Protein Kinase C Phosphorylation of Syntaxin 4 in Thrombin-activated Human Platelets*

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We postulated that the syntaxins, because of their key role in SNARE complex formation and exocytosis, could be important targets for signaling by intracellular kinases involved in secretion. We found that syntaxin 4 was phosphorylated in human platelets treated with a physiologic agent that induces secretion (thrombin) but not when they were treated with an agent that prevents secretion (prostacyclin). Syntaxin 4 phosphorylation was blocked by inhibitors of activated protein kinase C (PKC), and, in parallel assays, PKC inhibitors also blocked secretion from thrombin-activated platelets. In platelets, cellular activation by thrombin or phorbol 12-myristate 13-acetate decreased the binding of syntaxin 4 with SNAP-23, another platelet t-SNARE. Phosphatase inhibitors increased syntaxin 4 phosphorylation and further decreased syntaxin 4-SNAP-23 binding induced by cell activation. Conversely, a PKC inhibitor blocked syntaxin 4 phosphorylation and returned binding of syntaxin 4-SNAP-23 to that seen in nonstimulated platelets. In vitro, PKC directly phosphorylated platelet syntaxin 4 and recombinant syntaxin 4. PKC phosphorylation in vitro inhibited (71 ± 8%) the binding of syntaxin 4 to SNAP-23. These results provide evidence that extracellular activation can be coupled through intracellular PKC signaling so as to modulate SNARE protein interactions involved in platelet exocytosis.

In regulated exocytosis, the release of biologically important effector molecules (growth factors, cell activators, hormones, neurotransmitters, and others) from intracellular secretory vesicles is triggered by intracellular signaling. The fusion of secretory vesicle membranes with the plasma membrane is mediated by the core SNARE1 complex (1). This complex is a closely packed helical bundle formed by three membrane proteins derived from different gene families: the syntaxins, the VAMPs (or synaptobrevins), and the SNAP-25 family (2, 3). The SNARE proteins interact with each other through conserved sequences of about 60 amino acids, known as a SNARE motif (4). The syntaxins play a key role in SNARE complex formation and exocytosis, because they interact with several other proteins that are believed to modulate exocytosis such as the Sec1/Munc-18 proteins, Munc13, synaptotagmin, Ca2+ channels, and others (reviewed in Refs. 5–7).

Regulated exocytosis occurs after cell activation or membrane depolarization through intracellular signaling by Ca2+ and, potentially, by kinases, phospholipids, etc. In neurons, synaptotagmin I appears to be one of the molecules that transduces the Ca2+ signal to the exocytotic process through its interactions with syntaxin 1 (8). There is also a growing realization that phosphorylation could play a key role in regulating SNARE complex interactions. Recent in vitro studies suggest that the syntaxins also serve as direct substrates for intracellular kinases such as casein kinase II (CKII), protein kinase A (PKA), and Ca2+/calmodulin-dependent kinase II (CaMKII) (9–11). However, the physiologic significance of these observations is unclear because syntaxin phosphorylation has not been demonstrated in vivo.

Platelets are highly specialized secretory cells that have a well characterized pattern of cell activation and intracellular signaling. Platelet activation by specific extracellular agonists such as thrombin, collagen, or ADP triggers exocytosis of molecules from vesicles known as the alpha and dense granules (12). Platelet activation causes increased intracellular Ca2+, the production of inositol-1,4,5-trisphosphate, diacylglycerol (DAG), and the activation of PKC, as well as other kinases. Although increases in intracellular Ca2+ are sufficient to induce platelet exocytosis, there is evidence that PKC activity may interact with Ca2+ synergistically to amplify platelet secretion (13, 14). Recently, we and others have shown that platelets contain non-neuronal isoforms of v- and t-SNAREs, including syntaxin 4, SNAP-23, and platelet VAMP (15–17). Platelets also contain an Sec1 homologue that is likely to modulate platelet secretion through its binding to syntaxin 4 (15). The regulated exocytosis of alpha and dense granules requires N-ethylmaleimide-sensitive factor activity (18), and granule release is mediated in part by syntaxin 4, VAMP (17), and SNAP-23 (19).

