Effect of selective activation of phospholipase C and $G_{12/13}$ pathways on platelet dense granule release in $G\alpha_q$ null mouse platelets. Aspirin-treated and washed $G_q^{-/-}$ mouse platelets were stimulated with 80 μM m-3MFBS, 500 μM AYPGKF or 80 μM m-3MFBS + 500 μM AYPGKF (A and B), 100 nM 2 MeSADP, 500 μM AYPGKF or 100 nM 2MeSADP + 500 μM AYPGKF (C and D) at 37 °C while stirring. Platelet aggregation (A and C) and secretion (B and D) were measured in a Lumi-aggregometer. The tracings are representative of data from at least three independent experiments.

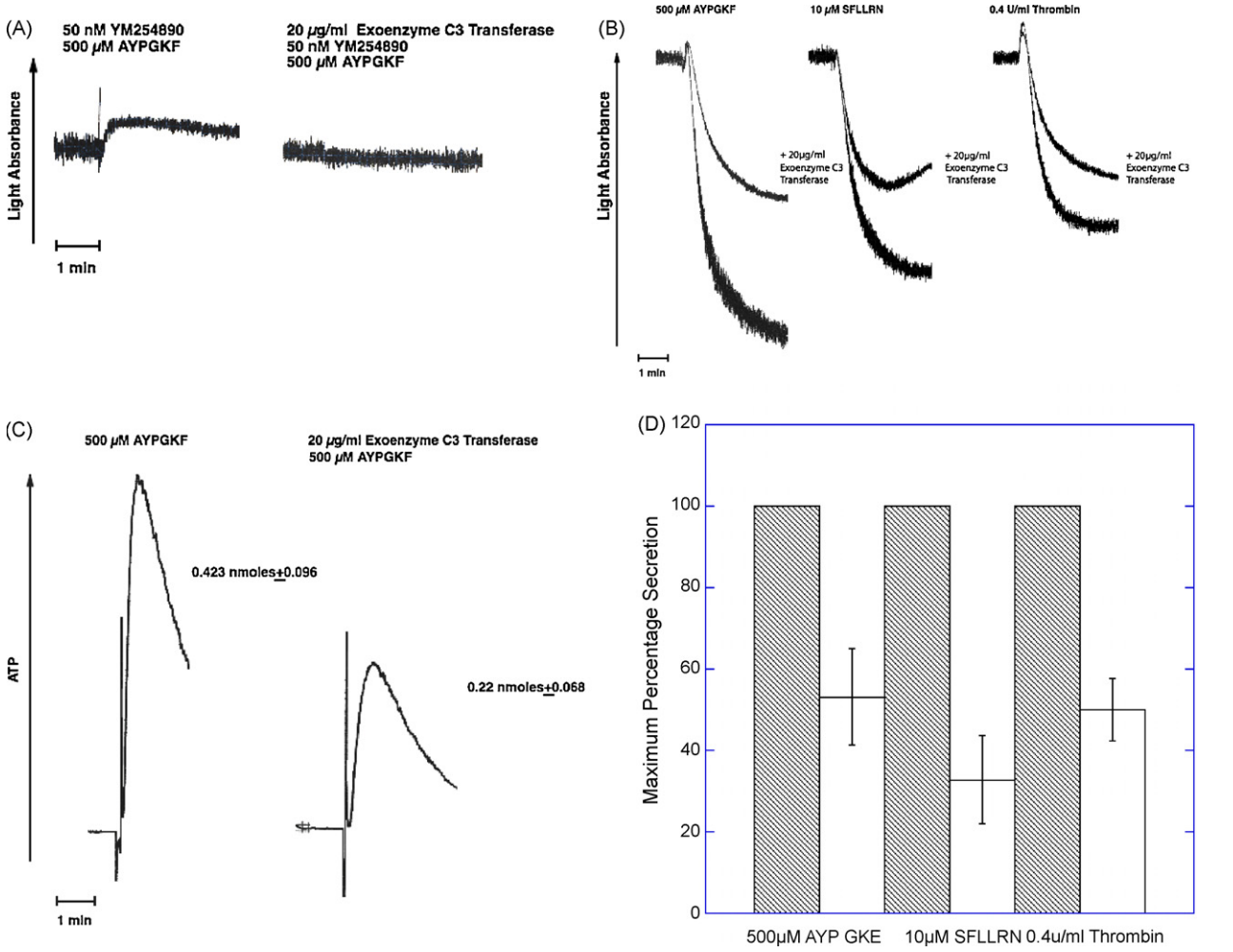

Fig. 4 – Effect of C3 transferase on PAR-mediated dense granule secretion in human platelets. Aspirin-treated and washed human platelets were stimulated with 500 μM AYPGKF, 10 μM SFLLRN or 0.2 U/ml thrombin at 37 °C while stirring. Platelet samples were incubated with 20 μg/ml exoenzyme C3 transferase at 37 °C for 4 h before the addition of agonists. Platelet shape change (A) aggregation (B) and secretion (C and D) were measured in a Lumi-aggregometer. The tracings are representative of data from at least three independent experiments. The secretion data in panel D were normalized to the maximum secretion (taken as 100%).

