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CXCL12 regulates platelet activation via the regulator of G-protein signaling 16

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Abstract

The regulators of G protein signaling (RGS) protein superfamily negatively controls G protein-coupled receptor signal transduction pathways. One of the members of this family, RGS16, is highly expressed in megakaryocytes and platelets. Studies of its function in platelet and megakaryocyte biology have been limited, in part, due to lack of pharmacological inhibitors. For example, RGS16 overexpression inhibited CXC chemokine receptor 4 (CXCR4)-mediated megakaryocyte migration. More recent studies showed that the chemokine stromal cell-derived factor (SDF1α or CXCL12) regulates platelet function via CXCR4. Based on these considerations, the present study investigated the capacity of RGS16 to regulate CXCL12-dependent platelet function, using the RGS16 knockout mouse model (*Rgs16*$^{-/-}$). RGS16-deficient platelets had increased protease activated receptor 4 and collagen-induced aggregation, as well as increased CXCL12-dependent agonist-induced aggregation, dense and alpha granule secretion, integrin αIIbβ3 activation and phosphatidylserine exposure compared to those from WT littermates. CXCL12 alone did not stimulate aggregation or secretion in either RGS16-deficient or WT platelets. Furthermore, platelets from *Rgs16*$^{-/-}$ mice displayed enhanced phosphorylation of ERK and Akt following CXCL12 stimulation relative to controls. Finally, we also found that PKCδ is involved in regulating CXCL12-dependent activation of ERK and Akt, in the Rgs16-deficient platelets. Collectively, our findings provide the first evidence that RGS16 plays an important role in platelet function by modulating CXCL12-dependent platelet activation.

Keywords

Platelets; Signal transduction; Regulators of G-protein signaling; Regulator of G-protein signaling 16; Stromal cell-derived factor; CXCL12

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Conflict of interest statement

The authors have no conflicts of interest to declare.

1. Introduction

Platelets are blood cells essential for normal hemostasis, but “tight” regulation of platelet function is required to avoid the destructive effects of either inappropriate activation or excessive responses to injury. In addition to their well-recognized role in hemostasis and acute thrombus formation, platelets are also thought to have proinflammatory and growth-regulatory properties that contribute to progression of atherosclerosis [1,2]. Platelets provide the first response at the site of vascular injury. During this process, platelets release multiple growth factors and inflammatory mediators, including chemokines into the vascular micro-environment [3]. While platelets contain a number of chemokines (e.g., CXCL12) [4,5], their role in regulating platelet function is unclear.

G-protein coupled receptors (GPCRs) are essential for cell to cell communications and are targeted by 60% of all clinically approved drugs [6]. Ligand-activated GPCRs, including those for chemokines, act as a guanine nucleotide exchange factor for the Gα subunit of the heterotrimeric G protein [7,8] and are linked to Gαi and possibly Gαq [9]. The primary signal transduction of GPCRs, the heterotrimeric G protein complex of α, β, and γ subunits, induces pathway activation through GDP–GTP exchange on Gα and stimulation of numerous effectors including kinases, phospholipases, and ion channels [10,11]. The intrinsic GTPase activity of α subunit, which promotes Gα reassociation with βγ to form an inactive heterotrimer, terminates ligand-induced signaling. The regulators of G protein signaling (RGS) superfamily, which has >30 members in mammalian cells, negatively regulate G protein activity [12,13].

Platelets secrete the chemokine CXCL12, which activates platelets in an autocrine/paracrine manner [14]. However, the regulatory role RGS16 plays in CXCL12-dependent platelet function remains to be elucidated. We found that in the presence of CXCL12, platelets from *Rgs16*$^{-/-}$ mice exhibited significantly enhanced agonist dependent platelet aggregation, dense granule and alpha granule release, integrin αIIbβ3 activation and phosphatidylserine (PS)-exposure, relative to WT controls. Our findings support the notion that RGS16 is directly involved in modulating platelet function, and it does so, at least in part, by regulating CXCL12-dependent platelet activation.

2. Materials and methods

2.1. Reagents and materials

Anti-RGS16 was from Santa Cruz Biotechnology (Dallas, TX), anti-ERK, anti-phospho-ERK, anti-Akt, anti-phospho-Akt, FITC-conjugated Annexin V and anti-P-selectin were from Cell Signaling Technology (Beverly, MA). JON/A antibody was from emfret analytics (Würzburg, Germany). CXCL12 (SDF1α) was from Abcam (Cambridge, MA). PAR4 peptide (TRAP4) was from Peptides International Inc. (Louisville, KY). Apyrase, phorbol 12-myristate 13 acetate (PMA), prostaglandin I_2 (PGI_2), and appropriate secondary antibodies coupled to HRP were obtained from Sigma-Aldrich (St. Louis, MO). Gö6976 was from Calbiochem (San Diego, CA). Type I collagen, stir bars and other disposables were from Chrono-Log Corporation (Havertown, PA). U73122 was from Alexis Biochemicals (San Diego, CA). Other reagents were of analytical grade.

2.2. Animals and genotyping

Rgs16$^{-/-}$ mice were generated as described previously [15]. Mice were housed in groups of 1–4 at 24 °C, under 12/12 h light/dark cycles, with access to water and food ad libitum. All experiments involving animals were performed in compliance with the institutional guidelines, and were approved by the Western University of Health Sciences Institutional Animal Care and Use Committee. For genotyping, PCR was performed on tail snip DNA using following WT primers: sense: 5′-GAAGCCACCTTTTATGGAACGC-3′ and antisense 5′-TTCACAGACAGACAACAGGGTCC-3′ and KO primers: sense: 5′-AGTCCCGAATCCACTAACCCTC-3′ and antisense 5′-GGTGACCTATGTCCTCTACAGCAAG-3′ with the following PCR conditions: denaturation: 94 °C for 1 min, amplification: 96 °C for 10 s, 64 °C for 15 s, 72 °C for 15 s for 30 cycles and finally extension at 72 °C for 2 min. After completion of the PCR cycle, amplified DNA samples were run in 2% agarose gel and visualized in the gel documentation system. The expected products per genotype are as follows: WT: 390 bp, Het: 290/390 bp, and mutant: 290 bp.

2.3. Platelet preparation

Mouse blood was collected from the ventricle and the citrated (0.38%) blood was mixed with phosphate-buffered saline, pH 7.4, and incubated with PGI_2 (10 ng/mL; 5 min), followed by centrifugation at 237 ×*g* for 10 min at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483 ×*g* for 10 min at RT. The pellets were resuspended in HEPES/Tyrode's buffer (HT; 20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM D-glucose) supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_2. Platelets were washed and resuspended in HT (pH 7.4) without EGTA, apyrase, or PGI_2. Platelets were counted with an automated hematology analyzer (Drew Scientific Dallas, TX) and adjusted to the indicated concentrations.

