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ROLES OF VESICLE-ASSOCIATED MEMBRANE PROTEIN, SNAP-23, AND SYNTAXIN 4*

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To understand the molecular basis of granule release from platelets, we examined the role of vesicle-associated membrane protein, SNAP-23, and syntaxin 4 in α-granule secretion. A vesicle-associated membrane protein, SNAP-23, and syntaxin 4 were detected in platelet lysate. These proteins form a SDS-resistant complex that disassembles upon platelet activation. To determine whether these proteins are involved in α-granule secretion, we developed a streptolysin O-permeabilized platelet model of α-granule secretion. Streptolysin O-permeabilized platelets released α-granules, as measured by surface expression of P-selectin, in response to Ca\(^{2+}\) up to 120 min after permeabilization. Incubation of streptolysin O-permeabilized platelets with an antibody directed against vesicle-associated membrane protein completely inhibited Ca\(^{2+}\)-induced α-granule release. Tetanus toxin cleaved platelet vesicle-associated membrane protein and inhibited Ca\(^{2+}\)-induced α-granule secretion from streptolysin O-permeabilized platelets. An antibody to syntaxin 4 also inhibited Ca\(^{2+}\)-induced α-granule release by approximately 75% in this system. These results show that vesicle-associated membrane protein, SNAP-23, and syntaxin 4 form a heterotrimeric complex in platelets that disassembles with activation and demonstrate that α-granule release is dependent on vesicle SNARE-receptor target SNAP receptor (vSNARE-tSNARE) interactions.

α-Granules are the most abundant platelet secretory granule. These granules contain many components that have been implicated in thrombosis and atherosclerosis, including adhesion molecules, coagulation factors, soluble mediators of inflammation, and growth factors (1). α-Granule constituents are released from the platelet after platelet stimulation. Secretion of α-granules is tightly controlled to prevent unregulated release. Although there are many known signal transduction pathways that are activated concurrently with α-granule secretion, little is known regarding the mechanisms that lead directly to membrane fusion between granules and surface-connected membranes. Ultrastructural studies have shown that the α-granules of platelets are secreted primarily via fusion with the surface-connected open canalicular system (2) after apparent centralization of granules and microtubule organization. Such observations have led to speculation that cytoskeletal reorganization is responsible for α-granule release. However, inhibition of actin polymerization (3) or microtubule organization (4, 5) does not inhibit granule secretion. Furthermore, granule secretion and shape change can be dissociated under several experimental conditions (6–8). Thus, α-granule secretion is not solely dependent on shape change, indicating that other mechanisms need to be explored to understand the molecular mechanisms of α-granule secretion.

The study of the secretory machinery using cell-free systems and permeabilized cells has demonstrated the existence of a class of proteins, termed SNARE1 proteins, that mediate vesicle secretion (9, 10). These proteins have been found to mediate vesicle secretion in essentially all organisms investigated, from yeast to human (9). SNARE family proteins have been found in numerous cell types including cells responsible for maintaining vascular integrity such as platelets (11), leukocytes (12–14), and endothelial cells (15). Three sets of SNARE proteins have been identified (16). vSNAREs are type II integral membrane proteins located on vesicles and oriented such that the majority of the protein resides within the cytosol. tSNAREs are membrane proteins that are associated with target membranes and are also oriented toward the cytoplasm. Soluble SNAREs are cytoplasmic proteins that associate with vSNARE-tSNARE complexes. Functional evaluation of these proteins has led to the SNARE hypothesis that states that interactions between vSNAREs and tSNAREs mediate vesicle fusion with target membranes (17). Certain vSNAREs and tSNAREs contain coiled-coil structures that bind in a parallel (18) manner to create a stable exocytic core complex (19). The soluble SNARE proteins that are subsequently recruited to this complex then mediate membrane fusion via a process that involves ATP hydrolysis (17).