RhoA activation occurs upstream of PLC and downstream of G_q stimulation. These data clearly show that ADP, through the P2Y1 receptor, activates RhoA. Thus, RhoA activation occurs in platelets through both G_q and $G_{12/13}$ pathways.

3.5. *Regulation of protein kinase C δ by RhoA pathways*

We and others have shown that PAR-mediated dense granule release is regulated by the PKCδ isoform [44,45]. Hence we investigated whether $G_{12/13}$ pathways regulate dense granule release through regulating the activity of PKCδ, by analyzing the activation-dependent phosphorylation of the threonine 505 residue as a measure of the activation of PKCδ [46–50]. As shown in Fig. 6, exoenzyme C3 transferase inhibited PAR-mediated PKCδ Thr 505 phosphorylation, indicating that RhoA regulates PKCδ activity. As PKCδ is known to play a positive role in PAR-mediated dense granule release [45], $G_{12/13}$ pathways, through RhoA, thus regulate dense granule release by modulating PKCδ activity.

4. Discussion

In this study we addressed a long-standing question in platelet physiology regarding dense granule release. Of the several physiological platelet agonists, thrombin, thromboxane A2, and collagen cause platelet dense granule release independently of thromboxane generation, whereas other agonists, mainly ADP, do not cause dense granule secretion when thromboxane generation is blocked. The reason for this difference between ADP and strong agonists is not understood to date.

A number of platelet agonists stimulate phospholipase C β2 through G_q activation resulting in an increase in intracellular concentrations of inositol-1,4,5-trisphosphate and diacylglycerol [18,20]. The formation of inositol-1,4,5-trisphosphate induces an increase in the cytosolic concentration of Ca^{2+} while the release of diacylglycerol activates PKC isoforms [20,51,52]. The intracellular signaling events contributing to platelet secretion have been identified as an increase in

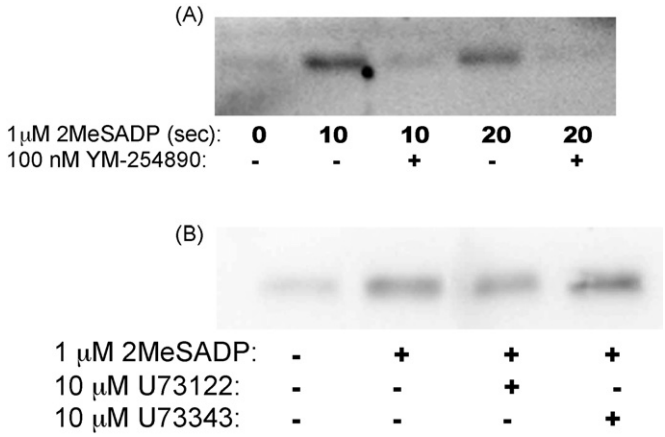

Fig. 5 – RhoA activation downstream of G_q pathways. (A) Aspirin-treated and washed human platelets were stimulated with 1 μM 2MeSADP at 37 °C for the indicated times with or without 5 min pre-incubation with the G_q inhibitor YM-254890. Active RhoA was then assayed using agarose-coupled GST-Rhotekin-RBD and Western blotting probing for RhoA. (B) GST-pull down of active RhoA was performed on lysates from unstimulated platelets or platelets pre-incubated with or without the PLC inhibitor U73122 or its control compound (U73343) for 8 min prior to activation with 2MeSADP for 10 s. Active RhoA was detected via Western blotting with an anti-Rho monoclonal antibody. The blot is representative of data from at least three independent experiments.