How regulated exocytosis is linked to cell activation through intracellular signaling by kinases remains an unanswered question. We hypothesized that the syntaxins, by virtue of their critical role in vesicle fusion, could be targets of intracellular kinases whose signaling activity is coupled to cell activation. We found that syntaxin 4 was phosphorylated when cells are activated by thrombin to induce secretion. PKC inhibitors significantly reduced both syntaxin 4 phosphorylation and platelet secretion. In contrast to previous in vitro studies (10, 11) we found that PKC directly phosphorylated syntaxin 4 with a

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‡ The abbreviations used are: SNARE, SNAP receptor; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; PKC, protein kinase C; PKA, protein kinase A; CKII, casein kinase II; CaMKII, Ca2+/calmodulin-dependent kinase II; VAMP, vesicle-associated membrane protein; SNAP-23, synaptosome-associated protein-23; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; r-, recombinant; PGI2, prostaglandin I2; PAGE, polyacrylamide gel electrophoresis; PVD, polyvinylidene difluoride; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; Ro-31-8220, (Bisindoylmaleimide I); 1H-indol-3-yl)-3-(1-methylindol-3-yl)-maleimide).
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stoichiometry of 0.8 mol of phosphate/mol of syntaxin 4. Stimulation of PKC in thrombin-activated platelets decreased the interaction of syntaxin 4 with SNAP-23 as detected by coimmunoprecipitation studies. In vitro phosphorylation of syntaxin 4 by PKC also decreased syntaxin 4 binding to SNAP-23. These studies provide the first demonstration of syntaxin 4 phosphorylation in vivo and indicate that intracellular signaling by kinases may play a modulatory role in linking the processes of cell activation to regulated exocytosis.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—Recombinant (r-) syntaxin 4 was produced in bacteria and purified on glutathione-Sepharose as described previously (15). Human r-SNAP-23 (Research Genetics, Huntsville, AL) was expressed as an N-terminal 6-His fusion protein via the pProEX HTA vector (Life Technologies, Inc., Rockville, MD) in bacteria and purified on Ni-agarose as described previously (18).

Phosphorylation in Intact Platelets Loaded with 32P—Freshly isolated platelets (18) were centrifuged and resuspended in buffer (25 mM HEPES, pH 7.3, 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose, 0.5 mM EDTA). Platelets (1 × 10^10 cells/ml) were metabolically loaded with 500 μCi/ml [32P]orthophosphate (NEN Life Science Products) at 30 °C for 1 h. Metabolically labeled platelets were incubated with and without inhibitors (see Table 1 for details, all inhibitors were from Calbiochem, San Diego, CA) for 15 min at 30 °C. Then thrombin (1 unit/ml), PGL (10 μM), or phorbol 12-myristate 13-acetate (PMA) (1 μM) was added to the cells. The platelets were promptly solubilized in lysis buffer (0.5% SDS, 50 mM NaH2PO4, 2 mM EDTA, pH 8.0, and 1 mM dithiothreitol). After boiling for 5 min, the samples were diluted with 1.25× radioimmunoprecipitation assay buffer (1.25% Nonidet P-40, 1.25% Triton X-100, 10 mM sodium vanadate, 5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl2, 0.1 mg/ml phosphatidylinerine, 15 mM MgCl2, and 100 μM of ATP) and with 500 μg/ml leupeptin, and 100 μg/ml aprotinin. Then 100 μl of the samples were fractionated on Ni-agarose as described previously (18).

