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ORIGINAL ARTICLE

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 $\alpha_{IIb}\beta_3$ 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.

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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 isozymes 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 chromaffin 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 (Bristol, UK). The GST-GGA3 construct was a generous gift from Professor Sidney Whiteheart (University of Kentucky, USA). Cross-linked collagen-related peptide (CRP) was synthesized by Professor Richard Farndale (University of Cambridge, UK). PE-P-selectin and FITC-PAC1 antibodies were from Emfret Analytics (Eibelstadt, Germany). Luciferin-luciferase was from Chronolog (Labmedics, 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 $\times g$ for 17 min. The platelet-rich plasma was subsequently centrifuged at 650 $\times g$ 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 Na_2HPO_4, 1 mM $MgSO_4$), 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 ($PKC\alpha^{-/-}$) 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 CO_2 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 μL of modified HEPES-Tyrode's buffer pH 7.2, and centrifuged at 180 $\times g$ for 8 min. PRP was removed and platelets were isolated by centrifugation at 520 $\times g$ for 10 min in the presence of 10 μM indomethacin and 0.02 U mL^{-1} apyrase. Pelleted platelets were resuspended to the required density in modified HEPES-Tyrode's buffer pH 7.2.

Platelet stimulation and lysis

Washed human platelets (4×10^8 mL^{-1}) or mouse platelets (2×10^8 mL^{-1}) were incubated for 15 min with the indicated inhibitor or 0.2% dimethylsulfoxide (DMSO) vehicle. Next, platelets were stimulated at 30 °C under non-stirring conditions. For IP, co-IP and ARF-GTP pull down studies, platelets were lysed with an equal 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 $MgCl_2$), respectively, to which protease inhibitors were added. Cell extracts were centrifuged at 10 000 $\times g$ at 4 °C, and the supernatant was taken for subsequent analysis. Alternatively, for western blotting (whole cell

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 2-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 (0.2% DMSO) or 15 μM SecinH3. Before stimulation, platelets were incubated with 5 μL luciferase-luciferin reagent at RT. Light transmission and luminescence were recorded in a Chronolog 590-2A aggregometer.

Flow cytometry

Platelets (2×10^7 mL^{-1}) were treated with vehicle or 15 μM SecinH3 for 15 min before stimulation in the presence 1 : 12 PE-conjugated anti-P-selectin antibody and 1 : 6 FITC-conjugated PAC-1 antibody, to assess α-granule secretion and $\alpha_{IIb}\beta_3$ integrin activation, respectively. Platelets were stimulated for 10 min at RT prior to fixation in 1% paraformaldehyde for 30 min. Fluorescent analysis was conducted by flow cytometry on a FACSCalibur flow cytometer.

Measurement of β-hexosaminidase release

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.

Statistics

Data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as the mean ± SEM of the indicated number of independent observations and *P*-values were calculated using Student's *t*-test. Differences between concentration-response curves were determined by *F* test, where the null hypothesis states that both datasets can be fitted using the same parameters.

Results

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-isoform-specific 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 phosphomotif 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 A_2 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 $P2Y_{12}$ 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

