The t-SNAREs Syntaxin 1 and SNAP-25 Are Present on Organelles That Participate in Synaptic Vesicle Recycling

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Abstract. Syntaxin 1 and synaptosome-associated protein of 25 kD (SNAP-25) are neuronal plasmalemma proteins that appear to be essential for exocytosis of synaptic vesicles (SVs). Both proteins form a complex with synaptobrevin, an intrinsic membrane protein of SVs. This binding is thought to be responsible for vesicle docking and apparently precedes membrane fusion. According to the current concept, syntaxin 1 and SNAP-25 are members of larger protein families, collectively designated as target-SNAP receptors (t-SNAREs), whose specific localization to subcellular membranes define where transport vesicles bind and fuse. Here we demonstrate that major pools of syntaxin 1 and SNAP-25 recycle with SVs. Both proteins cofractionate with SVs and clathrin-coated vesicles upon subcellular fractionation. Using recombinant proteins as standards for quantitation, we found that syntaxin 1 and SNAP-25 each comprise ~3% of the total protein in highly purified SVs. Thus, both proteins are significant components of SVs although less abundant than synaptobrevin (8.7% of the total protein). Immunoisolation of vesicles using synaptophysin and syntaxin specific antibodies revealed that most SVs contain syntaxin 1. The widespread distribution of both syntaxin 1 and SNAP-25 on SVs was further confirmed by immunogold electron microscopy. Botulinum neurotoxin C1, a toxin that blocks exocytosis by proteolyzing syntaxin 1, preferentially cleaves vesicular syntaxin 1. We conclude that t-SNAREs participate in SV recycling in what may be functionally distinct forms.

Norepinephrine release occurs via Ca2+-dependent exocytosis of synaptic vesicles (SVs).1 Whereas the mechanism of exocytosis is still unclear, rapid progress has recently been made in identifying proteins that are involved in membrane fusion. According to the current concept, the core of the exocytotic fusion machine consists of a set of highly conserved membrane proteins (for reviews see Rothman and Warren, 1994; Ferro-Novick and Jahn, 1994). In neurons, these proteins are the SV protein synaptobrevin (VAMP) (Trimble et al., 1988; Baumert et al., 1989) and the synaptic plasmamembrane proteins syntaxin 1 (Bennett et al., 1992; Inoue et al., 1992) and SNAP-25 (Oyler et al., 1989).

Rothman and coworkers have recently shown that these three proteins form a complex that interacts in an ATP-dependent manner with the soluble proteins N-ethylmaleimide sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs, Sollner et al., 1993a). Both NSF and SNAPs are highly conserved in evolution and are required for membrane fusion events from yeast to mammals. The ability of synaptobrevin, syntaxin 1, and synaptosomal-associated protein of 25 kD (SNAP-25) to bind SNAPs has led to their designation as SNAP receptors (SNAREs). Accordingly, the vesicle protein synaptobrevin was designated as vesicle-SNARE (v-SNARE) and the membrane proteins SNAP-25 and syntaxin 1 as target-SNAREs (t-SNAREs) (Sollner et al., 1993a). Based on their observations, Rothman and coworkers proposed that most, if not all, intracellular fusion events are mediated by SNARE-interactions (Sollner et al., 1993a). This model envisions that the membrane specificity of fusion results from the selective interactions of discrete pairs of v-SNAREs and t-SNAREs localized to specific membranes, whereas NSF and SNAPs may participate...
in all fusion events (for reviews see Rothman and Warren, 1994; Ferro-Novick and Jahn, 1994).

Several lines of evidence support the view that synaptobrevin, syntaxin, and SNAP-25 are essential components of membrane fusion. First, all three proteins exhibit sequence homologies to yeast proteins that have been demonstrated by genetic approaches to be required for intracellular fusion events. The highest degree of homology was found between gene products operating at the fusion of carrier vesicles with the plasma membrane. These proteins include Snc1 and Snc2 (homologous to synaptobrevin; Gerst et al., 1992; Protopopov et al., 1993), Sso1 and Sso2 (homologous to syntaxin; Aalto et al., 1993), and Sec9 (homologous, but to a lesser degree, to SNAP-25; Brennwald et al., 1994) of yeast. Second, more closely related homologues of syntaxin (Bennett et al., 1993) and synaptobrevin (McMahon et al., 1993) were found in nonneuronal cells where they are selectively localized to distinct intracellular membranes. Third, clathrioidal neurotoxins (tetanus and botulinum neurotoxins) were shown to exert their inhibitory action on neurotransmitter release by selectively cleaving synaptobrevin, syntaxin, and SNAP-25 (for review see Niemann et al., 1994). These findings provide the most direct evidence so far that these proteins are required for exocytosis.

