Cytohesin-2 phosphorylation by protein kinase C relieves the constitutive suppression of platelet dense granule secretion by ADP-ribosylation factor 6

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Summary. Background: Protein kinase C (PKC) is a major regulator of platelet function and secretion. The underlying molecular pathway from PKC to secretion, however, is poorly understood. By a proteomics screen we identified the guanine nucleotide exchange factor cytohesin-2 as a candidate PKC substrate. Objectives: We aimed to validate cytohesin-2 as a PKC substrate in platelets and to determine its role in granule secretion and other platelet responses. Methods and results: Immunoprecipitation was performed with a phosphoserine PKC substrate antibody followed by mass spectrometry, leading to the identification of cytohesin-2. By western blotting we showed that different agonists induced cytohesin-2 phosphorylation by PKC. Protein function was investigated using a pharmacological approach. The cytohesin inhibitor SecinH3 significantly enhanced platelet dense granule secretion and aggregation, as measured by lumi-aggregometry. Flow cytometry data indicate that α-granule release and integrin αIIbβ3 activation were not affected by cytohesin-2 inhibition. Lysosome secretion was assessed by a colorimetric assay and was also unchanged. As shown by western blotting, ARF6 interacted with cytohesin-2 and was present in an active GTP-bound form under basal conditions. Upon platelet stimulation, this interaction was largely lost and ARF6 activation decreased, both of which could be rescued by PKC inhibition. Conclusions: Cytohesin-2 constitutively suppresses platelet dense granule secretion and aggregation by keeping ARF6 in a GTP-bound state. PKC-mediated phosphorylation of cytohesin-2 relieves this inhibitory effect, thereby promoting platelet secretion and aggregation.

Keywords: ADP-ribosylation factor 6; cytohesin-2; platelets; protein kinase C; secretion.

Introduction

Platelet function and secretion are critically regulated by protein kinase C (PKC), which is activated downstream of a multitude of cell surface receptors [1]. The conventional isoforms PKCα and PKCβ are the major positive regulators of platelet function in human and mouse platelets [2,3]. PKCα knockout mice show reduced thrombus formation and platelet secretion, suggesting PKCα to be critically involved in these processes [4]. A similar role has been reported for the highly related PKCβ [5]. The mechanism by which PKC regulates platelet secretion is unclear. When activated, conventional PKC isoforms preferentially phosphorylate substrates containing serine or threonine within a defined consensus sequence, with arginine or lysine at the −3, −2 and +2 positions, and hydrophobic amino acids at position +1 [6]. A few PKC substrates in platelets are well characterized, such as pleckstrin [7] and MARCKS [8]. More recently, we showed that SHP-1 is phosphorylated by PKC [9] and that GSK3 and PDE3A are regulated in a PKC-dependent manner [10,11]. However, the majority of proteins phosphorylated by conventional PKC isoforms, and their relationship to platelet secretion, are presently undefined.

Using an anti-phosphopeptide antibody-based approach followed by a proteomics screen we identified cytohesin-2, also known as ARF nucleotide-binding site opener (ARNO), as a candidate conventional PKC substrate in platelets. Cytohesin-2 is a guanine-nucleotide exchange factor (GEF) for the small GTPase called ADP-ribosylation factor 6 (ARF6). The conserved SEC7 domain catalyses GDP release from, and GTP binding to, ARF6, resulting in its activation [12]. ARF6 regulates vesicle trafficking [13].
and has been shown to be located at the membrane and to regulate exocytosis in chromaffin cells [14], adipocytes [15] and neuroendocrine cells [16,17]. More importantly, ARF6 has been reported to be involved in collagen-induced platelet aggregation and spreading, and ARF6-GTP levels decrease upon stimulation of platelet activation in a PKC-dependent manner [18,19]. In cell lines, PKC phosphorylates Ser392 of cytohesin-2, causing its translocation to the cytosol [20]. Anti-cytohesin-2 antibodies were reported to inhibit catecholamine secretion in chronomorph cells [21] and cytohesin-2 co-localized with proteins involved in exocytosis in neuroendocrine cells [22]. The exact mechanism of cytohesin-2/ARF6 regulation in platelets, however, has not been established.