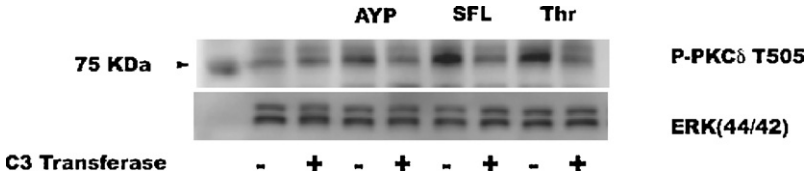

Fig. 6 – Effect of C3 transferase on PAR-mediated activation-dependent PKCδ phosphorylation. Aspirin-treated and washed human platelets were stimulated with 250 μM AYPGKF, 10 μM SFLLRN, or 0.2 U thrombin at 37 °C while stirring. Platelet samples were incubated with 20 μg/ml exoenzyme C3 transferase at 37 °C for 4 h before the addition of agonist. PKCδ activation was measured by detecting T-505 phosphorylation by Western blotting using phospho-specific antibodies. Total Erk1/2 was used as lane loading control. The blot is representative of data from at least three independent experiments.

intracellular calcium and activation of PKC, downstream events of phospholipase C activation [21,22]. We demonstrated that the PKCδ isoform plays an important role in regulating PAR-mediated dense granule secretion in human platelets [45]. Both G protein-coupled receptor (GPCR) and GPVI signaling pathways lead to activation of PKCδ. However, there are significant differences in the functional role of PKCδ downstream of PAR and GPVI signaling in platelets as demonstrated in our previous studies [45].

In human platelets deficient in G_q or PLCβ$_2$, thrombin and U46619 stimulation results in markedly decreased platelet secretion [18]. Similarly, G_q-deficient mouse platelets do not secrete in response to U46619 or thrombin [16]. Finally, collagen failed to cause dense granule release in PLCγ2-deficient mouse platelets [19]. These studies established the role of G_q, PLCβ$_2$, intracellular calcium increases, and PKC activation as important signaling molecules in U46619- or thrombin-induced platelet dense granule release. However, an important question remained unanswered. ADP, through activation of the P2Y$_1$ receptor stimulates G_q and PLCβ$_2$, and causes increases in intracellular calcium and activation of PKC [15,16,23,25,53], but fails to cause dense granule release in platelets under the conditions of thromboxane A$_2$ generation blockade [4]. What are the signaling differences between ADP and U46619 or thrombin that account for the lack of dense granule release by ADP?

It is known that G_q-signaling is required for platelet secretion [16]. Hence, we evaluated the role of $G_{12/13}$ pathways in dense granule release in platelets. We rationalized that because thromboxane A$_2$ and thrombin can activate *both* G_q and $G_{12/13}$, they cause platelet dense granule release, whereas ADP fails to cause platelet secretion from dense granules because it does not activate $G_{12/13}$.

We used three independent strategies to evaluate the effect of $G_{12/13}$ pathways on G_q and PLC-mediated effects in platelets. We have previously established the conditions for the use of YFLLRNP as a selective agonist for $G_{12/13}$ pathway stimulation in platelets [36]. Supplementing $G_{12/13}$ pathway activation resulted in dense granule release by ADP indicating that $G_{12/13}$ pathways contribute to platelet dense granule secretion. In addition, we blocked G_q pathways with YM254890 or used platelets from mice lacking $G\alpha_q$, and selectively stimulated $G_{12/13}$ pathways with AYPGKF. Under these conditions of selective $G_{12/13}$ stimulation, limited activation of PLC resulted

dense granule release, although limited PLC activation alone failed to cause dense granule secretion. The potentiation of ADP-induced platelet aggregation by $G_{12/13}$ pathways could be either because of their direct effect on fibrinogen receptor activation pathways [36] or due to positive feedback from the secreted ADP. These results demonstrated the role of $G_{12/13}$ pathways in dense granule release.

G_q pathways are absolutely required for dense granule release as this event does not occur in $G\alpha_q$ null mouse platelets or when G_q pathways are blocked with an inhibitor. Furthermore, when PLC is activated strongly by m-3M3FBS, we observed dense granule release without supplementation of $G_{12/13}$ pathways. These results indicate that when the G_q-PLC axis is strongly stimulated dense granule secretion occurs independently of $G_{12/13}$ pathways. Under these conditions, the $G_{12/13}$ pathways may not potentiate the dense granule release. This conclusion is consistent with the observations of Offermanns et al. that thrombin-induced dense granule release was unaffected in $G\alpha_{12}/G\alpha_{13}$ null mouse platelets [27]. We interpret that in these mouse platelets, strong stimulation of G_q pathways by thrombin eliminated the potentiating effect of $G_{12/13}$ pathways on dense granule release. We predict that a concentration–response curve in these mice would probably reveal a right ward shift compared to wild-type mouse platelets at lower concentrations.