As discussed above, syntaxin and SNAP-25 are thought to function as the receptors for SVs in the presynaptic plasma membrane. In support of this model, it was reported that the integral membrane protein syntaxin 1 is enriched in synapses and that it is associated with the plasma membrane (Bennett et al., 1992). Similarly, the peripheral membrane protein SNAP-25 has also been observed in the presynaptic membrane (Oyler et al., 1989). Using immunogold electron microscopy, however, syntaxin 1 has also been found on SVs (Koh et al., 1993). This finding raises the question whether t-SNAREs may undergo recycling in the nerve terminal. For these reasons, we have analyzed the localization of syntaxin 1 and SNAP-25 in recycling organelles of the nerve terminal. We found that significant pools of syntaxin 1 and SNAP-25 are localized to SVs since both comprise 3% of the total vesicle protein. We also report that recycling of these proteins occurs via clathrin-coated vesicles and that the vesicular pool of syntaxin 1 is a preferential target botulinum neurotoxin CI (BoNT/C1).

Materials and Methods

Materials

The monoclonal antibody directed against syntaxin (HPC-1) was a kind gift of Dr. C. J. Barnstable (Department of Ophthalmology and Visual Sciences, Yale University School of Medicine, New Haven, CT). Barnstable et al., 1985). This antibody reacted equally well with both neuronal isoforms of syntaxin 1A and B, henceforth collectively named as syntaxin 1 (see Bennett et al., 1993). In our experiments, the two isoforms were normally not resolved, however, occasional checks showed no difference in isoform distribution. Monoclonal antibodies directed against Na+/K-ATPase (α subunit) and ribophorin were generously provided by Drs. M. J. Caplan (Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT) and G. Kreibich (New York University, New York, NY). Pietrin et al., 1992; Kelleher et al., 1992). Monoclonal and polyclonal antibodies directed against the following proteins were described previously: synaptophysin (Jahn et al., 1985), rab3A (Matteoli et al., 1991), clathrin-light chain (Maycox et al., 1992), InsP3 receptor (Mignery et al., 1992), and Src9 receptor R1 subunit (Sucher et al., 1993). Monoclonal and polyclonal antibodies directed against synaptobrevin I and II and SNAP-25 will be described in detail elsewhere (Edelmann et al., 1995; Bruns, D., and R. Jahn, manuscript in preparation). BoNT/C1 was kindly provided by Dr. H. Niemann (Institut für Mikrobiologie, Tübingen, Germany).

Subcellular Fractionation

Synaptic vesicles were purified from isolated nerve terminals essentially as described (Nagy et al., 1976; Hutner et al., 1983) using chromatography on controlled pore glass beads as a final purification step. Preparation of immunobeads and immunoinosolization of organelles followed published procedures (Burger et al., 1989; Walch-Solimena et al., 1993). Clathrin-coated vesicles were purified from nerve terminals as described (Maycox et al., 1992). Briefly, synaptosomes were purified by differential and ficoll gradient centrifugation (see below), lysed by hypotonic shock and subfractionated by consecutive steps of differential centrifugation: sedimentation at 20,000 g for 20 min to sediment heavy membranes (fraction hm) and sedimentation at 55,000 g to sediment the bulk of coated and SVs (light membranes, lm). Fraction (Im) was further purified by ficoll gradient centrifugation, followed by centrifugation through a Dextran sulfate cushion, yielding purified clathrin-coated vesicles (CCV) (see Maycox et al., 1992) for full details of the procedure.