2.4. In vitro platelet aggregation

PRP from *Rgs16*$^{-/-}$ and WT mice was stimulated with the following agonists: 100 nM CXCL12 alone, 10 μg collagen or 80 μM thrombin receptor activating peptide 4 (TRAP4; agonist for protease-activated receptor 4) alone or in combination with 100 nM CXCL12, as described before [14]. Platelet aggregation was measured by the turbidometric method using a model 700 aggregometry system (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

2.5. Dense granule release

Platelets were prepared as described above (250 μL; 2.5 × 10^8/mL) before being placed into siliconized cuvettes and stirred for 5 min at 37 °C at 1200 rpm. The luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log) was then added, followed by the addition of CXCL12 alone (100 nM), either collagen (10 μg) or TRAP4 (80 μM) alone or after mixing these two agonists with 100 nM CXCL12.

2.6. Alpha granule release, αIIbβ3 activation and PS-exposure by flow cytometry

Flow cytometric analysis was carried out as discussed before [16–18]. Briefly, platelets (2×10^8) were stimulated for 3 min with either 10 μg collagen or 80 μM TRAP4, after mixing them with 100 nM CXCL12. Platelets were incubated with FITC-conjugated anti-P-selectin, JON/A or Annexin V antibodies at room temperature for 30 min in the dark. The platelets were then diluted 2.5-fold with HEPES/Tyrode's buffer (pH 7.4). The samples were transferred to FACS-tubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

2.7. Immunoblotting studies

Platelet proteins were separated on SDS-PAGE gels and electrophoretically transferred to Immobilon-P PVDF membranes, as previously described [16,17,19]. The blots were incubated with different antibodies: anti-ERK, anti-phosphoERK, anti-Akt, and anti-phosphoAkt. Following washing, the blots were incubated with HRP-labeled anti-rabbit IgG or anti-mouse IgG as required. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

2.8. Statistical analysis

All experiments were performed at least three times. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SEM. Analysis was conducted using t-test. Significance was accepted at $P < 0.05$ (two-tailed P value), unless stated otherwise.

3. Results

3.1. RGS16 protein deletion

To assess and better understand the role of RGS16 in platelets, we undertook a genetic approach, and employed an *Rgs16*$^{-/-}$ mouse line. We first confirmed the absence of RGS16 in platelets from these mice by PCR and Western blotting experiments. PCR of genomic DNA confirmed genetic deletion of *Rgs16* (Fig. 1A). We detected RGS16 protein in platelets from WT but not *Rgs16*$^{-/-}$ mice (Fig. 1B). Deletion of RGS16 did not have any apparent impact on the protein expression level of other RGS proteins known to be expressed in platelets (i.e., RGS10 and RGS18). *Rgs16*$^{-/-}$ mice were visibly indistinguishable from their WT littermate—appearing healthy without anatomical abnormalities, reduced lifespan, or fertility defects.

3.2. CXCL12-dependent platelet aggregation is enhanced in Rgs16$^{-/-}$ mice

It is well established that platelets and megakaryocyte store CXCL12 [13,14], which regulates megakaryocyte migration and activation [13,14]. However, whether RGS16 directly modulates platelet function, and the regulatory relationship between RGS16 and CXCL12 in platelets remains unknown. To investigate these issues, we stimulated platelets using two different agonists – the GPCR-dependent TRAP4, and the GPCR-independent collagen – to investigate RGS16's canonical and non-canonical signaling mechanisms,

respectively. Hence, platelets from WT or *Rgs16*$^{-/-}$ mice were stimulated with CXCL12 alone, either collagen or TRAP4 alone or together with CXCL12. Platelets from either mouse did not aggregate when treated with CXCL12 alone (Fig. 2A). However, platelets from *Rgs16*$^{-/-}$ mice, displayed enhanced aggregation in response to collagen (10 μg) or TRAP4 (80 μM) (Fig. 2B and C), as well as in response to either of these two agonists plus CXCL12 (100 nM), compared to platelets from WT littermates (Fig. 2D and E); data quantification is shown in Fig. 2F and G). Together, these data clearly indicate that RGS16 protein directly and negatively regulates platelet aggregation induced by both GPCR-dependent and -independent signals.

3.3. CXCL12-induced platelet dense and alpha granule secretion is enhanced in platelets from Rgs16$^{-/-}$ mice

Platelet secretion is a very important and early event in platelet activation and is essential for the aggregation response. Thus, we examined agonist-induced dense and alpha secretion granule release by measuring ATP secretion and P-selectin expression, respectively, in the presence of CXCL12. CXCL12 alone did not stimulate dense granule secretion in platelets from either WT or RGS16-deficient mice (Fig. 3A). However, platelets from RGS16-deficient mice displayed enhanced dense granule secretion in response to collagen or TRAP4 (Fig. 3B and C), when compared to WT controls. Furthermore, platelets from RGS16-deficient mice displayed a further enhanced dense and alpha granule secretion in response to a mixture of collagen and CXCL12 (Fig. 3D and E), when compared to WT controls. We also observed enhanced dense and alpha granule release in response to TRAP4 plus CXCL12 (Fig. 3F and G) compared to WT. These results support the hypothesis that RGS16 regulates CXCL12-dependent platelet secretion.

3.4. RGS16 regulates CXCL12-dependent integrin αIIbβ3 activation and PS exposure in platelets

Since we observed that the CXCL12-dependent, agonist-induced aggregation response is enhanced in the platelets devoid of RGS16, we investigated whether there is commensurate increase in integrin αIIbβ3 activation, which is used as a separate index of aggregation. Using flow cytometry, we found that CXCL12 significantly enhances integrin αIIbβ3 activation induced by TRAP4 or collagen in WT platelets, and the response is amplified in platelets from *Rgs16*$^{-/-}$ mice (Fig. 4A–B). Next, we assessed if CXCL12 participates in the regulation of the exposure of the proaggregatory molecule PS to the outer leaflet of the platelet membrane. Indeed, we observed enhanced PS expression in the platelets from *Rgs16*$^{-/-}$ mice compared to WT Fig. 4C and D) under the same conditions. Together, these provide evidence that RGS16 regulates CXCL12-dependent platelet function, as well as the platelet procoagulant activity.

3.5. CXCL12 mediated and PKCδ dependent phosphorylation of ERK and Akt in platelets from Rgs16$^{-/-}$ mice

In the next set of experiments, we investigated how agonist-triggered signaling events downstream of GPVI and PAR4 are affected by the loss of RGS16. We examined phosphorylation of ERK and Akt because they have been previously shown to play a critical role in platelet function downstream of GPVI and PAR4 [20]. Platelets from *Rgs16*$^{-/-}$ mice

had increased Erk and Akt phosphorylation in response to CXCL12 plus TRAP4 or collagen compared to WT controls whereas responses to either agonist alone were similar (Fig. 5A, B and C; collagen data not shown). These results indicate that, in the presence of CXCL12, agonist-stimulated signaling events downstream of GPVI and PAR4 are also enhanced in an RGS16-dependent manner.