Platelets (1 × 10^10 cells/ml) were centrifuged and resuspended in buffer (5 mM HEPES, pH 7.2, 140 mM NaCl, 4.8 mM KCl, 1 mM MgCl2, 5.5 mM glucose, 0.35% bovine serum albumin). PKC-phosphorylated or mock-phosphorylated (incubated with thrombin (1 unit/ml) for 1.5 min at 30 °C) platelets—Freshly isolated platelets (18) were incubated with and without inhibitors (Table 1) for 15 min at 30 °C. Secretion was induced by thrombin (1 unit/ml) for 30 s or 1 min at 30 °C. One volume of ice-cold 5% formaldehyde, 50 mM EDTA, pH 7.4, was added to stop the reaction, the sample was centrifuged at 3000 × g for 2.5 min, and the supernatants were analyzed by scintillation counting. Samples containing 0.2% Triton X-100 were used for the measurement of total cellular [3H]serotonin. Basal release, in the absence of stimulation, was typically less than 5% of total [3H]serotonin content.

Data are generally expressed as mean ± S.E. The statistical significance of differences between paired samples was assessed by a paired Student’s t test.

RESULTS

Syntaxin 4 Is Phosphorylated in Thrombin-activated Platelets—Platelet exocytosis is triggered by intracellular signals initiated by physiologic cell activators such as thrombin. To determine whether syntaxin 4 was a substrate for platelet kinases activated by thrombin, we examined phosphoproteins in cells metabolically loaded with [32P]PO4. First we established that syntaxin 4 was specifically immunoprecipitated from platelet lysates by our anti-syntaxin 4 antibodies but not by control IgG antibodies nor by anti-syntaxin 4 antibodies preadsorbed with syntaxin 4 proteins (Fig. 1, A and B, immunoblot). Platelets loaded with [32P]PO4 were incubated with PGL (an agent that activates PKA and blocks cell activation and secretion), thrombin, or no agent (resting). Treatment of platelets with PGL did not induce syntaxin 4 phosphorylation (Fig. 1A). Minimal if any syntaxin 4 phosphorylation was seen in unstimulated (resting) platelets. However, as shown in Fig. 1A, thrombin activation of platelets strongly induced syntaxin 4 phosphorylation. A similar pattern of syntaxin 4 phosphorylation was seen in permeabilized platelets loaded with [γ32P]ATP and treated with the same agents (Fig. 1B and data not shown). Effects of PKC Inhibitors on Syntaxin 4 Phosphorylation and Exocytosis in Platelets—Thrombin activation of platelets can induce protein phosphorylation by several different kinases. We examined the simultaneous effects of cell-permeable kinase inhibitors on both syntaxin 4 phosphorylation and secretion. Because our initial studies of thrombin-activated platelets with an anti-phosphotyrosine antibody did not detect tyrosine phosphorylation of syntaxin 4 (not shown), we focused our studies on inhibitors of serine/threonine kinases. Inhibitors of several different kinases (PKA, CaMKII, CKII, and others) had minimal if any effects on syntaxin 4 phosphorylation and secretion (data not shown). However, PKC inhibitors significantly decreased both syntaxin 4 phosphorylation and platelet secretion. Ro-31-8220, a general inhibitor of all PKC isozymes, reduced thrombin-induced 4-phosphorylated syntaxin 4 antibody by 48 ± 15% to the level of ATP, to the extent that it inhibited the phosphorylation of pleckstrin (49 ± 2.7%), a direct PKC substrate (Fig. 2A). Similarly, the general PKC inhibitor Gö-6983 also reduced syntaxin 4 phosphorylation by 52–57% in both intact and permeabilized cells. The selective inhibitor of Ca2+-dependent PKC enzyme Gö-6976 (21) had less inhibitory effects (19–30%). Finally, phorbol ester (PMA), a
PKC activator, induced the phosphorylation of syntaxin 4 (Fig. 2B), whereas PGI₂, a stimulator of PKA, had no effect (Fig. 1A).