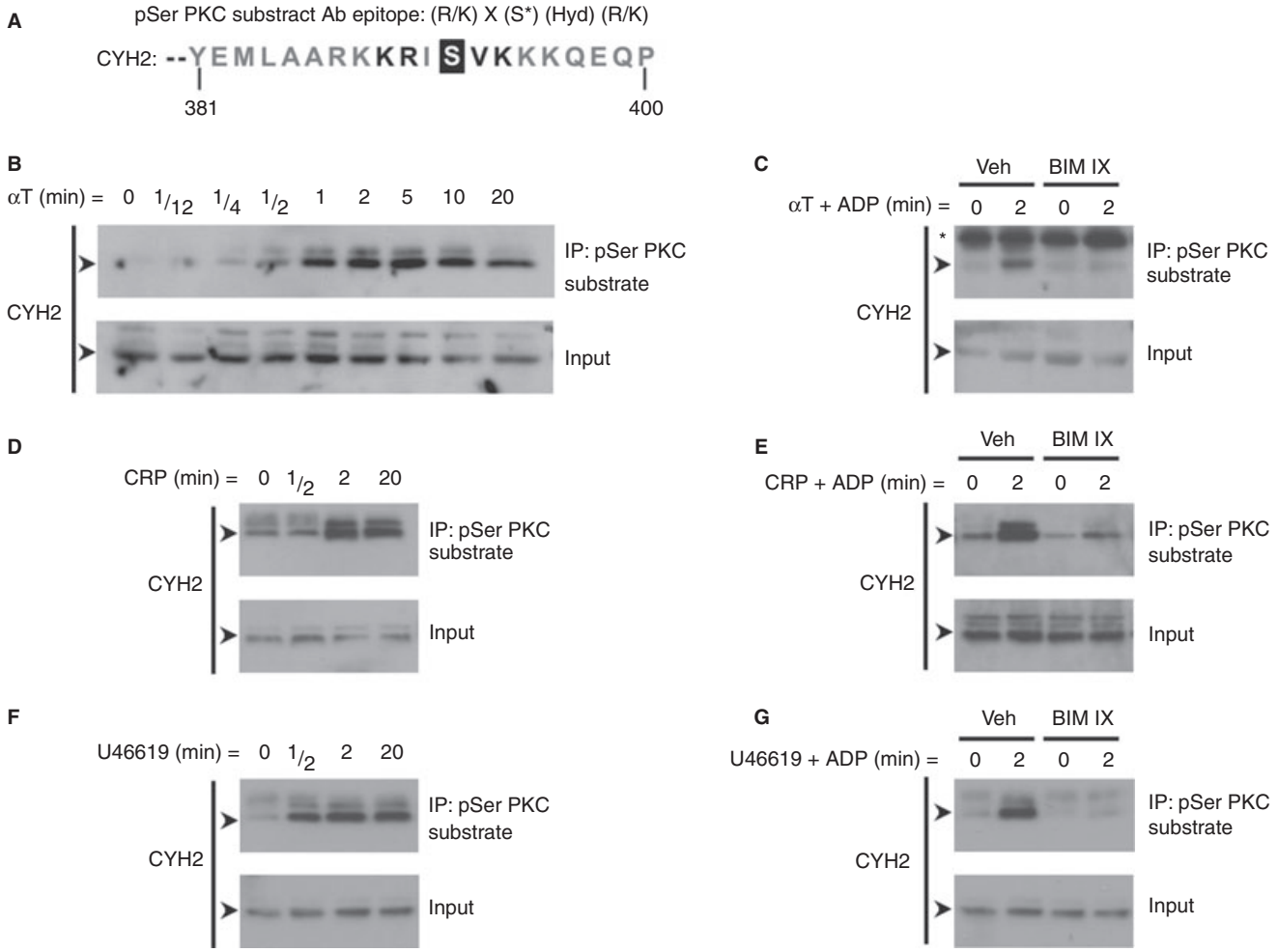

Fig. 1. PKC phosphorylates cytohesin-2 upon platelet activation. The C-terminus of cytohesin-2 (CYH2) contains the phospho-motif recognized by the pSer PKC substrate antibody (A). X, any amino acid. Hyd, any hydrophobic amino acid. Washed platelets (4×10^8 mL^{-1}) were stimulated with 0.2 U mL^{-1} α-thrombin (αT) (B), 5 μg mL^{-1} CRP (D) or 10 μM U46619 (F) and lysed at the indicated time-points. Alternatively, platelets were treated for 15 min with 0.2% DMSO vehicle (Veh) or the PKC inhibitor BIM IX (2 μM) prior to stimulation with the same agonists in the presence of 10 μM ADP (C, E, G). Clarified whole cell lysates (input) and immunoprecipitations (IP) using the pSer PKC substrate antibody were immunoblotted for CYH2. The arrows (➤) indicate the 47 kDa CYH2 band and the asterisk (*) indicates a non-specific band. Results (B–G) are representative of at least three independent experiments.