Also, it has been previously shown in platelets that agonist-mediated intracellular calcium is regulated by CXCL12 [14]. Since calcium leads to activation of PKC [19], we sought to gain more insight into CXCL12-dependent calcium signaling in the context of RGS16 and PKC. Given that ERK and Akt activation in platelets is known to be downstream of PKC [21–25], the next set of experiments sought to identify which isoform, if any, is involved in this process with regard to regulation by RGS16. Thus, using isoform-specific inhibitors, i.e., Gö6976 for α/β isoforms [26] and rottlerin for PKCδ [26], it was observed that Gö6976 (1 μM) did not produce any detectable effects on ERK and Akt phosphorylation when platelets from *Rgs16* gene-deleted mice were stimulated with a mixture of 80 μM TRAP4 and 100 nM CXCL12, whereas rottlerin (10 μM) did exert inhibitory effects (Fig. 5A, B and C). These data indicate that PKCδ, but not α/β, is involved in TRAP4-induced, CXCL12-dependent phosphorylation of ERK and Akt, in an RGS16-dependent fashion.

3.6. PMA-induced ERK and Akt phosphorylation is intact in the platelets from the Rgs16$^{-/-}$ mice

In control experiments, we assessed if the ERK and Akt responses would be enhanced in response to the PKC activator, PMA. As one might predict since PMA bypasses the G protein and directly activates PKC [27], we observed no detectable effects on ERK and Akt phosphorylation in platelets from the *Rgs16*$^{-/-}$ mice (Fig. 6A and B).

4. Discussion

The regulator of G-protein signaling 16 (RGS16) belongs to the B/R4 subfamily of RGS proteins [28] and is known to play essential regulatory roles (primarily) in the signaling of G protein-coupled receptors (GPCRs). Certain *RGS* genes co-evolved with those constituting the major histocompatibility complex and its paralogous genomic regions [12,29]. RGS16 is reportedly expressed by many cells in the immune system [30], and it is likely involved in the control of monocyte migration via its effects on GPCR signaling [31,32]. RGS16 may also modulate cytokine production during inflammatory responses [33] through inhibition of the MAPK and PI3K/Akt pathways [34–37]. Berthebaud et al. were the first to show that RGS16 is a negative regulator of CXCL12–CXCR4-mediated cell migration [13]. More recently, it was shown that CXCL12 is secreted by platelets, and acts in an autocrine manner through CXCR4 to potentiate platelet activation [14].

CXCL12 belongs to the chemokine family constitutively produced by bone marrow stromal cells and is the major chemo-attractant for hematopoietic stem cells [38] and is involved in the retention of hematopoietic precursor cells in the bone marrow [39,40]. The regulation of CXCL12-induced platelet activation, specifically, in the context of RGS16, is unknown. Our results revealed a gain of function phenotype in platelets from *Rgs16*$^{-/-}$ mice in the presence of CXCL12. Specifically, the aggregation response was found to be augmented when these

platelets were stimulated with a mixture of agonist (i.e., collagen and TRAP4) and CXCL12 compared to responses of WT platelets. This enhanced response was not limited to platelet aggregation, as the combination of CXCL12 and agonist induced dense and alpha granule secretion, integrin αIIbβ3 activation and PS-exposure were also found to be enhanced in platelets from *Rgs16*$^{-/-}$ mice. Of note, separate experiments indicated that CXCL12 alone does not have the capacity to stimulate the RGS16 null or WT platelets on its own, whereas platelet aggregation and dense granule secretion were enhanced in the RGS16-deficient platelets in the presence of agonists alone. Taken together, these data demonstrate that RGS16 regulates platelet function, at least in the context of CXCL12, and suggest that it plays a direct role in their activation. Furthermore, the fact that platelet activation induced by the non-GPCR-agonist collagen (GPVI-mediated; in the presence or absence of CXCL12) was also found to be enhanced in the RGS16-deficient platelets, suggests that RGS16 may play a non-canonical function in these cells. It is noteworthy that a non-canonical function for RGS proteins has also been documented in separate cell systems and with other RGS proteins [41,42].

It has been shown that CXCL12 is highly expressed in thrombotic coronary arteries after injury, which generates substantial localized levels that potentiate platelets [43]. Also, CXCL12 surface expression was found to be increased in platelets from individuals with acute coronary syndrome [44]. Therefore, these conditions will result in continuous exposure of platelets to the increased CXCL12 plasma concentrations, which acts as a priming agent, and therefore enhances their response to circulating agonists (e.g., thrombin) [45]. Given that CXCL12 enhances platelet function, our findings suggest that RGS16 serves to limit excessive platelet activation, thereby preventing the undesired consequences of an “over-activated” platelet, such as unnecessary vessel occlusion. In other words, while CXCL12 supports an efficient and a robust platelet response at sites of vascular injury, RGS16 acts as a brake to prevent excessive platelet responses occurring at sites of high intravascular CXCL12 concentrations.

Our studies also examined whether the enhanced platelet activation phenotype was also accompanied by enhanced activation of markers for platelet function. Indeed, we observed that, in the presence of CXCL12, agonist-induced phosphorylation of ERK and Akt was enhanced in RGS16-deficient platelets.

As for mechanistic insight, and given that PKC activation is known to regulate ERK and Akt phosphorylation, [23,24], we sought to investigate if it is involved in modulating CXCL12-RGS16-dependent platelet activation. Thus, using a pharmacological approach, we found that PKCδ is involved in agonist-induced, CXCL12-dependent and agonist-induced phosphorylation of platelet ERK and Akt, and that it does so in an RGS16-dependent fashion. However, this was not found to be the case when PMA was used as an agonist. This is not surprising because it is known to bypass G protein activation. The finding that PKCδ is the isoform involved may be surprising, but we speculate that it is due to, at least in part, hyper-stimulation of Gα/Gq as a result of lack of RGS16; which in turn depletes the typical downstream effector pool (PKCα/β), thereby resulting in the involvement of “non-canonical” PKC isoform. It is also possible, especially for non-GPCR pathways, that the PKC isoform involved may not depend on the G protein but rather vary from cell

to cell. Additional studies are, nonetheless, warranted to further delineate its signaling mechanisms in platelet biology. Collectively, the present studies highlight novel canonical and non-canonical roles for RGS16 in regulating platelet function, at least in the context of the chemokine CXCL12. Furthermore, our findings suggest that RGS16 may serve as a viable target for the management of thrombotic disorders.

