Adrenal chromaffin cells contain functionally different SNAP-25 monomers and SNAP-25/syntaxin heterodimers

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Abstract Syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), associated with the neuronal plasmalemma, and synaptobrevin, a membrane protein of synaptic vesicles, are essential components of the exocytotic apparatus of synaptic vesicles. All three can be proteolytically cleaved by tetanus and/or botulinum neurotoxins. As a consequence of their cleavage, exocytosis of neurotransmitters is blocked. In adrenal chromaffin cells botulinum neurotoxin A only incompletely inhibits exocytosis. This incomplete inhibition of exocytosis is associated with only partial cleavage of SNAP-25 by the toxin, indicating that distinct pools of SNAP-25 may exist in chromaffin cells which differ in their sensitivities to botulinum neurotoxin A. In line with this result we localized SNAP-25 by immunogold electron microscopy not only to the plasmalemma but also to the chromaffin vesicle membrane. Moreover, in addition to SNAP-25 monomers, stable SNAP-25/syntaxin heterodimers were found in chromaffin cells. Subfractionation studies revealed the presence of SNAP-25/syntaxin heterodimers in an enriched fraction of chromaffin vesicles. This complex proved to be stable in SDS, and SNAP-25 within heterodimers was resistant to proteolytic attack by botulinum neurotoxin A. We suggest that these preexisting heterodimers may serve as receptors of soluble NSF attachment proteins (SNAP receptors) during chromaffin vesicle exocytosis.

Key words: Exocytosis; Botulinum toxin A; Calcium; Secretion

1. Introduction

Tetanus and botulinum neurotoxins inhibit neurotransmitter release by cleaving essential components of the exocytosis apparatus in synapses. Tetanus toxin and botulinum neurotoxins B, D, F and G attack synaptobrevin (also referred to as VAMP, vesicle-associated membrane protein), botulinum neurotoxins A and E proteolyse synaptosome-associated protein of 25 kDa (SNAP-25), and botulinum neurotoxin C cleaves syntaxin (also referred to as HPC-1) (reviewed in [1]). Synaptobrevin, an integral membrane protein of synaptic vesicles, and the plasma membrane proteins SNAP-25 and syntaxin have also been identified as major components of a complex formed in detergent extracts of brain together with *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) [2]. Because NSF/SNAP couple vesicular synaptobrevin with SNAP-25 and syntaxin localized at the plasmalemmal target membrane, these proteins have been termed vesicular and target SNAP receptors (v-SNAREs and t-SNAREs), respectively. In detergents synaptobrevin, SNAP-25 and syntaxin first assemble as a 7S complex which, after binding of SNAPs and NSF, forms a 20S complex, which dissociates in the presence of ATP as a result of the ATPase activity of NSF [3–5]. Studies of recombinant proteins or solubilized brain in detergents revealed that monomers of SNAP-25 and syntaxin, heterodimers (between syntaxin and SNAP-25, synaptobrevin and syntaxin or synaptobrevin and SNAP-25) and heterotrimers between the three membrane proteins bind SNAPs [5–11]. From these studies carried out in the presence of detergents it is unknown whether such heterodimers or heterotrimers exist in intact membranes of secretory cells.

Tetanus and botulinum neurotoxins inhibit exocytosis not only of synaptic vesicles but also of secretory granules. Cleavage of the v-SNARE synaptobrevin results in the inhibition of exocytosis by adrenal chromaffin and pheochromocytoma cells [12,13]. Catecholamine release from adrenal chromaffin cells, vasopressin release from neurohypophysial terminals and insulin release from pancreatic β cells is inhibited by botulinum neurotoxin A [14–18]. However, the variable cleavage of SNAP-25 in pancreatic β cells, the incomplete inhibition of vasopressin and catecholamine release and the preferential attack of ATP-dependent priming by botulinum neurotoxin A suggested that this toxin may have molecular targets of different sensitivities [14–21].

Here, we report for the first time that besides SNAP-25 monomers adrenal chromaffin cells also contain SNAP-25/syntaxin heterodimers. The latter are stable in SDS and resistant to botulinum neurotoxin A. Interestingly, we also found SNAP-25/syntaxin heterodimers, but virtually no SNAP-25 monomers in the membrane of chromaffin secretory granules. However, the original SNARE hypothesis predicts that SNAP-25 and syntaxin play their roles at the plasmalemma as t-SNAREs [2,3,22]. Our finding that complexes between SNAP-25 and syntaxin exist at the membrane of the vesicular membrane and the observation that such complexes avidly bind SNAPs [4,7] suggest that SNAP-25/syntaxin heterodimers may serve as v-SNAREs during exocytosis of chromaffin granules.