Preparation and Incubation of Subcellular Fractions

Synaptosomes were prepared by differential and ficoll gradient centrifugation as described (Fischer von Mollard et al., 1991; McMahon et al., 1992). Synaptosomes were incubated in the presence or in the absence of BoNT/C1 (75 nM) for 90 min at 37°C. At the end of the incubation, the inhibition of glutamate release by the toxin was controlled using an online photometric assay (Nicholls and Shiara, 1986; Blasi et al., 1993b). After the release assay synaptosomes were centrifuged at 12,000 g for 12 min. The resulting pellets were resuspended in 0.3 ml of incubation buffer (20 mM Hepes, pH 7.4, 10 mM glucose, 5 mM KCl, 140 mM NaCl, 5 mM NaHCO3, 1 mM MgCl2, 1.2 mM Na3HPO4) and lysed by hypotonic shock and rapid homogenization. Lysate pellets (containing heavy membranes including the plasma membrane) were collected after centrifugation at 12,000 g for 20 min in the corresponding lysate supernatants (light membranes) were centrifuged at 200,000 g for 20 min in a TLA 100.3 rotor (Beckman Instruments, Palo Alto, CA) to obtain crude SV fractions.

For BoNT/C1 treatment of subcellular fractions, synaptosomal lysate pellets and crude SVs (see above) were incubated in the presence or in the absence of BoNT/C1 holotoxin (100 nM) for 90 min at 37°C in 5 mM Hepes, pH 7.4, containing 100 mM potassium gluconate, 30 mM NaCl and 10 mM dithiothreitol (for the release of the toxin light chain).

Preparation of Recombinant Proteins

cDNA encoding synaptobrevin I and II (VAMP 1 and 2; Elfertink et al., 1989), syntaxin 1A (Bennett et al., 1992), and SNAP-25 (Blasi et al., 1993a) were kindly provided by Drs. R. H. Scheller (Stanford University/HHMI, Stanford, CA) and T. C. Sudhof (University of Texas, Dallas, TX). Full-length synaptobrevin coding sequences were amplified using the PCR with primers that contained BamHI and EcoRI restriction sites. The PCR products were digested with BamHI and EcoRI and ligated, in frame, to the glutathione-S-transferase (GST) gene of pGEX-2T (Pharmacia LKB Biotechnology, Piscataway, NJ). The GST-fusion proteins were expressed in JM109 cells (500 ml) grown to an A600 of 0.4, by inducing with 0.4 mM IPTG. After 2–4 h, cells were pelleted, resuspended in 10 ml 20 mM Tris, pH 7.2, 150 mM NaCl (TS buffer) containing protease inhibitors (1 mM PMSF, 2 μg/ml pepstatin, and 20 μg/ml aprotinin) and sonicated on ice 3 × 30 sec with a probe sonicator. Triton X-100 was added to the lysate (0.5%), mixed for 20 min at 4°C, and the suspension centrifuged twice at 12,000 rpm for 20 min in an SS-34 rotor (Sorvall, Boston, MA). The supernatant was mixed with 2.5 U of thrombin (Sigma Chem. Co., St. Louis, MO) per 100 μl beads, for 1 h at room temperature in TS containing 0.5% Triton X-100 (Guan and Dixon, 1991). The supernatant containing the purified synaptobrevin was collected and the thrombin was inhibited by the addition of 1 mM PMSF.

Full-length syntaxin 1A and SNAP-25 coding sequences were amplified using primers that contained BamHI restriction sites. The PCR products were digested and subcloned into pProHIs (Invitrogen, San Diego, CA), resulting in fusion proteins which contain a six histidine tag at their...
NH2-termini. The fusion proteins were expressed and harvested as described above for the GST fusion proteins with the following differences. Expression was in TOP-10 cells (Invitrogen), bacteria were lysed in 20 mM Hepes, pH 7.4, 500 mM KCl and the fusion proteins were purified using immobilized metal affinity chromatography using ProBond (Invitrogen) nickel resin in 20 mM Hepes, 200 mM KCl with 0.5% Triton X-100. Purified fusion protein was eluted with an imidazole gradient (0–500 mM imidazole) and the pure fractions were pooled and dialyzed against 10 mM Hepes, pH 7.4, 140 mM KCl and 0.5% Triton X-100. The purity of the recombinant proteins was assayed by SDS-PAGE and Coomassie blue staining (BioRad Labs., Hercules, CA) and their concentration was determined (BioRad Labs., Hercules, CA) using BSA as a standard. Full-length SNAP-25 and the cytoplasmic domain of syntaxin (residues 1–265) were generated by in vitro translation in the presence of [35S]methionine (Blasi et al., 1993a,b). Before mixing with purified SVs, translated proteins were centrifuged for 15 min at 40,000 rpm in a Beckman TLA 100.2 rotor (see Fig. 3).