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References

[1]. Page CP, Platelets as inflammatory cells, Immunopharmacology 17 (1989) 51–59. [PubMed: 2651354]

[2]. Ross R, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature 362 (1993) 801–809. [PubMed: 8479518]

[3]. Jonnalagadda D, Izu LT, Whiteheart SW, Platelet secretion is kinetically heterogeneous in an agonist-responsive manner, Blood 120 (2012) 5209–5216. [PubMed: 23086755]

[4]. Bubel S, Wilhelm D, Entelmann M, Kirchner H, Kluter H, Chemokines in stored platelet concentrates, Transfusion 36 (1996) 445–449. [PubMed: 8693510]

[5]. Kameyoshi Y, Dorschner A, Mallet AI, Christophers E, Schroder JM, Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils, J. Exp. Med 176 (1992) 587–592. [PubMed: 1380064]

[6]. Gonzalez-Maeso J, Anxious interactions, Nat. Neurosci 13 (2010) 524–526. [PubMed: 20421895]

[7]. Hepler JR, Gilman AG, G proteins, Trends Biochem. Sci 17 (1992) 383–387. [PubMed: 1455506]

[8]. Neer EJ, Heterotrimeric G proteins: organizers of transmembrane signals, Cell 80 (1995) 249–257. [PubMed: 7834744]

[9]. Kehrl JH, Chemoattractant receptor signaling and the control of lymphocyte migration, Immunol. Res 34 (2006) 211–227. [PubMed: 16891672]

[10]. Gilman AG, G proteins: transducers of receptor-generated signals, Annu. Rev. Biochem 56 (1987) 615–649. [PubMed: 3113327]

[11]. Marinissen MJ, Gutkind JS, G-protein-coupled receptors and signaling networks: emerging paradigms, Trends Pharmacol. Sci 22 (2001) 368–376. [PubMed: 11431032]

[12]. Bansal G, Druey KM, Xie Z, R4 RGS proteins: regulation of G-protein signaling and beyond, Pharmacol. Ther 116 (2007) 473–495. [PubMed: 18006065]

[13]. Berthebaud M, Riviere C, Jarrier P, Foudi A, Zhang Y, Compagno D, Galy A, Vainchenker W, Louache F, RGS16 is a negative regulator of SDF-1-CXCR4 signaling in megakaryocytes, Blood 106 (2005) 2962–2968. [PubMed: 15998835]

[14]. Walsh TG, Harper MT, Poole AW, SDF-1alpha is a novel autocrine activator of platelets operating through its receptor CXCR4, Cell. Signal 27 (2015) 37–46. [PubMed: 25283599]

[15]. Shankar SP, Wilson MS, DiVietro JA, Mentink-Kane MM, Xie Z, Wynn TA, Druey KM, RGS16 attenuates pulmonary Th2/Th17 inflammatory responses, J. Immunol 188 (2012) 6347–6356. [PubMed: 22593615]

[16]. Karim ZA, Vemana HP, Alshbool FZ, Lin OA, Alshehri AM, Javaherizadeh P, Paez Espinosa EV, Khasawneh FT, Characterization of a novel function-blocking antibody targeted against the platelet P2Y1 receptor, Arterioscler. Thromb. Vasc. Biol 35 (2015) 637–644. [PubMed: 25593131]

[17]. Karim ZA, Vemana HP, Khasawneh FT, MALT1-ubiquitination triggers non-genomic NF-kappaB/IKK signaling upon platelet activation, PLoS One 10 (2015), e0119363. [PubMed: 25748427]

[18]. Lin OA, Karim ZA, Vemana HP, Espinosa EV, Khasawneh FT, The antidepressant 5-HT2A receptor antagonists pizotifen and cyproheptadine inhibit serotonin-enhanced platelet function, PLoS One 9 (2014), e87026. [PubMed: 24466319]

[19]. Karim ZA, Zhang J, Banerjee M, Chicka MC, Al Hawas R, Hamilton TR, Roche PA, Whiteheart SW, IkappaB kinase phosphorylation of SNAP-23 controls platelet secretion, Blood 121 (2013) 4567–4574. [PubMed: 23613522]

[20]. Senis YA, Sangrar W, Zirngibl RA, Craig AW, Lee DH, Greer PA, Fps/Fes and Fer non-receptor protein-tyrosine kinases regulate collagen- and ADP-induced platelet aggregation, J. Thromb. Haemost 1 (2003) 1062–1070. [PubMed: 12871378]

[21]. Hadas K, Randriamboavonjy V, Elgheznawy A, Mann A, Fleming I, Methylglyoxal induces platelet hyperaggregation and reduces thrombus stability by activating PKC and inhibiting PI3K/Akt pathway, PLoS One 8 (2013), e74401. [PubMed: 24058557]

[22]. Jiang F, Jia Y, Cohen I, Fibronectin- and protein kinase C-mediated activation of ERK/MAPK are essential for proplateletlike formation, Blood 99 (2002) 3579–3584. [PubMed: 11986211]

[23]. Lee JJ, Han JH, Jung SH, Lee SG, Kim IS, Cuong NM, Huong TT, Khanh PN, Kim YH, Yun YP, Ma JY, Myung CS, Antiplatelet action of indirubin-3′-monoxime through suppression of glycoprotein VI-mediated signal transduction: a possible role for ERK signaling in platelets, Vasc. Pharmacol 63 (2014) 182–192.

[24]. Moroi AJ, Watson SP, Akt and mitogen-activated protein kinase enhance C-type lectin-like receptor 2-mediated platelet activation by inhibition of glycogen synthase kinase 3alpha/beta, J. Thromb. Haemost (2015).

[25]. Nadal-Wollbold F, Pawlowski M, Levy-Toledano S, Berrou E, Rosa JP, Bryckaert M, Platelet ERK2 activation by thrombin is dependent on calcium and conventional protein kinases C but not Raf-1 or B-Raf, FEBS Lett 531 (2002) 475–482. [PubMed: 12435596]

[26]. Karim ZA, Choi W, Whiteheart SW, Primary platelet signaling cascades and integrin-mediated signaling control ADP-ribosylation factor (Arf) 6-GTP levels during platelet activation and aggregation, J. Biolumin. Chemilumin 283 (2008) 11995–12003.