2. Materials and methods

2.1. Cell culture, permeabilization and incubation of chromaffin cells with BoNT/A

Bovine adrenal chromaffin cells were isolated and grown as described [23]. The cells were plated at $3.5–7\times10^5$ cells/well. After 2–5 days in culture Ca^{2+} was removed from the cultures by three washes with a medium containing (in mM) 140 NaCl, 4.7 KCl, 1.2 KH_2PO_4,

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Abbreviations: BoNT/A, botulinum neurotoxin A; DDT, dithiothreitol; NSF, *N*-ethylmaleimide-sensitive fusion protein; OG, β-D-octylglucopyranoside; SNAP, soluble NSF attachment protein; S-25, synaptosome-associated protein of 25 kDa (SNAP-25); St, synaptotagmin; Sx, syntaxin; Sb, synaptobrevin; SLO, streptolysin O

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1.2 $MgSO_4$, 0.5 ascorbic acid, 11 glucose and 15 PIPES (pH 7.2) followed by washes with potassium glutamate medium (in mM: 150 potassium glutamate, 0.5 EGTA, 5 EDTA, 10 PIPES, pH 7.2). The osmolarity of the media was maintained at 300–310 mosmol/kg. The cells were permeabilized (at 30°C for 2 min) with streptolysin O (63 hemolytic units/well) in potassium glutamate medium containing 2 mM Mg^{2+}-ATP, 7.67 mM magnesium acetate, 0.1% bovine serum albumin (BSA) and 1 mM dithiothreitol [24]. The efficiency of permeabilization (more than 95% of cells permeabilized) was checked with 0.04% trypan blue in the above medium. The medium was then replaced by the same medium containing BoNT/A, followed by incubation for 25 min at 30°C. Finally, the cells were used in release experiments (see below) or the cells were lysed with 100 μl SDS-sample buffer per well. Proteins (determined with the BCA method, Pierce, Oud-Beijerland, Netherlands) were subjected to SDS-PAGE followed by immunoblotting.

2.2. Release of noradrenaline from permeabilized chromaffin cells

Prior to the release experiments adrenal chromaffin cells were incubated for 1 h with [^{3}H]noradrenaline (1.3 μCi/ml) in serum-free culture medium (DMEM) supplemented with 1 mM ascorbic acid. The cells were washed and permeabilized with SLO and incubated with BoNT/A as described above. Then the cells were stimulated for 15 min at 30°C with potassium glutamate medium containing 30 μM free Ca^{2+}. Basal release was determined in the same medium with no Ca^{2+} added. Free Ca^{2+} concentrations were calculated and checked with a Ca^{2+}-specific electrode as described [25].

[^{3}H]Noradrenaline released into the supernatant and remaining in the cells (after lysis with 0.2% SDS) was determined. Percentage of [^{3}H]noradrenaline released was calculated using catecholamine content of the lysate plus released catecholamines as 100%. The values given are means ± S.D. of three wells.

2.3. Purification of chromaffin vesicle membranes and incubation with BoNT/A

Chromaffin vesicles were isolated as described [26]. Briefly, bovine adrenal glands were perfused with 5 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM EDTA. The medullae were homogenized in (20% w/v) 20 mM MOPS (pH 7.0), 5 mM EDTA, 340 mM sucrose in a Teflon-to-glass homogenizer. From the postnuclear supernatant chromaffin vesicles were harvested by centrifugation for 20 min at 12000 × *g*. They were placed on a discontinuous sucrose gradient consisting of 4 ml of 2.4 M and 2 ml of each 2.2, 2.0, 1.8 and 1.6 M sucrose in 20 mM MOPS (pH 7.0), 5 mM EDTA. Centrifugation was done for 1 h at 40000 rpm in a 50.2 Ti rotor (Beckman). Absorbance at 280 nm after precipitation of the protein with 10% (w/v) TCA was measured to conveniently locate chromaffin vesicles in the gradient while arylsulfatase served to identify fractions containing lysosomes and other subcellular membranes [26]. The fractions enriched in chromaffin vesicles (fractions 11–14) were recovered from the gradient, diluted 1:4 with 20 mM MOPS (pH 7.0), 5 mM EDTA and centrifuged for 1 h at 100000 × *g*. The purified chromaffin vesicles were osmotically lysed by addition of a 10-fold excess of 20 mM MOPS (pH 7.0) with 5 mM EDTA. Chromaffin vesicle membranes were recovered by centrifugation for 1 h at 100000 × *g* and washed twice in potassium glutamate medium (see above) containing 6 mM magnesium acetate. The final pellet was suspended in the same medium and stored at −20°C until use. Chromaffin vesicle membranes (100 μg) were incubated in a volume of 50 μl for 30 min at 30°C in potassium glutamate medium (see above) containing 100 nM BoNT/A and the different chemicals to be tested. For solubilization we used 1% (w/v) β-D-octylglucopyranoside (OG). Lipids were extracted by chloroform/methanol [27] and the samples were boiled in SDS-sample buffer for 5 min or processed for without boiling.