Under the same conditions used to examine syntaxin 4 phosphorylation, the PKC inhibitor (Ro-31-8220) blocked platelet granule secretion as did Gö-6976 (Table I). However, Gö-6976 had no effect, suggesting that the Ca²⁺-independent PKC enzymes have a role in platelet secretion. The relationship between syntaxin 4 phosphorylation and platelet secretion is summarized in Table I. General inhibitors of PKC activity such as Ro-31-8220 and Gö-6893 significantly reduced both syntaxin 4 phosphorylation and secretion. The selective inhibitor of Ca²⁺-dependent PKC isoforms Gö-6897 had minimal effects on syntaxin 4 phosphorylation and no effect on secretion. In these studies general PKC inhibitors coordinately blocked syntaxin 4 phosphorylation and secretion, suggesting that there is a physiological linkage between these processes.

**Direct Phosphorylation of Syntaxin 4 by PKC—Previous in vitro reports have suggested that recombinant syntaxin 4 is not a direct substrate for PKC (10,11). Because these results may have been due to abnormal protein folding of bacterially expressed r-syntaxin 4, we first examined whether native syntaxin 4 from platelets could be directly phosphorylated by PKC. Fig. 2C shows that human platelet syntaxin 4 was phosphorylated by purified PKC (Fig. 2C). This prompted a critical re-examination of the phosphorylation of r-syntaxin 4 using different reaction conditions, times, and enzyme:substrate ratios. Using purified proteins in vitro we found that syntaxin 4 phosphorylation was time-dependent and maximal at a molar ratio of ~1:8 (purified PKC : r-syntaxin 4) yielding a stoichiometry of 0.8 mol of phosphate/mol of syntaxin 4 (Fig. 2D). The fact that syntaxin 4 phosphorylation was differentially inhibited by selective inhibitors of different PKC isoforms, Gö-6976 and Gö-6983 (Fig. 2B and Table I) prompted us to examine the phosphorylation of syntaxin 4 by various PKC isoforms. Fig. 2D shows that PKC isoforms α, β₁, γ, δ, ε, and ζ phosphorylated syntaxin 4 in vitro.

**PKC Phosphorylation of Syntaxin 4 in Vitro Reduces Its Binding to SNAP-23—**Syntaxin 4 contains several potential PKC sites. One site is within the H3 domain (residue 216), a predicted coiled-coil structure required for protein-protein interactions, including the assembly of binary and ternary SNARE complexes. To determine whether phosphorylation of syntaxin 4 regulates its interaction with other proteins in the exocytic pathway, we examined the effect of PKC-dependent syntaxin 4 phosphorylation on its binding with SNAP-23, another key component of SNARE complex in platelets (17). Under these experimental conditions, PKC phosphorylated syntaxin 4 to a stoichiometry of 0.8 mol of phosphate/mol of protein. When compared with the mock-phosphorylated syntaxin 4 (incubated with phosphorylation reagents but not PKC), the ability of PKC-phosphorylated syntaxin 4 to bind to SNAP-23 was reduced by 71 ± 8% (four independent experiments) (Fig. 3).