[27]. Bonilla L, O'Donnell VB, Clark SR, Rubbo H, Trostchansky A, Regulation of protein kinase C by nitroarachidonic acid: impact on human platelet activation, Arch. Biochem. Biophys 533 (2013) 55–61. [PubMed: 23500138]

[28]. Abramow-Newerly M, Ming H, Chidiac P, Modulation of subfamily B/R4 RGS protein function by 14–3-3 proteins, Cell. Signal 18 (2006) 2209–2222. [PubMed: 16839744]

[29]. Suurvali J, Robert J, Boudinot P, Ruutel Boudinot S, R4 regulators of G protein signaling (RGS) identify an ancient MHC-linked synteny group, Immunogenetics 65 (2013) 145–156. [PubMed: 23129146]

[30]. Kveberg L, Ryan JC, Rolstad B, Inngjerdingen M, Expression of regulator of G protein signalling proteins in natural killer cells, and their modulation by Ly49A and Ly49D, Immunology 115 (2005) 358–365. [PubMed: 15946253]

[31]. Dohlman HG, Thorner J, RGS proteins and signaling by heterotrimeric G proteins, J. Biolumin. Chemilumin 272 (1997) 3871–3874.

[32]. Ross EM, Wilkie TM, GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins, Annu. Rev. Biochem 69 (2000) 795–827. [PubMed: 10966476]

[33]. Shi GX, Harrison K, Han SB, Moratz C, Kehrl JH, Toll-like receptor signaling alters the expression of regulator of G protein signaling proteins in dendritic cells: implications for G protein-coupled receptor signaling, J. Immunol 172 (2004) 5175–5184. [PubMed: 15100254]

[34]. Buckbinder L, Velasco-Miguel S, Chen Y, Xu N, Talbott R, Gelbert L, Gao J, Seizinger BR, Gutkind JS, Kley N, The p53 tumor suppressor targets a novel regulator of G protein signaling, Proc. Natl. Acad. Sci. U. S. A 94 (1997) 7868–7872. [PubMed: 9223279]

[35]. Liang G, Bansal G, Xie Z, Druey KM, RGS16 inhibits breast cancer cell growth by mitigating phosphatidylinositol 3-kinase signaling, J. Biolumin. Chemilumin 284 (2009) 21719–21727.

[36]. Vasilatos SN, Katz TA, Oesterreich S, Wan Y, Davidson NE, Huang Y, Crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases mediates antineoplastic efficacy of

HDAC inhibitors in human breast cancer cells, Carcinogenesis 34 (2013) 1196–1207. [PubMed: 23354309]

[37]. Wiechec E, Wiuf C, Overgaard J, Hansen LL, High-resolution melting analysis for mutation screening of RGSL1, RGS16, and RGS8 in breast cancer, Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 202011 397–407.

[38]. Nagasawa T, Kikutani H, Kishimoto T, Molecular cloning and structure of a pre-B-cell growth-stimulating factor, Proc. Natl. Acad. Sci. U. S. A 91 (1994) 2305–2309. [PubMed: 8134392]

[39]. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC, The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood, J. Exp. Med 185 (1997) 111–120. [PubMed: 8996247]

[40]. Broxmeyer HE, Kohli L, Kim CH, Lee Y, Mantel C, Cooper S, Hangoc G, Shaheen M, Li X, Clapp DW, Stromal cell-derived factor-1/CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells, J. Leukoc. Biol 73 (2003) 630–638. [PubMed: 12714578]

[41]. Derrien A, Druey KM, RGS16 function is regulated by epidermal growth factor receptor-mediated tyrosine phosphorylation, J. Biolumin. Chemilumin 276 (2001) 48532–48538.

[42]. Sethakorn N, Yau DM, Dulin NO, Non-canonical functions of RGS proteins, Cell. Signal 22 (2010) 1274–1281. [PubMed: 20363320]

[43]. Massberg S, Konrad I, Schurzinger K, Lorenz M, Schneider S, Zohlnhoefer D, Hoppe K, Schiemann M, Kennerknecht E, Sauer S, Schulz C, Kerstan S, Rudelius M, Seidl S, Sorge F, Langer H, Peluso M, Goyal P, Vestweber D, Emambokus NR, Busch DH, Frampton J, Gawaz M, Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo, J. Exp. Med 203 (2006) 1221–1233. [PubMed: 16618794]

[44]. Stellos K, Bigalke B, Langer H, Geisler T, Schad A, Kogel A, Pfaff F, Stakos D, Seizer P, Muller I, Htun P, Lindemann S, Gawaz M, Expression of stromal-cell-derived factor-1 on circulating platelets is increased in patients with acute coronary syndrome and correlates with the number of CD34+ progenitor cells, Eur. Heart J 30 (2009) 584–593. [PubMed: 19109356]

[45]. Gresele P, Falcinelli E, Momi S, Potentiation and priming of platelet activation: a potential target for antiplatelet therapy, Trends Pharmacol. Sci 29 (2008) 352–360. [PubMed: 18539343]

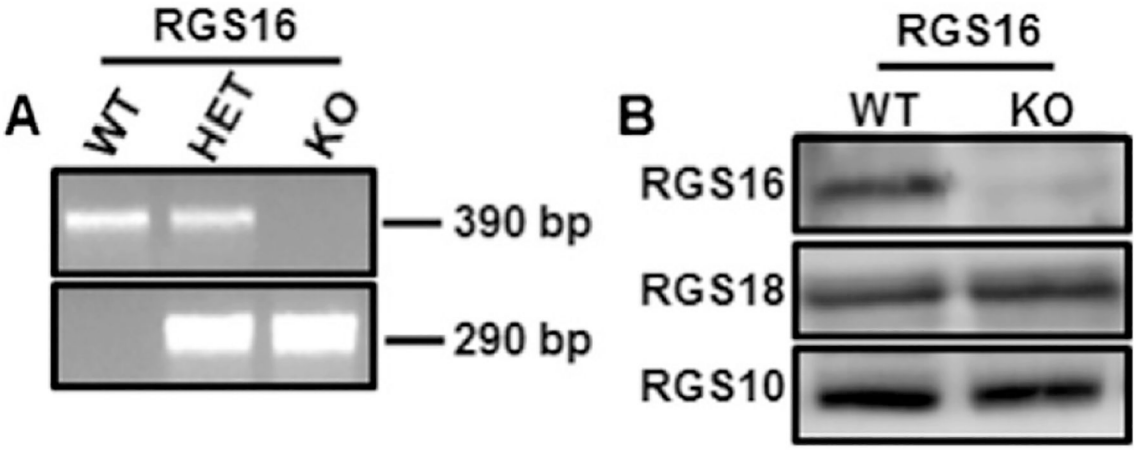

Fig. 1.

Deletion of RGS16 in platelets. (A) DNA was isolated and amplified by using PCR as described in the “Materials and methods” section. Amplified DNA were separated in 2% agarose gel and visualized in the gel documentation system (B) Platelet extracts (2×10^8/mL) were prepared from wild type (WT) and *Rgs16*$^{-/-}$ mice and the indicated proteins were detected by Western blotting.