The aims of our study were to elucidate how PKC regulates the cytohesin-2/ARF6 pathway and to examine the role of this pathway in granule secretion and function. Here we show that cytohesin-2 has a negative regulatory role in resting platelets, by keeping ARF6 in an activated GTP-bound state, which suppresses granule secretion and aggregation. Upon platelet activation, phosphorylation of cytohesin-2 by PKC causes it to dissociate from ARF6, which is then converted into its inactive GDP-bound form. This results in the relief of platelet inhibition, allowing granule secretion and aggregation to occur.

Materials and methods

Materials

The phospho-(Ser) PKC substrate (pSer PKC substrate) antibody was from Cell Signaling Technology (Danvers, MA, USA). The cytohesin-2 western blot antibody, ARF6 antibody and GAPDH antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cytohesin-2 immunoprecipitation antibody was from Thermo Scientific (Loughborough, Leicestershire, UK). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit, anti-mouse and anti-goat secondary antibodies were from Jackson ImmunoResearch Laboratories (Newmarket, UK). The PKC inhibitor bisindolylmaleimide (BIM) IX (Ro 31-8220) and the inactive analogue BIM V were from Calbiochem (Nottingham, UK). The PKC inhibitors BIM I (GF 109203X), Go 6983 and ruboxistaurin (LY 333531) and the cytohesin-2 inhibitor SecinH3 were from Tocris (Eidelberg, Germany). Luciferin-luciferase was from Chronolog (Lambeds, Stockport, UK). NuPAGE LDS sample buffer was from Invitrogen (Carlsbad, CA, USA). ECL reagent was from GE Healthcare (Amersham, UK). All other reagents were from Sigma-Aldrich (Poole, UK).

Human platelet preparation

Human blood was drawn from healthy volunteers, under local ethics committee agreement and after fully informed consent, and washed platelets were prepared as described previously [23]. In brief, blood anticoagulated with 0.4% (v/v) trisodium citrate and acidified with 16% (v/v) acidic citrate dextrose (85 mM trisodium citrate, 71 mM citric acid, 111 mM glucose) was centrifuged at 180 ×g for 17 min. The platelet-rich plasma was subsequently centrifuged at 650 ×g for 10 min in the presence of 10 μM indomethacin and 0.02 U mL⁻¹ apyrase. Platelets were resuspended to the required density in HEPES-Tyrode's buffer pH 7.2 (10 mM HEPES, 145 mM NaCl, 3 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄), modified with 0.1% (w/v) glucose, 10 μM indomethacin and 0.02 U mL⁻¹ apyrase. Platelets for use in immunoprecipitation (IP) studies were double washed.

Mouse platelet preparation

A colony of PKCα knockout (PKCα⁻/⁻) mice was kindly provided by Professor J. Molkentin (Cincinnati Children's Hospital, USA). Littermate PKCα wild-type (WT) mice were used as controls. Animals were sacrificed by CO₂ asphyxiation and blood was drawn by cardiac puncture under terminal anesthesia into 0.4% trisodium citrate. Blood was acidified with 20% ACD, diluted with 500 volume of ice-cold 2× RIPA buffer pH 7.4 (25 mM HEPES, 200 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM sodium β-glycerol phosphate, 10 mM sodium pyrophosphate, 1 mM benzamidine), NP40 buffer pH 7.5 (25 mM HEPES, 120 mM NaCl, 1 mM EDTA, 1% NP40, 20 mM sodium β-glycerol phosphate, 10 mM sodium pyrophosphate, 1 mM benzamidine) or ARF buffer pH 7.5 (50 mM Tris, 150 mM NaCl, 1% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl₂), respectively, to which protease inhibitors were added. Cell extracts were centrifuged at 10 000 ×g at 4 °C, and the supernatant was taken for subsequent analysis.
lysate), platelets were lysed in 4× NuPAGE LDS sample buffer, which was supplemented with 50 mM dithiothreitol (DTT).