2.4. SDS-PAGE and immunoblotting

The samples (15 μg/lane) of chromaffin vesicle membranes, chromaffin cells in culture or homogenates of adrenal medullary tissue were separated on 12.5% or 5–15% gradient gels by SDS-PAGE [28] and blotted onto nitrocellulose [29]. Binding of the monoclonal anti-synaptobrevin antibody (Cl. 10.1; diluted 1:2000) [30], of the monoclonal anti-synaptotagmin I antibody (Cl. 41.1; diluted 1:2000) [31], of monoclonal anti-SNAP-25 antibody (1:2000, SMI81, Sternberger Monoclonals Inc., Baltimore, MD, USA) and monoclonal anti-syntaxin antibody (1:2000, Cl. HPC-1, Sigma Immuno Chemicals, Deisenhofen, Germany) was detected by peroxidase-labelled anti-mouse IgG antibodies (1:3000, Dianova, Hamburg, Germany) and the enhanced chemiluminescence method (Amersham Buchler, Braunschweig, Germany). In some experiments after electrophoresis the bands of interest were cut out from the unstained acrylamide gels, left at room temperature or boiled for 5 min, placed on top of the stacking gel and subjected again to electrophoresis.

2.5. Immunolocalization of SNAP-25 at the ultrastructural level

Adrenal medullary tissue was fixed for 24 h at room temperature with 2.5% paraformaldehyde and 0.25% glutaraldehyde in 100 mM sodium cacodylate (pH 7.0). Then the tissue was dehydrated with ethanol (30% at 0°C, 50% at −20°C, 80% and 100% at −35°C) infiltrated at −35°C with the hydrophilic resin Lowicryl K4M, which was polymerized with ultraviolet light for 2 days at −35°C and 3 days at room temperature [32]. Ultrathin sections on 75 mesh nickel grids were rinsed with PBS, 50 mM glycine in PBS, 0.5% BSA in PBS, 5% normal goat serum followed by incubation (overnight at 4°C) with monoclonal anti-SNAP-25 antibody (SMI81, Sternberger Monoclonals Inc.) diluted 1:100 in PBS containing 1% BSA, monoclonal anti-synaptobrevin antibody (Cl. 10.1; diluted 1:50) [30], or monoclonal anti-syntaxin antibody (1:50, Cl. HPC-1, Sigma Immuno Chemicals). The sections were rinsed in PBS followed by incubation for 1 h at room temperature with Auro-Probe goat anti-mouse IgG-Au (5 nm) (Amersham Buchler) diluted 1:100 in PBS containing 1% BSA. Silver enhancement was done with Aurion R-gent (Biotrend, Cologne, Germany) according to the recommendation of the manufacturer containing in addition 30% gum arabic. Finally sections were contrasted with 2% uranylacetate (5 min) and lead citrate (3 min).