Downstream of $G_{12/13}$ pathways RhoA was established as an important signaling molecule that has an effect in platelet shape change [43]. Our studies with a pharmacological inhibitor indicate that RhoA contributes to PAR-mediated dense granule release as well as platelet aggregation. We interpret that such effect could involve direct contribution to dense granule release and hence an apparent role in αIIbβ3 integrin activation. Previous studies from Leng et al. [54] have implied that RhoA does not contribute to SFLLRN-induced fibrinogen receptor activation. We believe that there may be some differences in the experimental conditions, including the reagent used, that explain the contradicting data from our lab and Leng et al. [54].

One question arises from these data is that why elimination of $G_{12/13}$ pathways in mice has no effect on maximal G_q-mediated dense granule release [27], but blockade of downstream signaling molecules, viz. RhoA results in an inhibition. Our studies have revealed for the first time that RhoA pathways could be activated downstream of G_q as well (Fig. 5). Hence, when platelets are stimulated with ADP, the RhoA pathway is activated contributing to calcium-independent platelet shape change. Thus, as outlined in Fig. 7, RhoA pathways are activated downstream of both G_q and $G_{12/13}$ pathways and maximal stimulation of G_q alleviates the need for these signaling molecules downstream of $G_{12/13}$ pathways. However, when pharmacological inhibitors of RhoA are used, they block these signaling molecules downstream of both G_q and $G_{12/13}$ and hence affect agonist-induced dense granule release. In the case of ADP, as it is a weak stimulator of G_q, a threshold levels of calcium and RhoA, that are required for dense granule release, are not reached. Hence, dense granule release does not occur until the threshold is reached by supplementing the signaling with $G_{12/13}$ pathways.

In conclusion, we have shown that $G_{12/13}$ pathways contribute to PKCδ activation and platelet dense granule release through activation of RhoA. The potentiating effect of $G_{12/13}$ pathways is maximal when G_q/PLC stimulation is

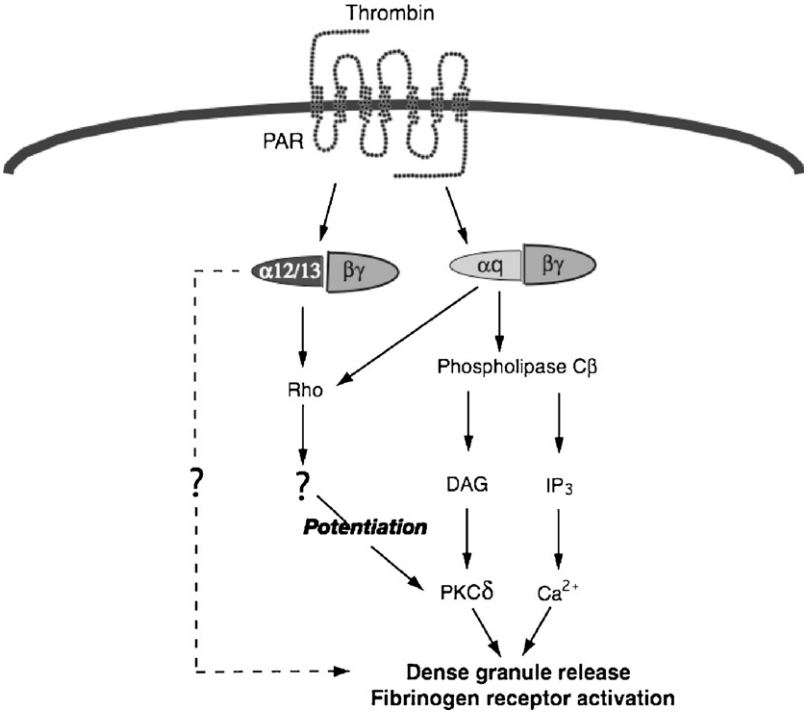

Fig. 7 – Model depicting RhoA pathways are activated downstream of both G_q and $G_{12/13}$ pathways. PAR activation by thrombin results in activation of G_q and $G_{12/13}$ pathways. $G_{12/13}$ pathways potentitate the G_q-mediated dense granule release and fibrinogen receptor activation. In the absence of G_q pathways these events do not occur. Maximum stimulation of G_q pathways by PARs can circumvent requirement of $G_{12/13}$ pathways due to possible direct activation of RhoA pathways.

limiting and becomes less important as the G_q/PLC pathways are maximally stimulated.

Acknowledgements

We thank Dr. Martin Schwartz, University of Virginia, for pGex-2T vector containing Rhotekin-Rho-binding domain. This work was supported by Research Grants HL81322, HL60683, and HL80444 from the National Institutes of Health (S.P.K.).

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