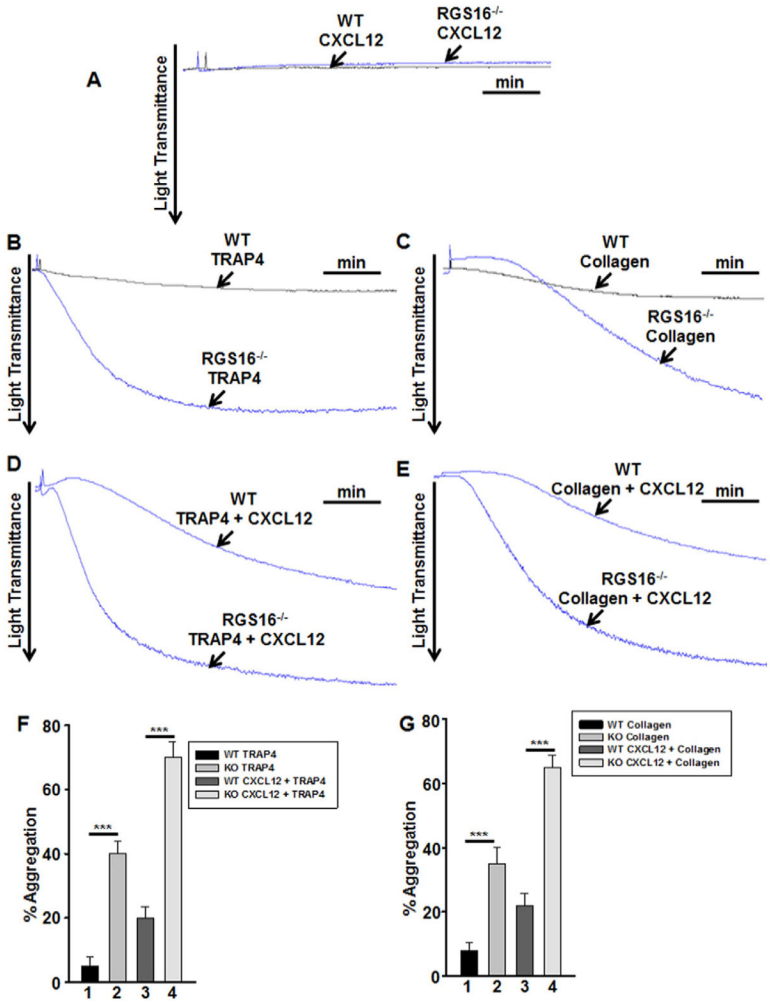

Fig. 2.

CXCL12 enhances platelet activation in RGS16-deficient platelets. Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared (2.5 × 10^8/mL), stimulated with either (A) CXCL12 alone, (B) TRAP4 (80 μM), or (C) collagen (10 μg) alone. Also platelets were stimulated with (D) TRAP4 peptide (80 μM) mixed with CXCL12 (100 nM) or (E) collagen (10 μg) mixed with CXCL12 (100 nM). Platelet aggregation was measured with constant stirring. These data are quantified in (F) and (G). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice (***P < 0.001, Mann–Whitney test).

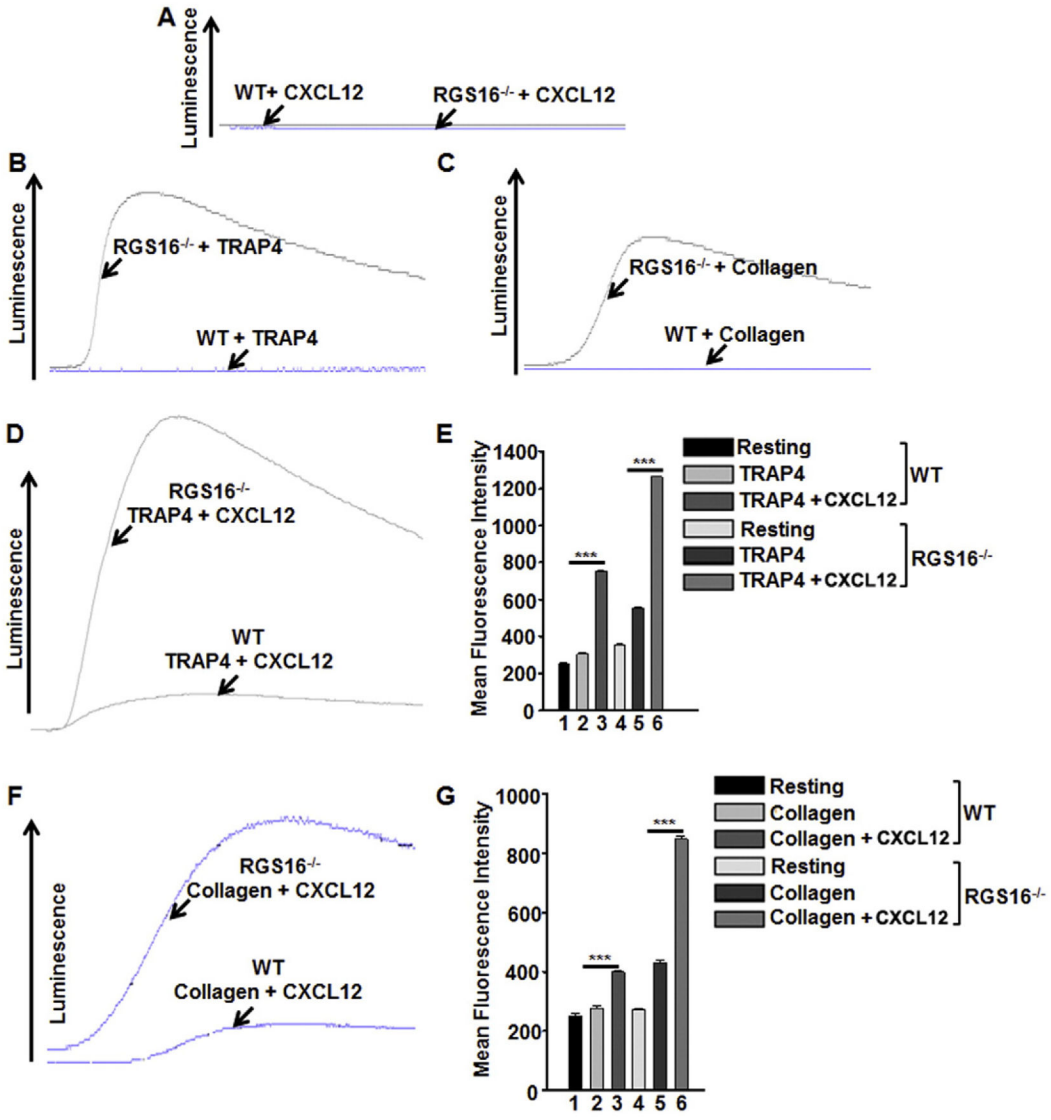

Fig. 3.