**Effect of PKC-dependent Phosphorylation on Syntaxin 4 and SNAP-23 Binding in Platelets—**The binding of syntaxin 4 to SNAP-23 in resting and stimulated platelets was assessed by coimmunoprecipitation of syntaxin 4-containing complexes with a monoclonal anti-syntaxin 4 antibody and quantitative immunoblotting with an anti-SNAP-23 antibody. To standardize the comparison between individual experiments, the pixel values for each SNAP-23 band were normalized to the corresponding pixel density of the syntaxin band in the same sample. Because the treatment of platelets with PGI₂ did not induce syntaxin 4 phosphorylation (Fig. 1A) we compared the binding of syntaxin 4 to SNAP-23 in PGI₂-treated and thrombin-activated platelets. In thrombin-activated platelets syntaxin 4-SNAP-23 binding was reduced by 12 ± 2% (four independent experiments, p < 0.01) (Fig. 4A). A similar reduction (14 ± 1.5%) in syntaxin 4 and SNAP-23 binding was observed in thrombin-activated platelets when compared with resting platelets (Fig. 4B). Initial studies indicated that a phosphatase inhibitor, okadaic acid, enhanced the syntaxin 4 phosphorylation in thrombin-activated platelets by more than 2-fold, suggesting that phosphosyntactin 4 may be subject to dephosphorylation. To maximize the effect of phosphorylation on the syntaxin 4-SNAP-23 interaction, the platelets were treated with phosphatase inhibitors (sodium fluoride and sodium vanadate) before cell activation. The phosphatase inhibitors further decreased syntaxin 4-SNAP-23 binding in thrombin-activated platelets by 25 ± 5.2% compared with resting platelets (without phosphatase inhibitors) (three independent experiments, p < 0.05). These phosphatase inhibitors also decreased the binding of syntaxin 4 to SNAP-23 by 16 ± 7.7% in thrombin-stimulated platelets compared with thrombin-stimulated platelets not treated with phosphatase inhibitors (three independent experiments, p < 0.05). These results indicate that thrombin activation of platelets, which endogenously phosphorylates syntaxin 4 through a PKC-dependent mechanism (Fig. 2), decreased the interaction of syntaxin 4 with SNAP-23.

To further examine whether PKC was responsible for the reduced interaction of syntaxin 4 with SNAP-23 in thrombin-stimulated platelets, a PKC inhibitor (Ro-31-8220) was used to block thrombin-induced phosphorylation. Thrombin activation of platelets decreased the binding of syntaxin 4 with SNAP-23 by 14% (see above) but Ro-31-8220 restored syntaxin 4-SNAP-23 binding to 95 ± 5.8% of that seen with resting platelets (Fig. 5A, three independent experiments). When PMA
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**Fig. 2. Syntaxin 4 is phosphorylated by a PKC-dependent mechanism.** A, platelets metabolically labeled with $^{32}$P were incubated with and without (none) 1 $\mu$M of Ro-31-8220 for 15 min before activation with 1 unit/ml of thrombin for 1 min at 30°C. After platelet lysis the proteins were immunoprecipitated with anti-syntaxin 4 antibodies. Left, upper panel shows an immunoblot with anti-syntaxin 4 antibody and the lower panel shows the level of pleckstrin phosphorylation on the same membrane. The right panel shows the level of pleckstrin phosphorylation in the crude lysates. The graph at bottom shows the PhosphorImager-derived quantitation of the phosphosyntaxin and phosphopleckstrin from three independent experiments. B, the metabolically labeled platelets were incubated with and without (none) 1 $\mu$M Go-6976 or Go-6983 and treated as above. In the experiments with PMA (a PKC activator), 1 $\mu$M PMA was used to activate platelets at 30°C for 5 min. The upper panel shows phosphoprotein and the lower panel shows an immunoblot of the same membrane with anti-syntaxin 4 antibodies. C, platelet syntaxin 4, immunoprecipitated from platelet lysates with control IgG antibody (control) or anti-syntaxin 4 antibody, were phosphorylated in vitro with purified PKC and $[\gamma^{32}$P]ATP for 40 min, subjected to SDS-PAGE, and phosphorimaging (upper panel). Immunoblotting with an anti-syntaxin 4 antibody of the same membrane is shown in the lower panel. D, indicated amounts of GST-cleaved r-syntaxin 4 (upper panel) or 10 pmol of GST-syntaxin 4 (lower panel) were phosphorylated in vitro with 0.18 pmol of PKC and $[\gamma^{32}$P]ATP for 1 h (upper panel) or 0.1 unit of the indicated PKC isozymes and $[\gamma^{32}$P]ATP at 30°C for 30 min (lower panel), subjected to SDS-PAGE, and phosphorimaging. The results shown are representative of two to six independent experiments.