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

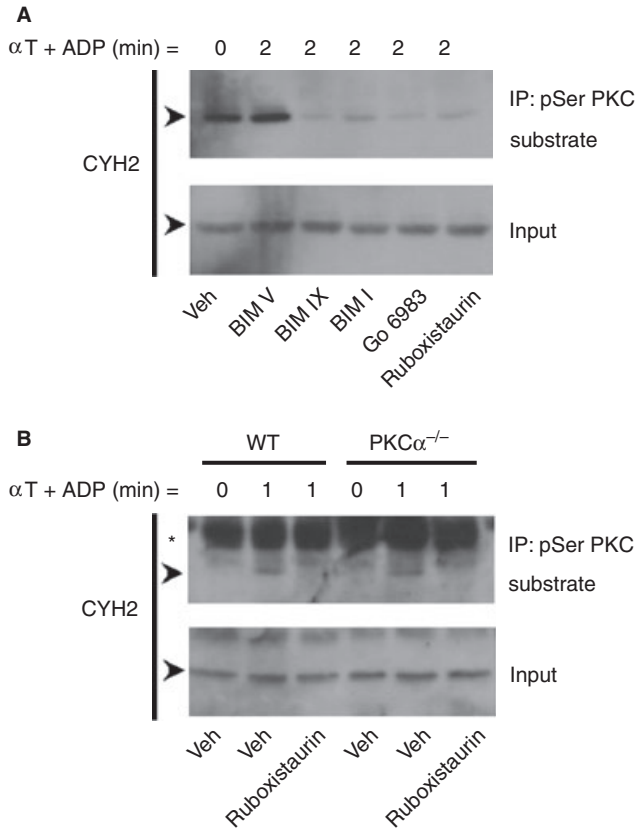

Fig. 2. Conventional PKC isoforms mediate the phosphorylation of cytohesin-2. Washed human platelets (4×10^8 mL^{-1}) were treated for 15 min with 0.2% DMSO vehicle (Veh), the control BIM compound BIM V (10 μM), the broad-spectrum inhibitors PKC inhibitor BIM IX (2 μM), BIM I (5 μM) and Go 6983 (10 μM), or the PKCα/β selective inhibitor ruboxistaurin (10 μM) (A). Alternatively, mouse wild-type (WT) and PKCα knockout (PKCα$^{-/-}$) mouse platelets (2×10^8 mL^{-1}) were used (B). Platelets were stimulated with 0.2 U mL^{-1} α-thrombin (αT) in the presence of 10 μM ADP and lysed at the indicated time-points. Clarified whole cell lysates and immunoprecipitations using the pSer PKC substrate antibody were immunoblotted for cytohesin-2 (CYH2). The arrows (➤) indicate the 47 kDa CYH2 band and the asterisk (*) indicates a non-specific band. Results are representative of at least three independent experiments.

optical aggregometry were performed to assess ATP secretion from dense granules and aggregation responses, respectively. Platelets were stimulated with a low agonist concentration to be able to detect both negative and positive functional changes. SecinH3 did not stimulate ATP secretion or aggregation by itself (data not shown). ATP secretion induced by 0.05 U mL^{-1} (Fig. 3Ai,ii), 0.075 U mL^{-1} (Fig. 3Ai,iii) and 0.2 U mL^{-1} (Fig. 3Ai,iv) thrombin was significantly enhanced by SecinH3 (Figure 3A). When platelets were stimulated with 0.3 μg mL^{-1} (Fig. 3Ci- ii) and 1 μg mL^{-1} (Fig. 3Ci,iii) CRP, but not 5 μg mL^{-1} CRP (Fig. 3Ci,iv), similar results were seen. Platelet aggregation in response to low and intermediate concentrations of both agonists was also significantly enhanced by SecinH3 (Fig. 3B,D). Using a higher concentration of SecinH3 did not result in a greater effect on ATP secretion or aggregation (Figure S2).