**Immunocytochemistry**

Negative staining and immunogold labeling of isolated SVs were performed as described (Jahn and Maycox, 1988). For synaptophysin labeling, the polyclonal antibody was affinity purified according to Navone et al. (1986).

**Other Methods**

SDS-PAGE was performed according to standard procedures (Laemmli, 1970) using the Protean II minigel system (BioRad Labs., Hercules, CA). Immunoblotting and visualization with $^{125}$I protein A (DuPont, Boston, MA) was performed as described (Jahn et al., 1985). For quantitation, the densities of the bands on the autoradiograms were integrated with a Visage 2000 scanner (Bio Image Products, MilliGen/Biosearch Division of Millipore, Ann Arbor, MI).

**Results**

**Syntaxin 1 and SNAP-25 Are Present on Purified Clathrin-coated Vesicles and Synaptic Vesicles**

We first examined whether syntaxin 1 and SNAP-25 participate in exo-endocytic membrane recycling. To address this issue, recycling organelles were isolated from nerve terminals and analyzed for the presence of both proteins.

Clathrin-coated vesicles were purified from nerve terminals using a previously described method (Maycox et al., 1992). This method yields a fraction of coated vesicles that is predominantly derived from nerve terminals and that is virtually free of contamination by other organelles, in particular SVs. Briefly, using a special buffer system, synaptosomes are subfractionated into a heavy membrane fraction (Fig. 1, hm) containing most of the synaptic plasma membrane and a light membrane fraction (Fig. 1, lm) that contains enriched clathrin-coated vesicles (20–50% of all organelles, as judged by electron microscopy; see Maycox et al. [1992]). The light membrane fraction is then subjected to two consecutive density gradients to remove contaminating membranes (mostly SVs) yielding a fraction of over 95% purity (not shown, see Maycox et al., 1992). Fig. 1 shows the distribution pattern of syntaxin 1 in the various fractions obtained during the isolation of clathrin-coated vesicles. Syntaxin was found to be a constituent of clathrin-coated vesicles which, as noted previously (Maycox et al., 1992), are virtually devoid of rab3A (Fig. 1, CCV). Similar results were obtained for SNAP-25 in independent experiments (data not shown). The clathrin light chain was used as a marker for clathrin-coated vesicles. Furthermore, the ω-conotoxin-binding Ca$^{2+}$ channel was not detected in this fraction (data not shown), demonstrating that syntaxin is not associated with N-type Ca$^{2+}$ channels in CCVs.

Most of the clathrin-coated vesicles in the nerve terminal are thought to be intermediates in the recycling of SVs (see synaptophysin inFig. 1), although additional recycling pathways may coexist (Heuser, 1989; Maycox et al., 1992). Therefore, it was important to clarify whether syntaxin 1 and SNAP-25 are sorted into SVs after endocytosis or whether their recycling pathway bypasses SVs. To address this issue, we prepared highly purified SVs and monitored the distribution of syntaxin 1 and SNAP-25 during the purification in comparison to SV markers. Synaptic vesicles were isolated according to Nagy et al. (1976) as modified by Huttner et al. (1983). In this procedure, crude synaptosomes (P2) are prepared by differential centrifugation and subsequently lysed to release SVs and other internal membrane compartments. Most large membranes are then removed by centrifugation for 15 min at 25,000 g (LP1). Synaptic vesicles are collected from the supernatant (synaptosomal lysate supernatant, LS1) by high speed centrifugation (LP2) and further purified by rate-zonal sucrose density gradient centrifugation and size-exclusion chromatography (controlled pore glass, CPG).