**IP and ARF-GTP pull down**

Protein A and G sepharose beads were used for IP studies with rabbit and mouse antibodies, respectively. The ARF activation was assessed as described previously [18]. In brief, GST-GGA3 fusion proteins, which specifically bind ARF-GTP, coupled to glutathione-agarose beads were prepared by E. Aitken in our laboratory. 250 μL platelet lysate was incubated overnight under constant rotation at 4 °C with 10 μL beads and, in the case of IP, 10 μL antibody. Beads were washed three times in 1× lysis buffer and bound proteins were eluted in 2× NuPAGE LDS sample buffer, which was supplemented with 50 mM DTT at 70 °C for 10 min.

**Electrophoresis and immunoblotting**

Samples were separated by SDS-PAGE on 10% polyacrylamide gels. Proteins were transferred at 100 V for 1 h to PVDF membranes in transfer buffer (22.5 mM Tris, 172.5 mM glycine, 20% methanol). The membranes were blocked using 1× Sigma blocking buffer or, in the case of ARF6 blotting, 1% milk in Tris-buffered saline with Tween (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween). Blots were probed with primary and horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using ECL reagents. Membranes were stripped in stripping buffer pH 6.8 (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol) and reprobed as appropriate.

**Platelet aggregation and ATP secretion assay**

Platelet aggregation and ATP secretion from dense granules were monitored simultaneously at 37 °C under constant stirring. Platelets (2 × 10^8 mL^-1) were treated for 15 min with vehicle or 15 μM SecinH3 prior to stimulation for 15 min and centrifuged at 650 × g for 5 min. Supernatants were taken and incubated with 20 μL of substrate (5 mM nitrophenyl-acetyl-glucosaminide) in citrate phosphate buffer pH 4.2 (0.2 M Na_2HPO_4, 0.1 M citric acid) in a 96-well plate. The reaction was stopped by the addition of 200 μL of 0.1 M NaOH. β-hexosaminidase release from lysosomes was assessed by measuring samples at 405 nm using a microplate spectrophotometer.

**PKC phosphorylates cytohesin-2 in platelets**

We used the commercially available phospho-(Ser) PKC substrate (pSer PKC substrate) antibody, raised against a set of peptides that correspond to conventional PKC consensus phosphorylation sites, to pull down potential non-isomeric conventional PKC substrates in thrombin-stimulated platelets. One of the proteins identified by mass spectrometry was cytohesin-2, a GEF for ARF6. Interestingly, Serine 392, which is located at the C-terminus of cytohesin-2, lies perfectly within the consensus phospho-motif of the pSer PKC substrate antibody (Fig. 1A). First, we validated that PKC phosphorylates cytohesin-2 in platelets upon cellular activation by immunoblotting pSer PKC substrate antibody immunoprecipitations for cytohesin-2. Cytohesin-2 was phosphorylated in platelets after stimulation with thrombin (Fig. 1B), collagen-related peptide (CRP) (Fig. 1D) and the thromboxane A2 mimetic U46619 (Fig. 1F), reaching a maximum at 2 min. A low agonist concentration was also able to induce cytohesin-2 phosphorylation (Figure S1). To address whether this phosphorylation event was dependent on PKC, platelets were pre-treated with the broad-spectrum PKC inhibitor BIM IX. PKC inhibition blocks platelet dense granule secretion, and thereby also the release of ADP, which mediates autocrine P2Y12 signaling. To avoid any potential side-effects resulting from the blockage of PKC-mediated autocrine-produced ADP, platelets were stimulated in the presence of ADP. Incubation of platelets with ADP alone did not cause cytohesin-2 phosphorylation (Figure S1). Phosphorylation...
of cytohesin-2 induced by thrombin (Fig. 1C), CRP (Fig. 1E) and U46619 (Fig. 1G) was completely abolished by PKC inhibition.