**TABLE 1.** The effects of PKC inhibitors on thrombin-induced syntaxin 4 phosphorylation and platelet secretion

| PKC inhibitors | Relative secretion in intact cells | Relative syntaxin 4 phosphorylation in intact cells | In permeabilized cells |
|----------------|-----------------------------------|-----------------------------------------------|----------------------|
| Ro-31-8220 (1 $\mu$M) | 0.24 ± 0.09 (3) | 0.52 ± 0.15 (3) | 0.47 ± 0.06 (3) |
| Go-6983 (3 $\mu$M) | 0.58 (2) | 0.43 (1) | 0.48 (2) |
| Go-6976 (1 $\mu$M) | 1.02 (2) | 0.70 (2) | 0.81 (2) |

was used to stimulate PKC activity in platelets, syntaxin 4 binding to SNAP-23 was inhibited by 18 ± 1.5% (Fig. 5B, two independent experiments). Phosphatase inhibitors further decreased syntaxin 4-SNAP-23 binding in PMA-activated platelets by 31 ± 7.8% compared with resting platelets (from three independent experiments, p < 0.05). Phosphatase inhibitors also decreased the binding of syntaxin 4 to SNAP-23 in PMA-activated platelets by 21 ± 6.7% compared with PMA-activated platelets not treated with phosphatase inhibitors (three independent experiments, p < 0.05). Ro-31-8220 blocked the inhibitory effect of PMA on syntaxin 4-SNAP-23 interactions and restored binding to 96 ± 7.5% of the level seen in resting platelets (three independent experiments). These results provide consistent evidence that PKC activity contributes to the reduced interaction of syntaxin 4 with SNAP-23 in thrombin- or PMA-stimulated platelets.

**DISCUSSION**

These studies establish that syntaxin 4, a key SNARE protein, is phosphorylated when platelets are activated by a physiologic agent that triggers platelet secretion. We found that PKC inhibitors blocked both syntaxin 4 phosphorylation and exocytosis. Studies with platelet syntaxin 4 confirmed that it was a direct substrate for purified PKC. Moreover, in contrast to other reports, we found that a mixture of purified PKC isoforms directly phosphorylated r-syntaxin 4 with a stoichiometry of 0.8 mol of phosphate/mol of protein. Studies with individual PKC isoforms confirmed that r-syntaxin 4 was a substrate for PKC $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and $\zeta$. PKC phosphorylation of syntaxin 4 was functionally important, because it decreased binary interactions of syntaxin 4 with SNAP-23, both in vitro and in vivo. Taken together, these results provide a novel link between platelet activation and exocytosis through intracellular...
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**Fig. 4.** Reduced interaction of syntaxin 4 with SNAP-23 in thrombin-activated platelets. Platelets were treated with 10 μM PGI2 (PGI2) for 1 h at 30 °C, or with 1 unit/ml thrombin (Thr) for 1 min at 30 °C, or kept resting (Rest). After lysis, platelet proteins were immunoprecipitated with a monoclonal anti-syntaxin 4 antibody, subjected to SDS-PAGE, transferred to PVDF membranes, immunoblotted with antibodies against syntaxin 4 and SNAP-23, and analyzed by phosphorimaging. A, representative immunoblots of syntaxin 4 (36 kDa) and SNAP-23 (28–29 kDa). The platelet lysates used for immunoprecipitations are shown. B, quantitation of SNAP-23 bound to syntaxin 4. To allow comparison between individual experiments, the pixel values for each SNAP-23 band (bracketed region) were normalized to the corresponding density of the syntaxin band in the same sample. The relative SNAP-23/syntaxin 4 was calculated by the ratio of SNAP-23/syntaxin 4 in thrombin-stimulated sample to that in PGI2-treated (left panel) or resting platelets (right panel). Data are mean ± S.E. The left panel is from four independent experiments in duplicate, and the right panel is representative from two independent experiments (four samples/experiment). ∗∗, p < 0.01 Thr versus PGI2; ∗, p < 0.05 Thr versus Rest.