**Figure 1.** Syntaxin 1 is present on clathrin-coated vesicles isolated from rat brain nerve terminals. Starting from brain homogenate (total homogenate, TH), synaptosomes (syn) were isolated and subfractionated by differential centrifugation, yielding a heavy membrane fraction (hm) and a light membrane fraction (lm) containing the bulk of clathrin-coated vesicles. The light membranes were further fractionated by consecutive Ficoll gradient and D$_2$O-sucrose gradient centrifugation, yielding purified clathrin-coated vesicles (CCV) (see Materials and Methods for details). All samples were analyzed for syntaxin 1, clathrin light chain, and the vesicle proteins rab3A and synaptophysin by SDS-PAGE and immunoblotting (10 μg protein/lane, $^{125}$I protein A procedure). Of note, the loss of rab3A during purification of clathrin-coated vesicles indicates that contamination with SVs is low (Maycox et al., 1992).
Syntaxin and SNAP-25 Are Major Constituents of Synaptic Vesicles

The results described above, however, do not distinguish whether the vesicular pools of syntaxin 1 and SNAP-25 represent only a negligibly small fraction of SV proteins or whether they are major vesicle constituents. For this reason, we quantitated both proteins in gradient-purified synaptosomes and in highly purified SVs and compared their relative abundance with synaptobrevin II. Quantitation was performed by radio immunoblotting, using highly purified recombinant proteins as standards. The data are summarized in Table I. Syntaxin 1 and SNAP-25 account for 4.3% and 4.9% of total synaptosomal protein, respectively. Thus they represent two of the most abundant constituents of isolated nerve terminals. In purified SVs, both syntaxin 1 and SNAP-25 accounted for ~3% of total protein. Thus, t-SNAREs are significant constituents of SVs. Synaptobrevin II amounted to 8.7% of the total vesicle protein. Thus, synaptobrevin is present in an approximately sixfold molar excess over each of the t-SNAREs and in threefold molar excess over synaptophysin (7% of total vesicle protein, Knaus et al., 1986; Jahn et al., 1987) and is probably the most abundant vesicle protein.

The data discussed so far document that a significant pool of syntaxin 1 and SNAP-25 are present on SVs. However it remains unclear whether syntaxin 1 and SNAP-25 are only present on a small population of SVs or whether the majority of SVs contains these proteins. To address this question, we performed organelle immunoisolation experiments using antibodies directed against syntaxin 1 covalently coupled to methacrylate microbeads (syntaxin beads). For comparison, synaptophysin-containing organelles were immunoisolated in parallel (synaptophysin beads). This method was previ-

Figure 2. Syntaxin and SNAP-25 are present on highly purified synaptic vesicles isolated from rat brain. Synaptic vesicles were isolated from rat brain homogenate according to Huttner et al. (1983). Briefly, synaptosomes (P2) were isolated from homogenate (H) by differential centrifugation after removal of large debris (P1), lysed by osmotic shock and subfractionated into a heavy membrane fraction (LP1) and a light membrane fraction enriched in SVs (LP2). LP2 was further purified by sucrose density gradient centrifugation and controlled pore glass (CPG) chromatography (see Huttner et al. [1983] for details). Three pools were collected from the CPG column (I, IA, and SV for SVs, see Fig. 2 b) with the SV pool consisting of highly purified SVs (Huttner et al., 1983). (a) Immunoblot analysis of fractions obtained during the purification of SVs for syntaxin 1 and SNAP-25, the vesicle marker synaptophysin, the plasma membrane markers Na/K-ATPase and NMDA-receptor, and a marker for the endoplasmic reticulum, ribophorin (10 μg protein/lane, visualization by the 125I protein A procedure). (b) Elution profile of the CPG column, indicating pools, I, IA, and SV.
Syntaxin and SNAP-25 are integral components of SVs. SVs (50 μg of protein, purified by CPG-chromatography) were incubated in 250 μl of 10 mM Hepes, pH 7.4, containing the additions indicated at the top of each lane (Hepes was absent when Na2CO3 was used). The samples were incubated for 15 min on ice and centrifuged for 30 min at 70,000 rpm in a Beckman TLA 100.3 rotor. Equal amounts of the supernatant and pellet fractions were subjected to SDS-PAGE and immunoblotting (lower panel). To test whether t-SNAREs bind to isolated SVs, recombinant syntaxin (lacking the transmembrane domain) and SNAP-25 (full-length), radiolabeled by [35S]methionine, were added before incubation and centrifugation. The distribution of these proteins was analyzed by autoradiography in the samples (upper panel). Note that the amount of recombinant protein was far lower than that of endogenous protein to avoid interference with the immunoblot detection of the endogenous pools.