Analysis of the components of the exocytotic core complex has demonstrated a large number of vSNARE and tSNARE isoforms. Such diversity may provide the specificity required to target vesicles to their appropriate destinations (20). vSNAREs include vesicle-associated membrane proteins (VAMPs) 1 and 2 (also known as synaptobrevins 1 and 2) as well as cellubrevin. Isoform-specific expression of VAMP-1 and VAMP-2 has been demonstrated in a number of tissues (21–23). Cellubrevin has been detected in both neuronal and nonneuronal tissue (24, 25). SNAP-23 and SNAP-25 are tSNAREs that associate with the inner leaflet of the plasma membrane via a palmitoylation

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1 The abbreviations used are: SNARE, SNAP receptor; SNAP-23, soluble N-ethylmaleimide-sensitive fusion protein) attachment protein 23; VAMP, vesicle-associated membrane protein; SL-0, streptolysin O; PE, phycoerythrin; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; NSF, N-ethylmaleimide-sensitive fusion protein.
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anchor. SNAP-23 is ubiquitously expressed, whereas SNAP-25 expression is somewhat more restricted (26). Syntaxins are a family of tSNAREs with multiple isoforms (26). Specific tSNAREs will only bind to a limited number of vSNAREs (19). Such restricted interaction of SNARE proteins is thought to form the basis for specificity of vesicle targeting. VAMP, SNAP-23, and syntaxin interact to form the heterotrimeric complex that serves as a receptor for soluble SNARE proteins. Exocytosis proceeds through this complex.

Understanding of the role of SNARE proteins in mediating vesicle secretion in mammalian cells is derived largely from studies in neuronal and neuroendocrine cell systems. To what extent the principles derived from these studies can be applied to platelet granule secretion is uncertain. Several fundamental differences exist between granule secretion by platelets and vesicle secretion by neurons and neuroendocrine cells. Platelets are anucleate cytoplasmic fragments derived from megakaryocytes. Although some α-granule proteins are acquired via both fluid phase and receptor-mediated endocytosis, the majority of α-granular proteins are incorporated during thrombopoiesis (27). In contrast, biogenesis of synaptic vesicles occurs de novo from newly synthesized proteins and from assembly of recycled vesicles (28). Furthermore, platelets have a unique secretory pathway. Morphologic studies have shown that platelet granules are secreted primarily via fusion with the open canalicular pathway (1). Such studies have also shown that platelet granules fuse with one another before exocytosis. However, platelet granule fusion with plasma membrane occurs only occasionally. In contrast, vesicles from neurons and neuroendocrine cells are exocytosed via fusion with plasma membrane exclusively. Whether the molecular mechanisms mediating vesicle membrane fusion in platelets and cells of neural crest origin also differ is not well characterized. An exocytotic complex consisting of VAMP 2, SNAP-23, and syntaxin 4 has been found in several nonneuronal cell types. However, these individual components failed to form a heterotrimeric complex when co-incubated in vitro (29). Furthermore, a previously published study failed to demonstrate some of the essential components of the exocytotic machinery (e.g. SNAP-25 and VAMP-2) in platelets (11). In this report, we demonstrate the presence of an exocytotic core complex consisting of a VAMP, SNAP-23, and syntaxin 4 in platelet lysate and show that platelet vSNARE-tSNARE interactions mediate α-granule secretion from platelets.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All buffer constituents and detergents were purchased from Sigma. Sepharose 2B was obtained from Amersham Pharmacia Biotech. Reduced streptolysin O (SL-O) was purchased from Murex (Dartford, UK). PE-conjugated AC1.2 anti-P-selectin antibody was purchased from Becton Dickinson. Micro-BCA protein assay kit was purchased from Pierce and used according to the manufacturer’s instructions. Serine-phenylalanine-leucine-leucine-arginine (SFLLR) was synthesized using solid phase Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on an Applied Biosystems model 430A peptide synthesizer. Bovine brain homogenate was obtained from StressGen (Victoria, Canada). Human endothelial cell homogenate was obtained from Transduction Laboratories (Lexington, Kentucky). All solutions were prepared using water purified by reverse-phase osmosis on a Millipore Milli-Q purification Water System.

Antibodies and Toxins—Rabbit polyclonal and mouse monoclonal anti P-selectin antibodies were obtained from Transduction Labs (Lexington, Kentucky), reacts specifically with syntaxin 4 and has no cross-reactivity with syntaxin 2. Anti-P-selectin cytoplasmic tail antibody, a gift from Dr. Michael Berndt, is a rabbit polyclonal antibody directed against an 11-amino acid fragment of the P-selectin cytoplasmic tail (30). Tetanus toxin was obtained from List Biologies, Inc. (Crawford, CA).