α-granule secretion, integrin $\alpha_{IIb}\beta_3$ activation and lysosome secretion are not affected by cytohesin-2 inhibition

Next, we investigated whether cytohesin-2 inhibition also affects α-granule secretion. Therefore, we assessed P-selectin exposure, a marker of α-granule release, by flow cytometry. Platelet pre-incubation with SecinH3 did not alter the expression of P-selectin induced by a range of concentrations of thrombin (Fig. 4A) and CRP (Fig. 4D). Integrin $\alpha_{IIb}\beta_3$ activation (Fig. 4B,E), which is required for crosslinking of platelets by binding to fibrinogen, was measured simultaneously and was also not affected. Furthermore, we analyzed the secretion of β-hexosaminidase from lysosomes using a colorimetric assay. SecinH3 had no effect on lysosome secretion in response to platelet stimulation with various concentrations of thrombin (Fig. 4C) or CRP (Fig. 4F), compared with the vehicle control.

PKC negatively regulates the cytohesin-2/ARF6 pathway upon platelet stimulation

As cytohesin-2 is a GEF for the small GTPase ARF6, we assessed the kinetics of ARF6 activation upon platelet stimulation with thrombin using a GGA3-GST pull down method [30]. Under basal conditions ARF6 was detected in an active GTP-bound state (Fig. 5A). Platelet stimulation with thrombin induced a decrease in active ARF6, which was minimal after 2–5 min and largely restored after 20 min. We investigated the role of PKC in this process by treating platelets with BIM IX. PKC inhibition completely rescued the observed decrease in ARF6-GTP levels upon platelet activation (Fig. 5B).

To investigate whether ARF6 binds to cytohesin-2, we performed immunoprecipitation studies using a cytohesin-2 antibody. ARF6 bound to cytohesin-2 under basal conditions and this interaction was significantly reduced upon platelet stimulation (Fig. 5C). This was most obvious when comparing the ratio of co-immunoprecipitated ARF6 with the levels of immunoprecipitated cytohesin-2, as cytohesin-2 levels were generally slightly enhanced under stimulated conditions (Fig. 5Ci). PKC inhibition with BIM IX completely reversed the decrease in interaction of cytohesin-2 with ARF6 observed upon platelet activation (Fig. 5C).

Discussion

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).