To allow for direct quantitative comparison, a defined volume of synaptosomal lysate supernatant (LS1, a fraction of small vesicular membranes containing SVs), was incubated with excess beads. The bead pellets were then washed and resuspended in the same volume as the starting fraction. As shown in Fig. 4, synaptophysin beads bound most of the synaptophysin- and synaptotagmin-containing membranes in agreement with previous results (Walch-Solimena et al., 1993). Syntaxin was also present in the organelles bound to synaptophysin beads, although the enrichment was lower compared to synaptophysin or synaptotagmin. The selectivity of the method is demonstrated by the lack of Na/K-ATPase in the synaptophysin bead pellet. Syntaxin beads, on the other hand, bound most syntaxin-containing membranes. Similarly, they bound the majority of membranes containing synaptophysin, demonstrating that there is considerable overlap of the localization of syntaxin 1 with synaptophysin. In contrast to synaptophysin beads, syntaxin beads bound most of the Na/K-ATPase present in the starting material, indicating that in addition to SVs, syntaxin beads bound plasma membrane fragments still contained in this membrane fraction. The immunoisolation procedure exhibits very low non-specific binding as demonstrated by the absence of significant amounts of the endoplasmic reticulum marker inositol 1,4,5-trisphosphate (InsP3) receptor, in both synaptophysin and syntaxin bead pellets. These data indicate that

Table I. Amount of Syntaxin, SNAP-25, and Synaptobrevin II (% of Total Protein) in Highly Purified Synaptosomes and Synaptic Vesicles

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<th>Synaptosomes</th>
<th>Synaptic vesicles</th>
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<tr>
<td>Syntaxin</td>
<td>4.3 ± 0.6†</td>
<td>3.1 ± 0.6 (9)</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>4.9 ± 0.9</td>
<td>3.4 ± 1.8 (7)</td>
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<tr>
<td>Synaptobrevin II</td>
<td>1.8 (2)</td>
<td>8.7 ± 0.8 (3)</td>
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Synaptosomes were purified by differential and Ficoll gradient centrifugation, yielding a fraction containing at least 70% resealed nerve terminals as judged by electron microscopy. Synaptic vesicles were >95% pure (Fig. 2, see also Huttner et al. [1983]).

* Percent of total protein, determined within the linear range of the standard curves obtained using purified recombinant proteins (see Materials and Methods for details).
† Standard deviations.
‡ Number of determinations.
syntaxin 1 is not only present on a small subpopulation but rather on the majority of SVs. We were unable to monitor SNAP-25 due to interference by the IgG light chain of the bead-coupled antibodies in immunoblots.

To confirm the observations of the subcellular fractionation and immunoisolation, we performed immunogold labeling for both syntaxin 1 and SNAP-25 using a preparation of highly purified SVs. As shown in Fig. 5, A and C, antibodies directed against both proteins resulted in widespread labeling of membrane profiles with the morphological characteristics expected for SVs. Although SNAP-25 and syntaxin 1 are present in approximately stoichiometric amounts,

Figure 5. Immunogold labeling for SNAP-25, syntaxin 1, synaptobrevin II, and synaptophysin on negatively stained synaptic vesicles (purified by CPG-chromatography). Immunogold labeling was performed with polyclonal antibodies against SNAP-25 (A) and synaptophysin (F) directly detected by protein A-gold or with monoclonal antibodies against syntaxin 1 (C) and synaptobrevin (E) through a rabbit-to-mouse IgG bridge antibody and protein A-gold. As controls, protein A-gold labeling was performed with SNAP-25 preimmune serum (B) and monoclonal anti-NMDA receptor antibody (D), respectively. Widespread labeling of synaptic vesicle membrane profiles was found using antibodies against the t-SNAREs SNAP-25 (A) and syntaxin 1 (C). The t-SNARE labeling was less intense, however, than the labeling for the vesicle proteins synaptophysin (F) and synaptobrevin (E). Virtually no immunogold labeling is observed in the polyclonal (B) and monoclonal (D) controls. Bar, 100 nm.
SNAP-25 labeling was less intense than syntaxin 1 labeling. This can be explained by the fact that the polyclonal antibody to SNAP-25 was detected directly by protein A-gold while the visualization of the monoclonal anti-syntaxin antibody required the use of a bridge antibody (rabbit-to-mouse IgG) resulting in amplification of the signal. No significant labeling was observed when preimmune serum (SNAP-25, Fig. 5 B or a monoclonal antibody specific for the NMDA receptor, a plasmalemmal protein (Fig. 5 D) was used. As expected, labeling of the t-SNAREs (Fig. 5 A and C) was less intense, however, than that for synaptobrevin (Fig. 5 E) or synaptophysin (Fig. 5 F).