Platelet Preparation—Blood from healthy donors who had not ingested aspirin in the two weeks before donation was collected by venipuncture into 0.4% sodium citrate. Citrate-anticoagulated blood was centrifuged at 200 × g for 20 min to prepare platelet-rich plasma. Platelets were then purified by gel-filtration using a Sepharose 2B column equilibrated in PIPES/EGTA buffer (25 mM PIPES, 2 mM EGTA, 137 mM KCI, 4 mM NaCl, 0.1% glucose, 0.1% bovine serum albumin, pH 6.4). Final gel-filtered platelet concentrations were 1–2 × 10^8 platelets/ml.

Platelet Permeabilization with SL-O—Gel-filtered platelets (20 μl) were incubated with 25 μM indicated dye in the presence or absence of 2 units/ml SL-O reduced with 5 mM dithiothreitol (1 μl). After a 15-min incubation, platelets were diluted in phosphate-buffered saline (500 μl) and analyzed immediately for fluorescence. Results are expressed as the fluorescence of platelets exposed to SL-O divided by the fluorescence of unexposed platelets. A value of 1 represents no permeabilization.

Analysis of P-selectin Surface Expression—Gel-filtered platelets (20 μl) were exposed to 2 units/ml SL-O. After the indicated amount of time, 5 mM MgATP was added to the reaction mixture at pH 6.4. We have previously demonstrated that maximal α-granule secretion occurs in permeabilized platelets at pH 6.4. After a 15-min incubation, Ca2+ was added to the reaction tube until otherwise indicated. The amount of CaCl2 required to give a free Ca2+ concentration of 10 μM in the presence of 2 mM EGTA was calculated for each condition using a computer program (gift from Dr. P. J. Padfield) based on the algorithm described by Fabiato and Fabiato (32). After an additional 5-min incubation, PE-conjugated AC1.2 anti-P-selectin antibody (10 μl) was transferred to the reaction tube. Phosphate-buffered saline (500 μl) was added to the sample after a 15-min incubation, and the platelets were analyzed immediately by flow cytometry as described below. In experiments designed to determine the efficacy of various inhibitors, the inhibitor was added at 45 min before the addition of MgATP. Tetanus toxin was activated with the addition of 5 mM dithiothreitol.

Immunoblot Analysis—In experiments evaluating the presence of individual proteins in platelets, samples were heated to 100 °C before electrophoresis. In experiments evaluating SNARE protein complexes, 500 μl of resting platelets or platelets exposed to 100 μM SFLLR were pelleted and solubilized in 250 μl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 0.5% NP-40, 0.5% β-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) at 37 °C. SDS-PAGE using either 10%, 12% gels, or 8%/15% discontinuous gels, immunoblotting, and enhanced chemiluminescence were carried out using standard protocols (33, 34). Densitometry was performed using Gelbase/Gelblot-Pro software on a Macintosh PowerPC.

Flow Cytometry—Flow cytometry was performed on gel-filtered platelet samples using a Becton-Dickinson FACSCalibur flow cytometer. Fluorescent channels were set at logarithmic gain. Ten thousand particles were acquired for each sample. A 530/30 band pass filter was used for FL-1 fluorescence, and a 585/42 band pass filter was used for FL-2 fluorescence. Platelet-associated fluorescein isothiocyanate was measured in the FL-1 channel. PE was measured in the FL-2 channel. Data were analyzed using CellQuest software on a Macintosh PowerPC.

RESULTS

Platelet VAMP, SNAP-23, and Syntaxin 4—To determine whether an exocytotic core complex consisting of VAMP, SNAP-23, and syntaxin 4 exists in platelets, we assayed for the presence of these proteins in platelet lysate. As illustrated in Fig. 1A, all three proteins are detected in platelet lysates subjected to immunoblotting. In these experiments, solubilized lysates were heated at 100 °C before loading onto the gel. The VAMP antibody used in this experiment was a polyclonal IgG directed against a peptide fragment of VAMP-2. This sequence is located in the SNAP-23/syntaxin binding domain, a region conserved among vesicle-associated membrane proteins (35) (Fig. 1A). This antibody recognized a single protein in bovine brain

2 Flamenhaft, R., Furie, B., Furie, B. C., J. Cell. Physiol., in press.
ents of the exocytotic core complex serve a functional role in platelets—