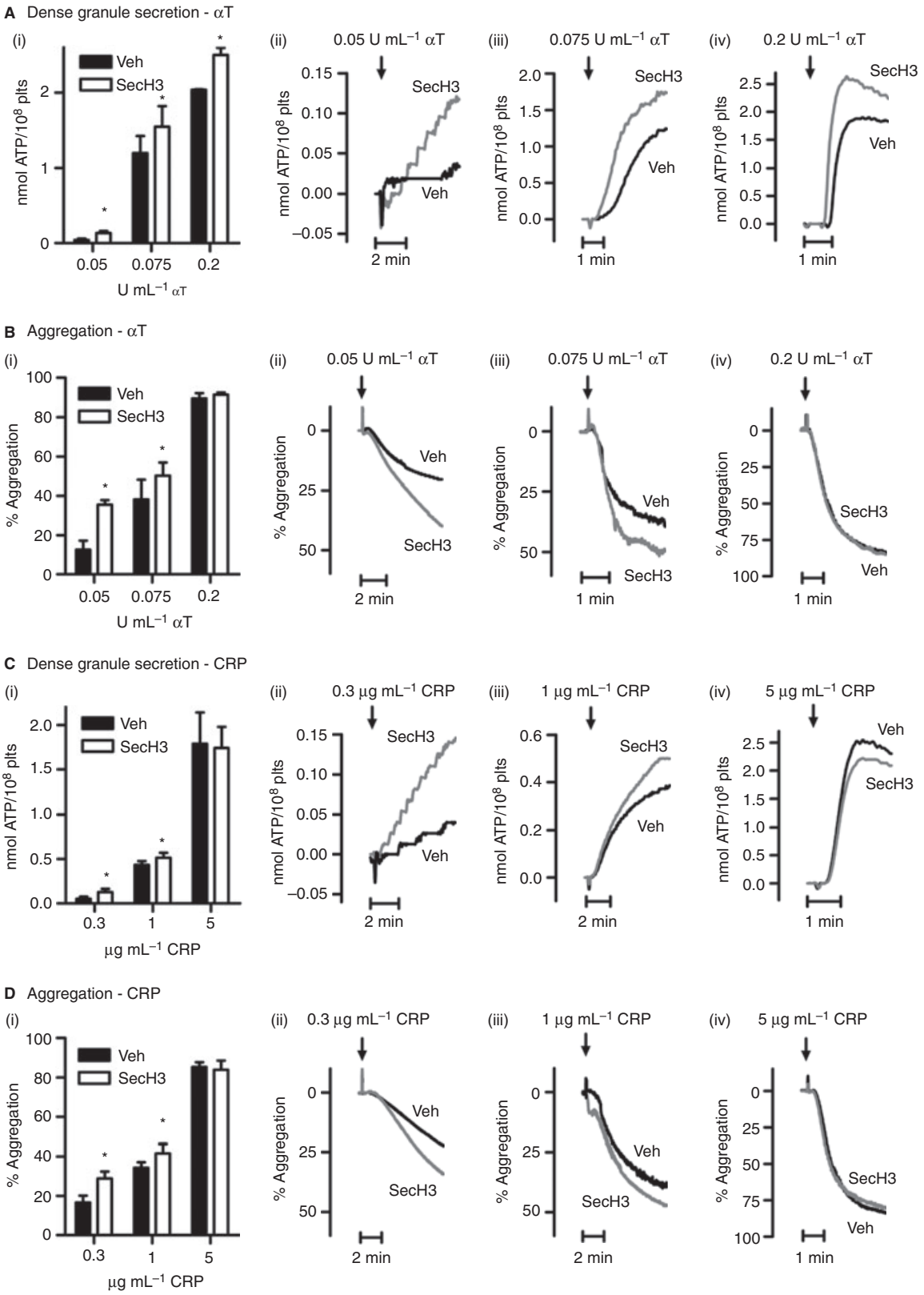

Fig. 3. Cytohesin-2 inhibition by SecinH3 enhances dense granule secretion and platelet aggregation. Washed platelets (2×10^8 mL^{-1}) were treated for 15 min with 0.2% DMSO vehicle (Veh) or 15 μM SecinH3 (SecH3). ATP release from dense granules (A, C) was assessed by luminometry, simultaneously with 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, $n \geq 3$) show ATP release (nmol ATP/10^8 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).

