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

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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

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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