FIG. 1. Detection of VAMP, SNAP-23, and syntaxin 4 in platelet lysates. a, proteins from bovine brain (20 µg) and human platelets (50 µg) were solubilized at 100 °C in sample buffer, separated by SDS-PAGE on a 12% gel, and electrophoretically transferred to polyvinylidene difluoride membranes. Immunoblotting was performed with anti-VAMP antibody. b, proteins from bovine brain (20 µg) and human platelets (1 µg) were prepared as in a, and immunoblotting was performed with anti-SNAP-23 antibody. c, proteins from human endothelial cells (20 µg) and human platelets (3 µg) were prepared as in a, and immunoblotting was performed with anti-syntaxin 4 antibody. Bands were visualized using enhanced chemiluminescence for detection. The position of the molecular mass standards used are indicated on the right.

lysatess and in human platelet lysates. However, the VAMP species in bovine brain had a molecular mass of 18 kDa, whereas the VAMP species in platelets migrated on the gel with an apparent molecular mass of 14 kDa. The VAMP species in platelets was not recognized by a monoclonal antibody specific to VAMP-2 (11) nor by an antibody specific to VAMP-1 (data not shown). These results suggest the platelet VAMP is yet another VAMP isoform. The antibody to SNAP-23 is a polyclonal antibody raised against the C-terminal end of SNAP-23. This antibody recognized a protein of approximately 23 kDa in both bovine brain and in human platelet lysates. The syntaxin 4 antibody is a monoclonal antibody raised against syntaxin 4. Proteins of approximately 34 kDa were detected by this antibody in both human endothelial cell and human platelet lysates.

VAMP, SNAP-23, and Syntaxin 4 Complex Formation in Platelets—An SDS-resistant, heat-sensitive complex consisting of VAMP, SNAP-23, and syntaxin 4 has been described in neurons (36, 37). To determine whether such a complex exists in platelets, platelet lysate was solubilized in SDS-containing sample buffer at 37 °C for 30 min and analyzed by SDS-PAGE followed by immunoblotting using the anti-VAMP-2, anti-SNAP-23, and anti-syntaxin 4 antibodies. A doublet of approximately M, 70,000 was recognized by antibodies directed against VAMP (Fig. 2a) and SNAP-23 (Fig. 2b). A band of approximately M, 70,000 was also recognized by the anti-syntaxin antibody (Fig. 2c). As has been shown in neuronal tissue, the high molecular weight complex was not observed when platelet lysate was subjected to 100 °C (36, 37). Nonimmune antibody did not detect any bands in the platelet lysate.

To determine whether disassembly of this complex occurred with platelet activation, platelets were exposed to SFLLR and subsequently assayed for the presence of the 70-kDa SDS-resistant, heat-sensitive complex. Disassembly of the complex was observed under these conditions upon platelet activation with SFLLR (Fig. 3). The amount of talin in the gels containing sample from resting and activated platelets was determined to assure equal protein content. The amount of talin present in each of the lanes did not vary by more than 10% (data not shown), indicating that differences in intensity of the 70-kDa band in resting samples compared with activated samples was not secondary to differences in protein loading. Activation of platelets upon exposure to SFLLR was confirmed by demonstrating increases in P-selectin surface expression in the SFLLR-exposed platelets using flow cytometry (data not shown).

Ca²⁺-induced α-Granule Secretion in SL-O-permeabilized Platelets—We next sought to determine whether the components of the exocytotic core complex serve a functional role in α-granule secretion. To access the platelet cytoplasm in whole, functional platelets with specific inhibitors of SNARE proteins such as antibodies and clostridial toxins, we exposed platelets to SL-O. SL-O was used for this purpose because it inserts preferentially into plasma membrane without permeabilizing granules; it creates stable, uniform pores, large enough to permit molecules of greater than 150 kDa into the cytoplasm. The efficacy of permeabilization was assessed by incubating SL-O-permeabilized platelets with fluorescent compounds of
Gel-filtered platelets (50 μg) were incubated with 2 units/ml SL-O or buffer in the presence of fluorescein isothiocyanate-dextran sulfate of the indicated molecular mass. Fluorescent compounds were at 25 μM. After 15 min, platelets were diluted in phosphate-buffered saline (500 μl) and analyzed immediately by flow cytometry. Incorporation of the indicated compounds into platelets is expressed as the fluorescence of SL-O-exposed platelets relative to nonpermeabilized platelets. Error bars represent the S.E. of four independent experiments.