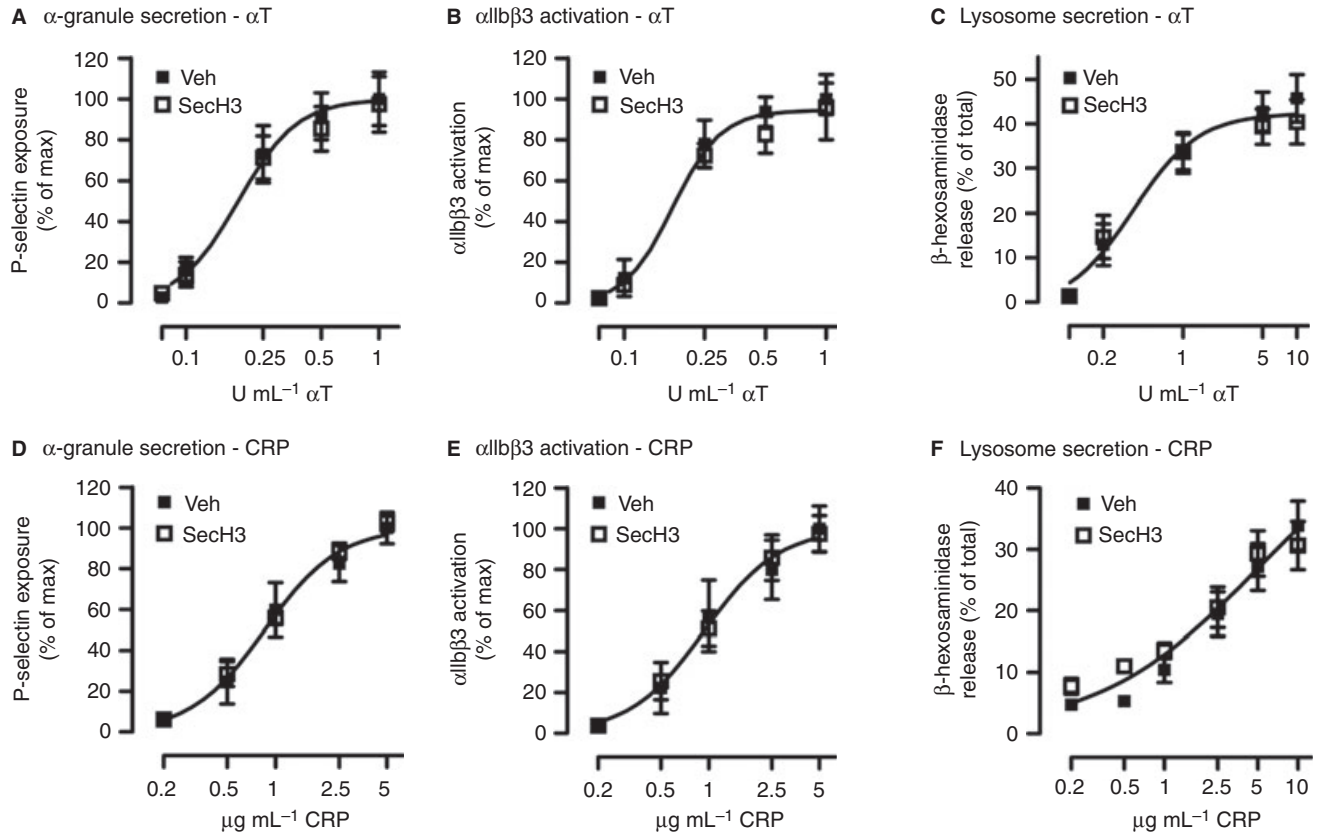

Fig. 4. Alpha granule secretion, integrin activation and lysosome release are not affected by SecinH3. Washed platelets (4×10^7 mL^{-1}) were treated with 0.2% DMSO vehicle (Veh) or 15 μM SecinH3 (SecH3) before stimulation for 10 min with a range of concentrations of α-thrombin (αT) (A, B) or CRP (D, E) in the presence of 1 mM $CaCl_2$. P-selectin expression (A, D) as a result of α-granule secretion and integrin $\alpha_{IIb}\beta_3$ activation (B, E) were measured by flow cytometry. Data are shown as the percentage of maximal response. Alternatively, washed platelets (2×10^8 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 $CaCl_2$ 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, $n \geq 4$.

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 PKCα 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. 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