BoNT/C1 Preferentially Cleaves the Pool of Syntaxin 1 on Synaptic Vesicles

The finding that syntaxin 1 is an abundant protein of SV membranes raises the question whether this pool is functionally distinct from that of the plasmalemma. To obtain insight into this issue we compared the ability of BoNT/C1 (Blasi et al., 1993b) to cleave the vesicular and plasma membrane pools of syntaxin 1. We analyzed the effect of toxin poisoning on both functionally active synaptosomes and on isolated heavy and light membranes. Although it is not possible to obtain synaptic plasma membranes free of SVs (since the heavy membrane fraction contains significant amounts of tightly bound SVs), the crude vesicle fraction is essentially free of plasma membrane markers (data not shown).

For the analysis of toxin action in intact nerve terminals, aliquots of the synaptosomes were preincubated for 90 min at 37°C with BoNT/C1 holotoxin to allow toxin uptake, and then depolarized to control for inhibition of neurotransmitter release. No Ca2+-dependent glutamate release was observed after BoNT/C1 incubation (not shown, see Blasi et al., 1993b). Synaptosomes were then washed to remove excess toxin and rapidly subfractionated into heavy membranes and crude SV pellet, followed by immunoblot analysis. As shown in Fig. 6 A, syntaxin 1 was almost completely degraded in the vesicle fraction after toxin poisoning. The degree of cleavage in the crude membrane fraction (lysate pellet) was significantly lower, being hardly detectable in some experiments. While this demonstrated a preference of BoNT/C1 for the vesicular pool of syntaxin, it cannot be excluded that during the onset of the toxin effect, cleaved and non-cleaved syntaxin is redistributed. Therefore, we examined whether the vesicular pool of syntaxin was also cleaved preferentially when synaptosomes were first subfractionated, and then treated with reduced BoNT/C1. As shown in Fig. 6 B, the result was similar. Again, vesicular syntaxin 1 was effectively cleaved. In this experiment, however, partial cleavage was also observed in the membrane-enriched fraction. While these results cannot exclude that the plasmalemmal pool of syntaxin 1 is partially cleaved, they clearly demonstrate that the vesicular pool of syntaxin 1 is the primary target for toxin cleavage.

Discussion

In the present study, we have shown that significant amounts of both syntaxin 1 and SNAP-25 are present on clathrin-coated vesicles and SVs of neurons. Furthermore, we have demonstrated that the vesicular pool of syntaxin 1 is preferentially cleaved by BoNT/C1. Syntaxin and SNAP-25 have been described as resident proteins of the plasma membrane. In fact, an enrichment of syntaxin 1 in synaptic areas has been reported (Bennett et al., 1992). These observations are in agreement with the proposed function of syntaxin 1 and SNAP-25 as the plasmalemmal partners for the vesicle protein synaptobrevin in forming the SNARE complex. Furthermore, syntaxin 1 binds to additional proteins such as ω-conotoxin-binding Ca2+-channels and the vesicle protein synaptotagmin (Bennett et al., 1992; Leveque et al., 1992, 1994; Yoshida et al., 1992) as well as munc-18, the mammalian homolog of the unc-18 and SEC1 gene products, respectively (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994a). Our subcellular fractionation studies confirm that the majority of syntaxin 1 is localized to the plasma membrane. Upon purification of SVs from synaptosomes, the syntaxin 1 content decreases from 4.4% to 3.0% and the SNAP-25 content from 4.9% to 3.4% whereas the synaptobrevin content increases from 1.8% to 8.7%. From these values it can be estimated that ~15% of both proteins present in purified synaptosomes is localized on SVs. These findings are consistent with immunofluorescence studies which revealed predominant labeling for syntaxin and SNAP-25 on the plasma membrane.