Many SL-O-permeabilized systems lose the ability to secrete in response to Ca2+ as a function of time after permeabilization (40). To determine whether SL-O-permeabilized platelets lose their responsiveness to Ca2+ over time, we permeabilized platelets with SL-O and exposed them to Ca2+ at various times after permeabilization. SL-O-permeabilized platelets express P-selectin on their surface in response to Ca2+ up to 2 h after permeabilization (Fig. 6). There is no detectable loss of responsiveness to Ca2+ over this period. Furthermore, no spontaneous P-selectin expression was observed in SL-O-permeabilized platelets that were not exposed to Ca2+. Thus, SL-O-permeabilized platelets secrete α-granules in response to Ca2+, and this response is stable for at least 2 h.

**Effect of Anti-VAMP Antibodies and Tetanus Toxin on Ca2+-induced α-Granule Secretion**—We next sought to determine whether platelet VAMP plays a role in mediating α-granule secretion. In these experiments, we incubated SL-O-permeabilized platelets with polyclonal anti-VAMP-2 antibody for 1 h before exposure to Ca2+. Anti-VAMP-2 antibody inhibits Ca2+-mediated P-selectin surface expression from SL-O-permeabilized platelets by greater than 95% (Fig. 7). In contrast, rabbit nonimmune IgG had no effect on Ca2+-induced P-selectin surface expression. To determine whether an antibody directed at an α-granule membrane protein thought not to be involved in α-granule secretion affects secretion (41), we exposed SL-O-permeabilized platelets to a polyclonal antibody directed against the cytoplasmic tail of P-selectin (30). This antibody had no effect on Ca2+-induced P-selectin surface expression. This result argues that the P-selectin cytoplasmic tail is not involved in secretion despite its phosphorylation during activation (42). It also suggests that an antibody directed at an α-granule surface protein will not nonspecifically interfere with secretion.

Tetanus toxin cleaves VAMP family proteins specifically (43). The next series of experiments were performed to determine the sensitivity of platelet VAMP to tetanus toxin. We exposed permeabilized platelets to tetanus toxin and assayed for VAMP by immunoblot analysis using the polyclonal anti-VAMP-2 peptide antibody. The VAMP family protein that is recognized by the polyclonal anti-VAMP-2 peptide antibody is
sensitive to degradation by tetanus toxin (Fig. 8a). Tetanus toxin had no effect on VAMP from nonpermeabilized platelets. Given the observation that tetanus toxin degrades the VAMP family protein found in platelets, we sought to determine whether tetanus toxin would inhibit Ca\textsuperscript{2+}-mediated \(\alpha\)-granule secretion. In these experiments, SL-O-permeabilized platelets were exposed to 2 \(\mu\)M tetanus toxin for 1 h before exposure to Ca\textsuperscript{2+}. Tetanus toxin inhibited Ca\textsuperscript{2+}-mediated surface P-selectin surface expression by approximately 70% (Fig. 8b). Dithiothreitol, which was used to reduce the tetanus toxin, had no significant effect on Ca\textsuperscript{2+}-induced P-selectin surface expression. Tetanus toxin that had been inactivated by boiling for 10 min had no effect on \(\alpha\)-granule secretion (data not shown). The effect of tetanus toxin was dose-dependent (Fig. 8c).

**Fig. 5.** Ca\textsuperscript{2+} stimulates \(\alpha\)-granule secretion from SL-O-permeabilized platelets. Gel-filtered platelets (20 \(\mu\)l) were incubated with buffer (nonpermeabilized) or 2 units/ml SL-O (permeabilized) for 5 min. Permeabilized samples were then incubated for 15 min with 5 mM MgATP at pH 6.4. Buffer, 10 \(\mu\)M Ca\textsuperscript{2+}, or 100 \(\mu\)M SFLLR was then added to the nonpermeabilized and permeabilized platelets as indicated, and the reaction mixtures were allowed to incubate for 5 min. P-selectin surface expression was assayed by incubating samples with a PE-conjugated AC1.2 anti-P-selectin antibody (10 \(\mu\)l) for 15 min. Phosphate-buffered saline (500 \(\mu\)l) was added to the sample after a 15-min incubation, and the platelets were analyzed immediately by flow cytometry as described under “Experimental Procedures.” P-selectin expression is indicated by PE-AC1.2 fluorescence. Data represent the distribution of the relative fluorescence of 10,000 platelets.