In addition to BIM IX, other pharmacological inhibitors of PKC were used to assess phosphorylation of cytohesin-2. The broad-spectrum PKC inhibitors BIM I and Go 6983, as well as the PKCα/β selective inhibitor ruboxistaurin [24], blocked phosphorylation of cytohesin-2 upon stimulation (Fig. 2A). These results suggest that cytohesin-2 phosphorylation is mediated through the conventional PKC isoform PKCα/β. Moreover, the inactive analogue (BIM V) did not affect cytohesin-2 phosphorylation, thereby serving as a control for non-specific effects. In addition, to further explore which PKC isoform is responsible for the phosphorylation of cytohesin-2, we performed similar experiments using PKCα knockout (PKCα−/−) mouse platelets (Fig. 2B). Cytohesin-2 phosphorylation was, however, not affected in PKCα−/− platelets, but was blocked by ruboxistaurin, suggesting that PKCβ may be principally responsible for the phosphorylation of cytohesin-2.

**Cytohesin-2 inhibition enhances dense granule secretion and aggregation**

To investigate the role of cytohesin-2 in platelet function, we used the pharmacological ARF-GEF inhibitor SecinH3, which displays selectivity for the cytohesin family, mainly cytohesin-2 [25]. SecinH3 was shown to potently inhibit receptor-mediated ARF6 activation in multiple cells, including platelets [26–29].

We incubated platelets with SecinH3 prior to stimulation with thrombin and CRP to analyze the function of the cytohesin-2/ARF6 pathway. Platelet luminometry and
PKC plays a central role in platelet granule secretion, but the downstream pathways that regulate this process are poorly understood. In this study, we aimed to elucidate the underlying mechanism by which PKC regulates secretion and established a novel mechanism of PKC-regulated dense granule secretion in human platelets (Fig. 6).
**Fig. 3.** Cytohesin-2 inhibition by SecinH3 enhances dense granule secretion and platelet aggregation. Washed platelets (2 x 10^8 mL⁻¹) were treated for 15 min with 0.2% DMSO vehicle (Veh) or 15 μM SecinH3 (SecH3). ATP release from dense granules (A, C) was assessed by luminometry, simultaneously with platelet aggregation (B, D). Platelets were stimulated with the indicated concentrations of α-thrombin (αT) (A, B) or CRP (C, D). The bar graphs (i, mean ± SEM, n ≥ 3) show ATP release (nmol ATP/10⁸ plts, A, C) and aggregation (% of maximal aggregation, B, D), whereas ii–iv (A–D) show representative traces. *P < 0.05 (Student’s paired t-test).

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We used a proteomic approach with an anti-PKC substrate antibody to identify novel PKC substrates that may be involved in platelet activation, and identified cytohesin-2 as a novel conventional PKC substrate. Cytohesin-2 becomes phosphorylated in a PKC-dependent manner upon platelet activation by thrombin, CRP and the TxA2 analogue U46619. Using different PKC inhibitors we confirmed that the conventional PKC isoforms α and β are responsible for the phosphorylation of cytohesin-2. Interestingly, cytohesin-2 phosphorylation was unchanged in αIIbβ3 knockout mouse platelets, suggesting that cytohesin-2 phosphorylation may be mediated by PKCβ. However, a redundant role for PKCα cannot be excluded.

As cytohesin-2 is a GEF for the small GTPase ARF6, PKC phosphorylation of cytohesin-2 is likely to be involved in regulating ARF6 activity in human platelets. The regulation and activity of ARF6 in platelets, however, is not firmly established. Choi et al. (2006) found that ARF6-GTP is present in resting platelets and diminishes rapidly upon activation with collagen or thrombin, thereby allowing collagen-induced aggregation, platelet adherence and spreading [18,19]. In contrast, Kanamarlapudi et al. (2012) reported that activation of human platelets with ADP promoted a transient but robust increase in ARF6-GTP levels [27], similar to findings in pancreatic β-cells [26]. Our findings correspond to the observations of Choi et al. (2006), showing that under basal conditions ARF6-GTP levels are maximal and decrease upon platelet stimulation with thrombin. Importantly, we also demonstrated that ARF6 interacted with cytohesin-2 in resting platelets and that this interaction decreased upon platelet stimulation with thrombin. Previous publications have described that phosphorylation of cytohesin-2 by PKC diminishes its membrane affinity, causing it to translocate from the membrane [20,31]. As ARF6 has been shown to be predominantly membrane-bound, this translocation in turn leads to the dissociation of cytohesin-2 from ARF6, resulting in the inactivation of ARF6. The mechanism in platelets may differ slightly, because although we were able to show that phosphorylation of cytohesin-2 by PKC affects its interaction with ARF6 and the activation state of ARF6, cytohesin-2 did not