**Fig. 5.** The interaction of syntaxin 4 with SNAP-23 in platelets is mediated by PKC-dependent phosphorylation. A, effects of thrombin and Ro-31-8220 on syntaxin 4 and SNAP-23 binding. B, effects of PMA, phosphatase inhibitors, and Ro-31-8220. Platelets were pretreated with and without 1 μM Ro-31-8220 (Ro) for 30 min or phosphatase inhibitors (PI, 5 mM sodium fluoride and 5 mM sodium vanadate) for 5–10 min at 30 °C and subsequently activated as described in the legend to Fig. 4. The upper panel shows representative immunoblots of syntaxin 4 (∼36 kDa) and SNAP-23 (28–29 kDa). The lower panel shows quantitation of SNAP-23 bound to syntaxin 4. The results shown here are representative of two (A) or three (B) independent experiments with four samples per experiment. a, p < 0.05 Thr versus Rest; b, p < 0.05 Thr/Ro versus Thr; c, p < 0.01 PMA/PI versus Rest and p < 0.01 PMA/PI versus PMA; d, p < 0.05 PMA versus Rest; e, p < 0.05 PMA/Ro versus PMA.

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There is a growing appreciation that intracellular signaling by kinases and second messengers plays an important role in exocytosis. Studies in synaptosomes, neuroendocrine cells, and pancreatic cells have shown that activated PKC stimulates secretion (22–24). Previous studies in platelets have also indicated that PKC has an amplifying or synergistic interaction with Ca2+ to stimulate secretion (14, 25). Platelets contain conventional PKCs (α, β, βII), which are activated by Ca2+ and DAG, the novel PKCs (δ, ε, η, and θ), which require only DAG for activation, and an atypical PKC (ζ), which is not activated by either Ca2+ or DAG (26). Although recombinant syntaxin 4 was a substrate for PKC α, β, γ, δ, ε, and ζ (Fig. 2D), the limited specificities of PKC inhibitors make it difficult to determine which PKC isoforms are involved in secretion in vivo. The fact that a selective inhibitor of Ca2+-dependent PKC isoforms (21) had no effect on platelet granule secretion and minimal effects on syntaxin phosphorylation (Table I) suggests that the Ca2+-independent PKC isoforms play a significant role in platelet secretion. It is also possible that different Ca2+-dependent or Ca2+-independent PKC isoforms act at specific steps in the secretory pathway.

Despite the growing evidence that PKC modulates the level of secretion induced by intracellular Ca2+ signaling, the mechanism of this effect remains poorly understood. We focused on syntaxin 4 phosphorylation, because the syntaxins play a central role in mediating molecular interactions that may affect exocytosis. The notion that PKC promotes exocytosis through syntaxin phosphorylation is consistent with a recent report (23) that the PKC-mediated effects on norepinephrine release in PC 12 cells could be completely blocked by the addition of exogenous syntaxin H3 domain, a region that mediates SNARE protein interactions (27, 28). Among potential phosphorylation sites in syntaxin 4, the site at residue 216 in the H3 domain is conserved among syntaxins (6, 29, 30). Mutations within the H3 domain affect the ability of syntaxin 1 to interact with SNAP-25 (31). Although the PKC phosphorylation site of syntaxin 4 remains to be determined, if syntaxin 4 is phosphorylated in this critical H3 domain, it should have effects on syntaxin 4-SNAP-23 interactions. Indeed, we found that PKC-mediated phosphorylation of syntaxin 4 blocks its binding with SNAP-23 by 71 ± 8% (Fig. 3), which represents almost complete inhibition of this 1-SNARE interaction when we consider that PKC maximally incorporated 0.8 mol of phosphate per mol of syntaxin 4.