**Fig. 6.** SL-O-permeabilized platelets secrete \(\alpha\)-granules in response to Ca\textsuperscript{2+} for 2 h after permeabilization. Gel-filtered platelets (20 \(\mu\)l) were incubated with 2 units/ml SL-O for 5 min. 5 mM MgATP was then added to the reaction mixture at pH 6.4. After the indicated amount of time after permeabilization, Ca\textsuperscript{2+} (○) or buffer (■) was added to the reaction tube. After an additional 5-min incubation, PE-conjugated AC1.2 anti-P-selectin antibody (10 \(\mu\)l) was transferred to the reaction tube. Phosphate-buffered saline (500 \(\mu\)l) was added to the sample after a 15-min incubation, and the platelets were analyzed immediately by flow cytometry. P-selectin expression is indicated by PE-AC1.2 fluorescence. Error bars represent the S.E. of three independent experiments.
Effect of Antibodies Directed against Syntaxin 4 on Ca\(^{2+}\)-induced \(\alpha\)-Granule Secretion—Syntaxin 4 is found in platelets (11) and is thought to play a role in the regulated secretion of granules from hematopoietic cells (14). To determine whether this tSNARE mediates \(\alpha\)-granule secretion, we incubated SL-O-permeabilized platelets with an anti-syntaxin 4 monoclonal antibody for 1 h before exposure to Ca\(^{2+}\). Anti-syntaxin 4 antibody inhibited Ca\(^{2+}\)-induced P-selectin surface expression by approximately 75% (Fig. 9). Nonimmune mouse IgG\(_1\) had no effect.

**DISCUSSION**

An understanding of the mechanisms that mediate \(\alpha\)-granule release has evolved over the past two decades. Morphologic studies demonstrated that platelets undergo shape change upon activation that results in the apparent centralization of granules and in microtubular band reorganization (44–46). These observations have led investigators to evaluate the roles of actin and microtubule reorganization in granule secretion. However, experiments using cytochalasin E at concentrations that inhibit actin polymerization and platelet shape change have no effect on \(\alpha\)-granule release (3). Similarly, although pharmacological agents that affect microtubule organization inhibited granule secretion in some studies, further investigation suggested that these agents worked through mechanisms other than disrupting microtubule reorganization (4, 5). Furthermore, granule secretion and shape change can be dissociated under several experimental conditions (6–8). Subsequent detailed ultrastructural studies demonstrated that 71% of dense granules are within 12.5 nm of the plasma membrane and that this population is preferentially released upon platelet activation (47, 48). Thus, it is not certain that granule movement is required for platelet granule secretion. Some of the protein components, termed SNARE proteins, of the secretory machinery that mediate vesicle secretion in other cell types have been shown to exist in platelets (11). However, several of the components thought to be essential for granule release were not found. The present study demonstrates that three SNARE proteins, VAMP, SNAP-23, and syntaxin 4, are found in platelets and form an exocytic core complex that disassembles upon platelet activation. This study also uses a
functional assay to show that vSNARE-tSNARE interactions mediate α-granule secretion.

Of the exocytotic core components evaluated in this study, only syntaxin 4 has been previously demonstrated in platelets. Lemons et al. (11) did not find VAMP-2 in a preparation from outdated platelets enriched for SNARE proteins by immunoprecipitation of a 20 S complex that typically includes a VAMP species. They probed for VAMP-2 using a monoclonal antibody (clone C141.1). In contrast, we detected VAMP in platelets using a polyclonal antibody directed against a peptide fragment of VAMP-2 and a different monoclonal antibody (clone SP10) that recognizes both VAMP-1 and VAMP-2. Platelet VAMP has an apparent molecular weight that is distinct from VAMP species found in brain (Fig. 1). In addition, greater concentrations of tetanus toxin are required to cleave and inactivate platelet VAMP (>0.5 μM) than are required to cleave neuronal VAMP (<50 nM) (Fig. 8) (49, 50). Furthermore, platelet VAMP is not recognized by monoclonal antibodies specific to either VAMP-2 (Lemons et al. (11)) or VAMP-1 (data not shown). For these reasons, we believe that platelet VAMP represents a novel species of VAMP. VAMP species with reduced or absent sensitivity to clostridial toxins have been identified in pancreatic zymogen granules (21), enterochromaffin cells (51), and adipocytes (52). However, the apparent molecular weight of the VAMP species described in these studies was similar to those of VAMP-1 and VAMP-2.