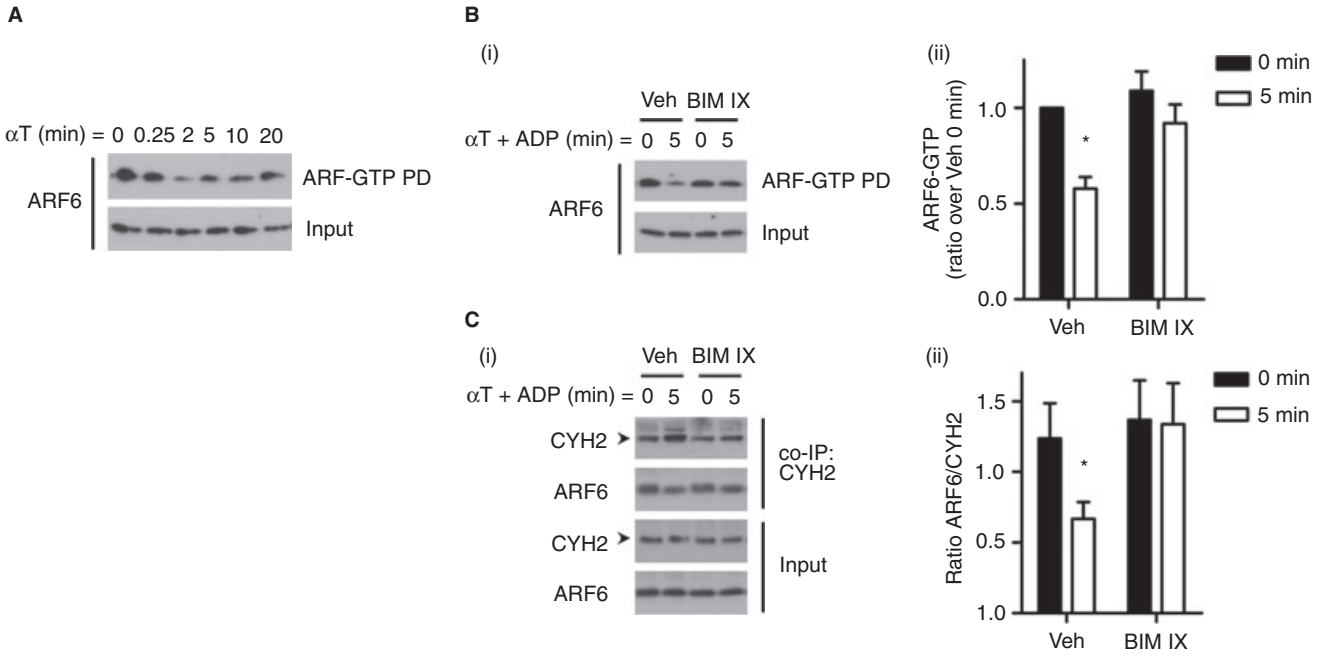

Fig. 5. ARF6 activation and interaction with cytohesin-2 decrease upon platelet stimulation. Washed platelets (4×10^8 mL^{-1}) were stimulated with 0.2 U mL^{-1} αT and lysed at the indicated time-points and ARF6-GTP was pulled down (PD) using GGA3-GST beads (A). Alternatively, platelets were incubated for 15 min with 0.2% DMSO vehicle (Veh) or 2 μM BIM IX prior to stimulation with 0.2 U mL^{-1} αT and 10 μM ADP, followed by ARF6-GTP pull down (Bi) or cytohesin-2 co-IP (Ci). Samples were immunoblotted for ARF6 and cytohesin-2 (CYH2). Densitometry was performed using ImageJ software and levels of ARF6-GTP (Bii) and ratios of ARF6 over CYH2 were calculated (Cii). *$P < 0.05$ (Student's paired t-test), mean ± SEM, $n \geq 4$. Shown are representative blots. The arrows (➤) indicate the 47 kDa CYH2 band.

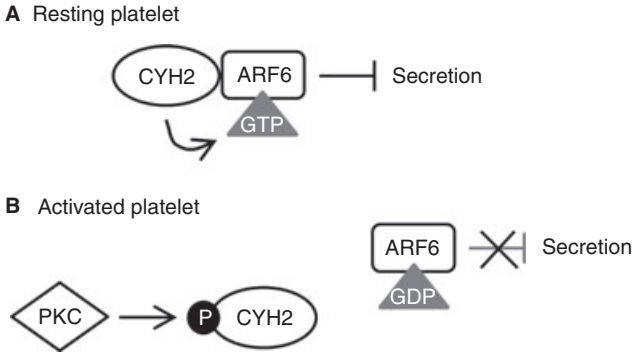

Fig. 6. Model of regulation of the cytohesin-2/ARF6 pathway by PKC. (A) In resting platelets, cytohesin-2 (CYH2) is associated with ARF6 and maintains it in an active GTP-bound state, which constitutively suppresses dense granule secretion. (B) Upon platelet stimulation PKC is activated and phosphorylates cytohesin-2. This results in the dissociation of cytohesin-2 from ARF6, which therefore becomes inactive and GDP bound, allowing dense granule secretion to occur.

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

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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