Commmunoprecipitation experiments in thrombin- or PMA-activated cells provided consistent evidence that PKC-dependent phosphorylation of syntaxin 4 reduced its binding to SNAP-23. Phosphatase inhibitors enhanced syntaxin 4 phosphorylation and further decreased syntaxin 4-SNAP-23 binding induced by cell activation. Conversely, the PKC inhibitor Ro-31-8220 blocked syntaxin 4 phosphorylation in thrombin- or PMA-activated platelets and restored syntaxin 4-SNAP-23 binding to levels seen in resting platelets. In cells the maximal inhibitory effects on syntaxin 4-SNAP-23 binding induced by thrombin or PMA activation in the presence of phosphatase inhibitors (31%) was less than that induced by direct phosphorylation of syntaxin 4 by PKC in vitro. This may be due to the fact that a proportion of platelet syntaxin 4 is engaged in interactions with other molecules such as platelet Sec1 protein that interfere with its phosphorylation. Also, phosphatase inhibitors may not completely block dephosphorylation of phospho-syntaxin 4 in vivo.

Ternary SNARE complex formation has been identified in platelets, and the functional role of the component SNARE proteins in exocytosis has been demonstrated (17, 19). We found that anti-syntaxin 4 antibodies communoprecipitate SNAP-23 (Figs. 4 and 5) and VAMP (data not shown). However, we were unable to comment on the stoichiometry of these complexes (i.e. binary versus ternary), because the molecular identity of the VAMP in platelets is still not known. Binary t-SNARE complexes (e.g. syntaxin 4 and SNAP-23 or syntaxin 36 kDa) and SNAP-23 (28–29 kDa) and SNAP-23-23 (28–29 kDa). The platelet lysates...
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1 and SNAP-25) have not been detected in platelets or other cells. Perhaps this is because in vitro t-SNARE binary interactions occur slowly, are less stable, and promote the fast formation of the ternary complex (32, 33). Although formation of the ternary complex is required for exocytosis, it has not been established at which stage in secretion that the SNARE complex acts (e.g. formation of a fusion stalk between membranes, enlargement of the fusion pore, full membrane fusion, and discharge of contents, etc.) (6, 34–36). Recent data suggest that SNARE complexes form early and by themselves cannot drive a fusion reaction to completion (reviewed in Ref. 6). Our experiments are consistent with the findings of Flamenhaft et al. (17) that SNARE complexes are dissociated after resting platelets are stimulated to secrete. A similar pattern of SNARE complex dissociation is seen after maximal secretion of the cortical vesicles in sea urchin eggs, a cell that resembles platelets because it has a single round of exocytosis that is not complicated by other vesicle-trafficking events (36). Similarly, SNARE complexes both assemble and can completely disassemble before vacuolar fusion in yeast (35). Thus, in addition to SNARE complex formation, SNARE complex dissociation may be an important step in exocytosis. PKC phosphorylation of syntaxin 4 may contribute to exocytosis by promoting disassembly of the SNARE complex.

The finding that PKC phosphorylates syntaxin 4 in thrombin-activated platelets, promotes exocytosis, and blocks syntaxin 4-SNAP-23 interactions confirms and extends observations in other cells that PKC plays a critical role in modulating exocytosis. Recent studies have shown that PKC phosphorylation of other proteins alters their interactions with syntaxins: SNAP-25 (37), n-Sec1/Munc-18 (38), human platelet Sec1/Munc-18 (15), and SNARE-interacting calcium channel (39). In addition, synaptotagmin I, a putative Ca²⁺ sensor, which interacts with syntaxin and SNAP-25, is a substrate for PKC both in vitro and in vivo (40, 41). This suggests that PKC signaling provides a link between changes in the external milieu, sensed by ligand-membrane receptor interactions, and the secretory machinery, which allows the cell to dynamically modulate secretion in response to changes in its environment.

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