Dense and alpha granule secretion in response to CXCL12 and platelet agonists are enhanced in Rgs16$^{-/-}$ platelets. Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared (2.5 × 10^{8}/mL), stimulated with either (A) CXCL12 alone, (B) TRAP4 (80 μM) or (C) collagen (10 μg) alone. Also platelets were stimulated with (D) TRAP4 peptide (80 μM) mixed with CXCL12 (100 nM), (F) collagen (10 μg) mixed with CXCL12 (100 nM) for 3 min. Release of ATP (for dense granule release) as a luminescence was measured by aggregometer. Each experiment was repeated at least 3 times (***P < 0.001, Mann–Whitney test). P-selectin (for alpha granule release) expression was measured by flow cytometry. Washed platelets were stimulated with either (E) TRAP4 peptide (80 μM) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test) or (G) collagen (10 μg) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test) for 3 min. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Platelets were incubated with

FITC-conjugated anti-P-selectin antibody (for alpha granule). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

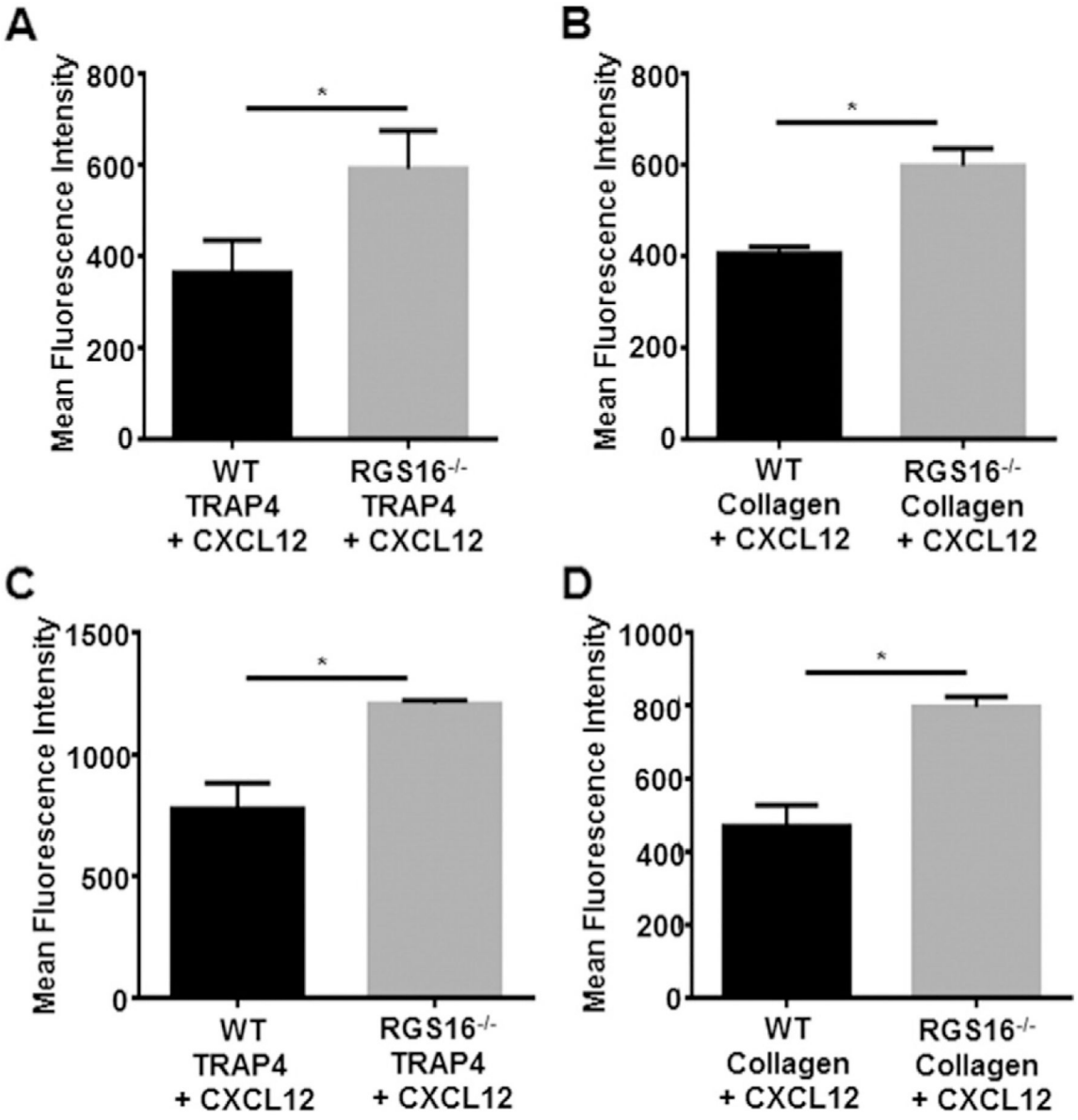

Fig. 4.

CXCL12 mediated integrin αIIβ3 activation and PS-exposure are increased in RGS16-deficient platelets. Integrin αIIbβ3 expression was measured by flow cytometry. Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared (2.5×10^8/mL), stimulated with either (A) TRAP4 peptide (80 μM) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test) or (B) collagen (10 μg) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test) for 3 min. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Platelets were incubated with FITC-conjugated anti-JON/A antibody (αIIbβ3). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice. PS exposure was measured by flow cytometer. Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared (2.5×10^8/mL), stimulated with either (C) TRAP4 peptide (80 μM) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test)

or (D) collagen (10 μg) mixed with CXCL12 (100 nM; *P < 0.05, Mann–Whitney test) for 3 min. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Platelets were incubated with FITC-conjugated anti-Annexin V antibody (PS expression). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