3. Results

In agreement with previous reports [14–16] we found that exocytosis of secretory granules by adrenal chromaffin cells is only incompletely inhibited by BoNT/A. Ca^{2+}-induced catecholamine release from bovine adrenal chromaffin cells permeabilized by SLO was significantly blocked by BoNT/A in concentrations as low as 1 nM. Increasing the amounts of the neurotoxin 10- or 100-fold did not proportionately affect the release (Fig. 1). In neurons blockade of transmitter release by BoNT/A is associated with cleavage of SNAP-25 [33,34]. In order to examine whether SNAP-25 is also cleaved by BoNT/A in adrenal chromaffin cells we analyzed SNAP-25 in permeabilized cells by immunoblotting. As shown at the bottom of Fig. 1 we found a conversion of the SNAP-25 immunoreactive band of 25 kDa to a slightly smaller fragment at all BoNT/A concentrations tested. The extent of cleavage paralleled the inhibition of exocytosis by the toxin and, even more important, was as incomplete as the blockade of catecholamine release. Higher concentrations of the toxin or longer periods of incubation did not result in further cleavage of SNAP-25. We interpreted these findings as evidence for the occurrence of SNAP-25 resistant to BoNT/A as well as SNAP-25 non-resistant to the toxin. If so, the non-resistant form should be rapidly cleaved within the chromaffin cells while the resistant form remains intact. Indeed we observed cleavage by 100 nM BoNT/A of 40% cellular SNAP-25 already after 5 min and of 72% after 15 min, that is roughly the same extent of cleavage seen after 25 min (see Fig. 1). Together our data suggest that the incomplete inhibition of exocytosis by BoNT/A is due to the occurrence of resistant or masked forms of SNAP-25 in adrenal chromaffin cells.

We detected two forms of SNAP-25 with different electrophoretic mobilities in adrenal medullary chromaffin cells by immunoblotting. In unboiled samples we observed immunoreactive bands of 25 and 60 kDa. By contrast, when the same fractions were boiled prior to SDS-PAGE, a single band of 25

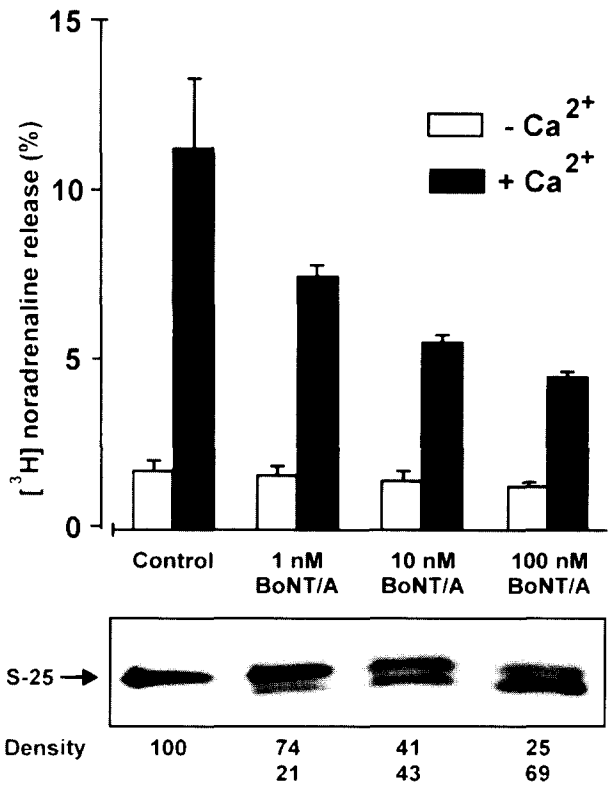

Fig. 1. Inhibition of exocytosis by botulinum neurotoxin A is paralleled by cleavage of SNAP-25 in permeabilized adrenal chromaffin cells. After permeabilization the cells were incubated for 25 min at 30°C with potassium glutamate medium (control), 1, 10, or 100 nM BoNT/A in potassium glutamate medium (see Section 2). Then the cells were stimulated for 15 min at 30°C with potassium glutamate medium containing 30 μM free Ca^{2+} (filled bars) or no Ca^{2+} (open bars) and the percentage of [^{3}H]noradrenaline released into the supernatant was determined. Note that the inhibition of Ca^{2+}-induced catecholamine release was incomplete, even at the highest BoNT/A concentration used. Data are means ± S.D. of three wells. After permeabilization with SLO and incubation of the cells with different concentration of BoNT/A for 25 min at 30°C, SDS-sample buffer was added followed by boiling, SDS-PAGE and immunoblotting. SNAP-25 (S-25) was visualized using the enhanced chemiluminescence method. SNAP-25 is incompletely cleaved in a dose-dependent manner by BoNT/A yielding in a slightly smaller product. The relative amounts of SNAP-25 and the cleavage as determined by densitometry are given at the bottom.

kDa was found (Fig. 2). The nature of the two forms of SNAP-25 became apparent by probing blots of chromaffin cells with an antibody directed against syntaxin. We found an immunoreactive band of 60 kDa if unboiled samples were analyzed but an immunoreactive band of 35 kDa if the samples were heated prior to PAGE (Fig. 2). These observations are compatible with the idea that SNAP-25/syntaxin heterodimers of 60 kDa are present in adrenal chromaffin cells which are stable in SDS at room temperature but are broken down upon heating. A band indicating syntaxin monomers is barely detectable in blots of unboiled samples. In contrast SNAP-25 monomers are present indicating the existence of a pool of SNAP-25 monomers in addition to SNAP-25/syntaxin heterodimers in adrenal chromaffin cells.