Fig. 4. Alpha granule secretion, integrin activation and lysosome release are not affected by SecinH3. Washed platelets (4 × 10^7 mL^-1) were treated with 0.2% DMSO vehicle (Veh) or 15 μM SecinH3 (SecH3) before stimulation for 10 min with a range of concentrations of α-thrombin (αT) (A, B) or CRP (D, E) in the presence of 1 mM CaCl₂. P-selectin expression (A, D) as a result of α-granule secretion and integrin αIIbβ3 activation (B, E) were measured by flow cytometry. Data are shown as the percentage of maximal response. Alternatively, washed platelets (2 × 10^9 mL^-1), treated with Veh or 15 μM SecH3, were stimulated for 15 min with αT (C) or CRP (F) in the presence of 1 mM CaCl₂ and the release of β-hexosaminidase from lysosomes was measured by a colorimetric assay. Data are expressed as the percentage of total content. Curves (A-F) were fitted by F-test. Mean ± SEM, n ≥ 4.
apparently translocate from membrane to cytosol in these cells (data not shown). A different molecular mechanism of disengaging cytohesin-2 and ARF6 may therefore apply in platelets, mediated by PKC. PKC inhibition completely rescued both the decrease in ARF6-GTP and the dissociation of cytohesin-2 from ARF6 upon platelet stimulation. PKC-mediated cytohesin-2 phosphorylation can thus directly regulate ARF6 activity in human platelets, by decreasing the association of cytohesin-2 with ARF6. At present, we cannot exclude that, in addition to this regulatory mechanism, PKC may also directly regulate the GEF activity of cytohesin-2.

A role for cytohesin-2 in granule secretion has previously been described for chromaffin and β-cells [21,26]. The recent development of the ARF-GEF inhibitor SecinH3 [25] allowed us to study the role of cytohesin-2 in platelet granule secretion and function. SecinH3 significantly enhanced platelet dense granule secretion induced by thrombin and CRP, suggesting that GTP-bound ARF6 restrains granule secretion. Aggregation was also elevated, which is likely to be due to the enhanced release of autocrine compounds from dense granules, because cytohesin-2 inhibition did not affect other platelet responses such as alpha granule secretion and integrin activation. Despite the considered relation of lysosomes to dense granules [32], lysosome release was unchanged by cytohesin-2 inhibition. Given the different secretion kinetics of these two granule types, this supports a different molecular mechanism of regulation [33]. Taken together, our results show that active GTP-bound ARF6 has a negative regulatory role in platelet dense granule secretion and aggregation. In line with this, it has previously been reported that the GTP-bound form of the related Golgi-regulating small GTPase ARF1 inhibits vesicle traffic in normal rat kidney cells [34]. The mechanism whereby active ARF negatively regulates vesicle transport

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has not been established yet, but it is postulated that association of ARF with specific membrane components prevents membrane fusion [35]. In summary, we propose a model for regulation of the GEF cytohesin-2, where cytohesin-2 in resting platelets keeps the small GTPase ARF6 in an active state, thereby constitutively suppressing dense granule secretion. Cytohesin-2 phosphorylation by a conventional PKC isoform, upon platelet stimulation, causes cytohesin-2 to dissociate from ARF6, which then becomes inactive, resulting in the relief of the constitutive inhibition of dense granule secretion (Fig. 6).

Addendum

M. T. J. van den Bosch designed, performed and analyzed the experiments and wrote the manuscript. A. W. Poole conceived the experiments, supervised the project and edited the manuscript. I. Hers conceived the experiments, supervised the project and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. ADP does not induce cytohesin-2 phosphorylation.
Fig. S2. SecinH3 dose-response.

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