Lemons et al. (11) similarly did not find SNAP-25 in platelets. However, we found SNAP-23, which has a wider tissue distribution (53), in platelets (Fig. 1). Platelets contain both syntaxin 2 and 4 but not syntaxins 1, 2, and 5 (11). The anti-syntaxin 4 antibody used in these studies to detect platelet syntaxin was raised to syntaxin 4, which has less than 50% amino acid sequence homology with syntaxin 2. Furthermore, this antibody does not cross-react with purified syntaxin 2. Therefore, the syntaxin isoform relevant to these studies is syntaxin 4.

The novel VAMP, SNAP-23, and syntaxin 4 assemble into a heterotrimeric complex in platelets as evidenced by the presence of a SDS-resistant, heat-sensitive complex in platelets. This complex contains platelet VAMP, SNAP-23, and syntaxin 4 in α-granule secretion.

**Fig. 8.** Effect of tetanus toxin on platelet VAMP and on P-selectin surface expression in SL-O-permeabilized platelets. A, gel-filtered platelets (250 μl) were exposed to either 2 units/ml SL-O or buffer for 5 min. Platelets were then incubated with 1 μM tetanus toxin or buffer for 45 min. Platelet proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. VAMP was visualized by immunoblotting using an anti-VAMP peptide antibody and enhanced chemiluminescence for detection. B, gel-filtered platelets (20 μl) were incubated with 2 units/ml SL-O for 5 min. Platelets were then incubated for 45 min with buffer (panels a and b), 5 mM dithiothreitol (panel c), or 2 μM tetanus toxin (panel d). MgATP (5 mM) was added to the reaction mixture at pH 6.4, and the reaction mixtures were incubated for 15 min. To stimulate platelet activation, either buffer (panel a) or Ca²⁺ (panels b, c, and d) was added to the reaction tube. Samples were assayed for P-selectin surface expression as described previously. Histograms represent the distribution of the relative fluorescence of 10,000 platelets. P-selectin expression is indicated by PE-AC1.2 fluorescence. C, SL-O-permeabilized platelets were exposed to increasing concentrations of reduced tetanus toxin for 45 min, exposed to MgATP at pH 6.4, and stimulated with Ca²⁺. α-Granule secretion was then assessed by assaying for P-selectin surface expression. Data are expressed as percent P-selectin expression compared with Ca²⁺-stimulated samples exposed to buffer alone.
as demonstrated by the fact that bands of approximately 70 kDa are detected by anti-VAMP, anti-SNAP-23, and anti-syntaxin 4 antibodies. The fact that the anti-VAMP and anti-SNAP-23 antibodies detect a doublet whereas the anti-syntaxin 4 antibody does not (Fig. 2) suggests that the doublet may represent complexes containing syntaxin 2 and syntaxin 4, which differ in molecular mass. A recently published study by Foster et al. (29) using recombinant VAMP-2, SNAP-23, and syntaxin 4 in an in vitro binding assay did not demonstrate a SDS-resistant complex of these three proteins (29). The discrepancy between these results and the detection of a SDS-resistant complex containing a novel VAMP isoform, SNAP-23, and syntaxin in platelets may result from the fact that only this novel VAMP can participate in the complex. Alternatively, the differences in experimental conditions between an in vitro assay using recombinant proteins and detecting native complex

in vivo may account for the difference in results. The fact that this complex disassembles upon platelet activation is consistent with observations in neuronal and neuroendocrine systems that the exocytotic core complex is disassembled upon membrane fusion. In these other systems, soluble SNARE proteins including N-ethylmaleimide-sensitive fusion protein (NSF) and α-SNAP are thought to mediate core complex disassembly via NSF hydrolysis of ATP. This mechanism has yet to be evaluated in platelets. However, the experiments performed in this study do demonstrate that constituents of this core complex participate in α-granule secretion.