Figure 6. BoNT/C1 cleavage of syntaxin 1 from both a plasma membrane fraction and synaptic vesicles after poisoning of isolated nerve terminals and synaptosomal subfractions. (A) Gradient-purified synaptosomes were incubated with 75 nM BoNT/C1, a concentration sufficient to completely inhibit Ca2+-dependent glutamate release (not shown), washed extensively, and then rapidly subfractionated on ice into a heavy membrane fraction containing plasma membranes and docked SVs (heavy membranes) and a light membrane fraction containing unbound SVs (synaptic vesicles). All samples were analyzed for syntaxin 1 and synaptophysin by SDS-PAGE and immunoblotting (10 μg protein/lane, 125I protein A procedure). (B) Heavy and light membrane fractions were incubated with 100 nM reduced BoNT/C1 and all samples processed as in A.
vesicle, thus poisoning the fusion complex. BoNT/C1 may be explained by the fact that truncated synapto-}

protease to syntaxin in the heavy membranes since syntaxin presence on SVs was confirmed by immunogold electron microscopy. For comparison, the SV proteins synapsin I, synaptophysin, synaptotagmin, and synaptobrevin II comprise 6%, 7%, 7.5%, and 8.7%, respectively, of total vesicle protein (Goelz et al., 1981; Jahn et al., 1987; Knaus et al., 1986; Chapman and Jahn, 1994, this study). Thus, the number of molecules per vesicle (average) of the t-SNAREs equals that of synapsin I but is sixfold less than for synaptobrevin, the most abundant vesicle protein. This would result in an average of approximately three copies of both syntaxin I and SNAP-25 per vesicle, assuming that the combined molecular mass of all vesicle proteins in a single vesicle is in the range of $3 \times 10^3$ kD (Jahn and Südhoff, 1993). Syntaxin I and SNAP-25 were always colocalized which may be explained by the fact that the two proteins bind directly to each other (Pevsner et al., 1994b; Chapman et al., 1994) and may be complexed to each other for most of their life cycle.

Syntaxin and SNAP-25 are the first examples of plasmalemmal proteins that recycle through SVs. This is remarkable since more available data indicate that SVs maintain their membrane composition efficiently during continuing rounds of exo-endocytosis (Valtorta et al., 1988; Torri-Tarelli et al., 1990). In fact, typical residents of the plasma membrane such as Na,K-ATPase, receptors or Na-dependent neurotransmitter transporters are selectively and efficiently excluded during endocytosis as demonstrated by their absence from purified SVs (Fig. 2a and unpublished observations).

BoNT/C1 preferentially cleaved the SV pool of syntaxin I in both intact synaptosomes and in isolated membrane fractions. These findings are not due to lack of access of the toxin protease to syntaxin in the heavy membranes since syntaxin I was equally degraded by trypsin in both fractions (data not shown). The preferential cleavage of syntaxin I localized to SVs implies that the protein undergoes changes in its conformation and/or its interaction with other proteins during the cycle of exo- and endocytosis. Inhibition of exocytosis by BoNT/C1 may be explained by the fact that truncated syntaxin I reaches the plasmalemma after being cleaved on the vesicle, thus poisoning the fusion complex.

What is the functional significance of vesicular syntaxin I and SNAP-25, and how can this unexpected localization be reconciled with their function as t-SNAREs specific for the plasma membrane? Furthermore, how are these proteins internalized? At present, answers to these questions are not available. Several alternatives are possible: (a) the vesicular pool represents an "overflow" from the synaptic plasma membrane. For instance, it can be envisioned that the t-SNAREs are internalized by a piggyback mechanism via synaptobrevin due to incomplete v-SNARE-t-SNARE-dissociation after fusion. In this scenario one would expect that syntaxin I would be inactivated, perhaps by associating with another protein, e.g., a protein of the Sec1 family and/or a conformational change. (b) Recycling of syntaxin I and SNAP-25 may be important for their function. For example, t-SNAREs may be reactivated by recycling. (c) Both proteins may perform specific functions on SVs. Such functions may include the fusion of additional vesicles to a docked vesicle immediately after opening of the fusion pore (compound exocytosis). Future work is required to distinguish between these possibilities and to elucidate the mechanisms by which the activity of syntaxin I and SNAP-25 in different stages of membrane traffic are regulated.

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