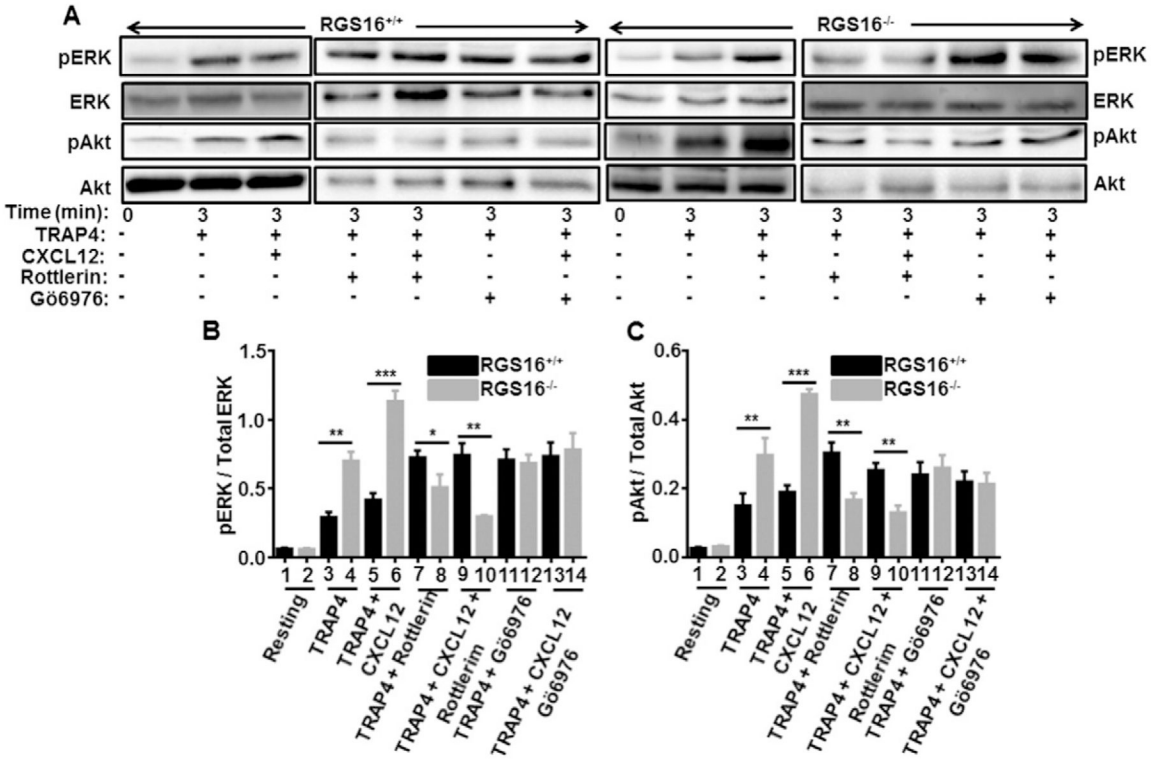

Fig. 5.

CXCL12 mediated and PKC δ dependent phosphorylation of ERK and Akt in *Rgs16*$^{-/-}$ platelets. (A-B) Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared, count adjusted to 2.5 × 10^8/mL, preincubated with rottlerin (10 μM) or Gö6976 (1 μM) for 3 min and stimulated with TRAP4 (80 μM); and TRAP4 (80 μM) mixed with CXCL12 (100 nM) for 3 min and proteins were lysed using 5× sample buffer. Proteins were separated by SDS-PAGE and immunoblotted using antibodies to pERK, ERK, pAkt, and Akt (*P < 0.05, **P < 0.01 and ***P < 0.001 Mann–Whitney test). Bar graphs in (B–C) represent densitometric analysis of protein phosphorylation (mean ± S.E.M. of three independent experiments).

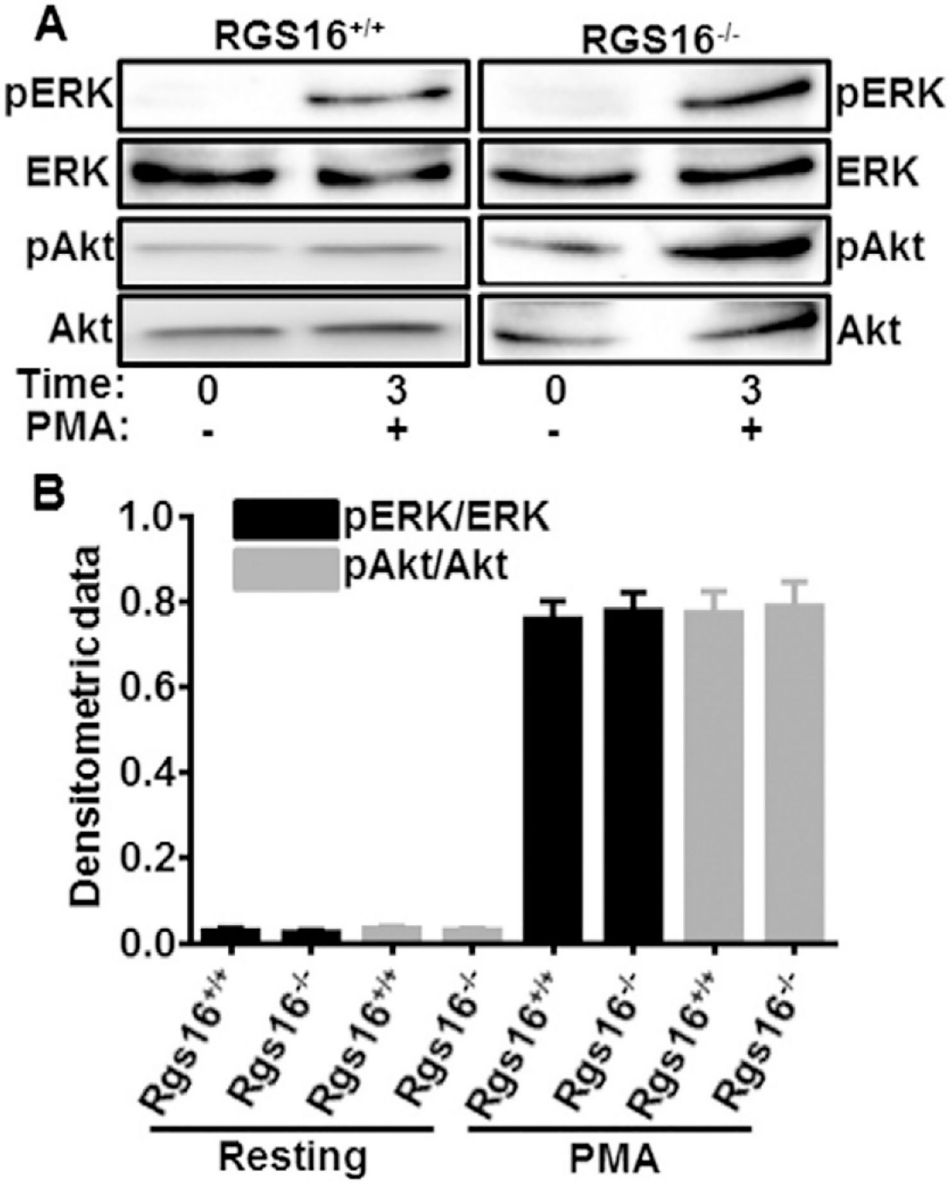

Fig. 6.

PMA induced phosphorylation of ERK and Akt in *Rgs16*$^{-/-}$ platelets. Platelets from WT and *Rgs16*$^{-/-}$ mice were prepared (2.5×10^8/mL), stimulated with PMA (100 nM) for 3 min and proteins were lysed using 5× sample buffer. Proteins were separated by SDS-PAGE and immunoblotted using antibodies to pERK, ERK, pAkt, and Akt.