SL-O-permeabilized cell models have been used to define the molecular mechanisms of various secretory events in neuronal, neuroendocrine, and endocrine cells. For example, incubation of SL-O-permeabilized pancreatic β cells with an antibody directed at synaptotagmin III inhibited insulin secretion (54).
Incubation of SL-O-permeabilized enterochromaffin cells and islet β cell lines with tetanus toxin inhibited Ca^{2+}-evoked release of amylase (55) and insulin (56) release, respectively. The finding that the Ca^{2+}-mediated secretory response of SL-O-permeabilized platelets is stable over time has certain implications with regard to the organization of the secretory machinery in platelets. For example, the SNARE hypothesis maintains that after interactions between vSNAREs and tSNAREs, cytoplasmic factors associate with these membrane proteins and mediate fusion events (17). The concentration of such cytoplasmic factors is decreased by several orders of magnitude upon permeabilization. In many systems, cytosol must be included in the permeabilization buffer to retain the secretory response to Ca^{2+} (40, 57, 58). The fact that platelets retain the ability to secrete α-granules upon exposure to Ca^{2+} after SL-O permeabilization without added cytosol raises the possibility that either cytoplasmic factors are not required for α-granule secretion or that such factors are already bound to the vSNARE-tSNARE complexes before permeabilization. Further studies will be required to differentiate between these two possibilities.

In this study, we have used a SL-O-permeabilized platelet model to demonstrate that both vSNAREs and tSNAREs mediate α-granule secretion. Platelet VAMP mediates α-granule secretion, as evidenced by the fact that both an antibody directed against the protein and tetanus toxin, which cleaves VAMP (31), inhibit α-granule secretion. The concentration of tetanus toxin required to achieve 50% inhibition of α-granule secretion in this system was approximately 900 nM. This concentration is more than 10-fold greater than that required to inhibit catecholamine release from permeabilized chromaffin cells (49) or acetylcholine release from synaptosomes (50). However, the concentration of tetanus toxin required to inhibit α-granule release are similar to those required to inhibit Ca^{2+}-induced histamine release from permeabilized enterochromaffin cells (51). These differences may be because platelets and enterochromaffin cells contain VAMP species that are relatively poor substrates for tetanus toxin compared with VAMP-1 and VAMP-2.

With regard to tSNAREs, we assessed the function of syntaxin 4 in α-granule secretion. Both syntaxins 2 and 4 are found in platelets (11). Syntaxin 2 is widely expressed and is thought to participate in constitutive secretion (20). In contrast, syntaxin 4 is more specifically expressed. Other hematopoietic cells, including macrophages and neutrophils, synthesize syntaxin 4 (13, 14). Syntaxin 4 may play a role in the regulated secretion of lysosomes by neutrophils (14). For these reasons, we choose to target syntaxin 4 to determine whether or not syntaxin family proteins function in α-granule secretion. The fact that this antibody inhibits α-granule secretion in SL-O-permeabilized platelets implicates this syntaxin isoform in α-granule secretion. However, this antibody does not completely block α-granule secretion. This observation suggests that either other molecules (e.g. syntaxin 2) can partially compensate for syntaxin 4 activity or that this particular monoclonal antibody is not capable of inhibiting syntaxin 4 activity completely.

The presence of the exocytotic core complex in platelets (11) and the demonstration that constituents of this complex mediate α-granule secretion suggest a model for α-granule secretion from platelets. According to this model, platelet VAMP, SNAP-23, and syntaxin 4 form a complex that docks α-granules to surface-connected membranes, primarily the open canalicular system. The heterotrimeric complex is then decorated with soluble SNARE proteins such as NSF and α-SNAP, both of which have been found in platelets (11). Binding of these solvable factors to the heterotrimeric complex may occur before activation, because permeabilization of platelets does not create a requirement for exogenously added factors. With activation, hydrolysis of ATP by NSF and release of Ca{sup 2+} into the cytosol cause complex disassembly and membrane fusion. Further investigation will be required to evaluate this model and define the relationship between platelet signaling events and SNARE protein rearrangements leading to membrane fusion.

In summary, we have detected a heterotrimeric exocytotic core complex in platelets and have demonstrated that a platelet VAMP and syntaxin 4 mediate α-granule secretion. These observations suggest that despite morphologic differences in granule secretion between platelets and nucleated cells, α-granule secretion shares several fundamental mechanisms with neuronal and neuroendocrine cell vesicle secretion at the molecular level. An understanding of the molecular mechanisms of α-granule secretion is not only of interest in platelet biology but is also of pharmacological interest given the possibility that proteins mediating α-granule secretion may serve as appropriate targets for anti-thrombogenic agents.
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Proteins of the Exocytotic Core Complex Mediate Platelet α-Granule Secretion: ROLES OF VESICLE-ASSOCIATED MEMBRANE PROTEIN, SNAP-23, AND SYNTAXIN 4

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