Observations at the light microscopic level have suggested that in chromaffin cells SNAP-25 is primarily found on the plasmalemma [35,36]. At the ultrastructural level we confirmed this localization (Fig. 3a). However, in addition we found SNAP-25 associated with chromaffin vesicle membranes (Fig. 3b,c). As positive controls we used antibodies directed against synaptobrevin, an established protein of the chromaffin vesicle membrane [12,37] as well as syntaxin [38]. Both antibodies decorated chromaffin vesicle membranes (Fig. 3d,e). In controls, in which the first antibodies were omitted, none of these specific labeling features were observed. Thus our ultrastructural data show that SNAP-25 and syntaxin are associated with chromaffin vesicle membranes.

We have subsequently purified chromaffin vesicles by differential and density gradient centrifugation and analyzed fractions recovered from sucrose gradients for the presence of the putative members of the core of the exocytosis apparatus, synaptobrevin, syntaxin and SNAP-25. We were interested to know whether SNAP-25 monomers and the stable SNAP-25/syntaxin heterodimers detected in adrenal chromaffin cells (see above) are present in chromaffin vesicles and whether SNAP-25 present in this subcellular location can be cleaved by BoNT/A. We found (see Fig. 4) that in addition to synaptobrevin chromaffin vesicles (fractions 11–14) possess SNAP-25 and syntaxin, confirming our ultrastructural data (see above) and earlier observations [12,38]. Chromaffin granules purified after differential centrifugation, first on a Percoll gradient and subsequently on a sucrose gradient [26], also contained synaptobrevin, syntaxin and SNAP-25 (not shown). The distribution of SNAP-25 in the gradient (see Fig. 4, lower

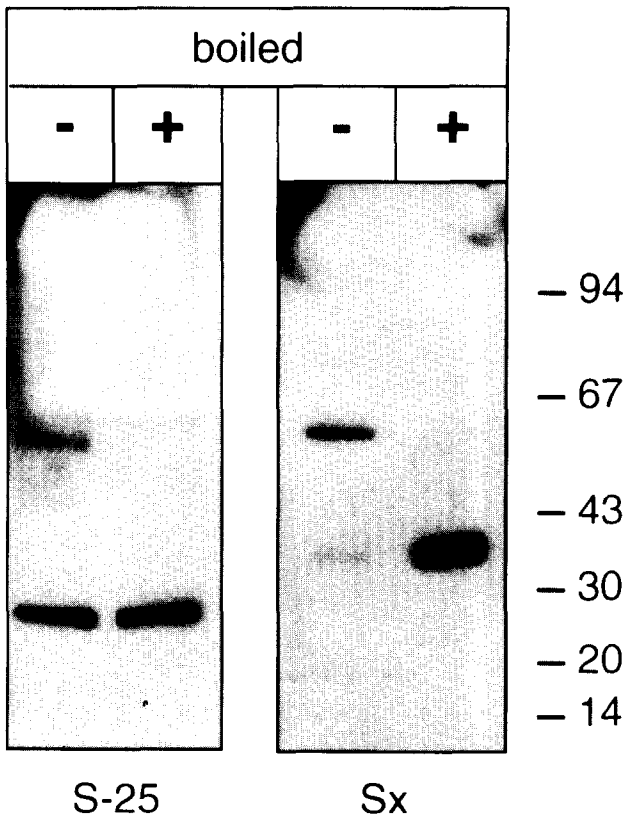

Fig. 2. Heterodimers of SNAP-25 and syntaxin are present in adrenal chromaffin cells. In unboiled samples of chromaffin cells SNAP-25 (S-25) predominates. However, an additional immunoreactive band of 60 kDa is detectible. By contrast in boiled samples only SNAP-25 is observed. In unboiled samples the major syntaxin (Sx)-immunoreactive band exhibits a molecular weight of 60 kDa and a faint band of 35 kDa. A major band of authentic syntaxin is only found in boiled samples.

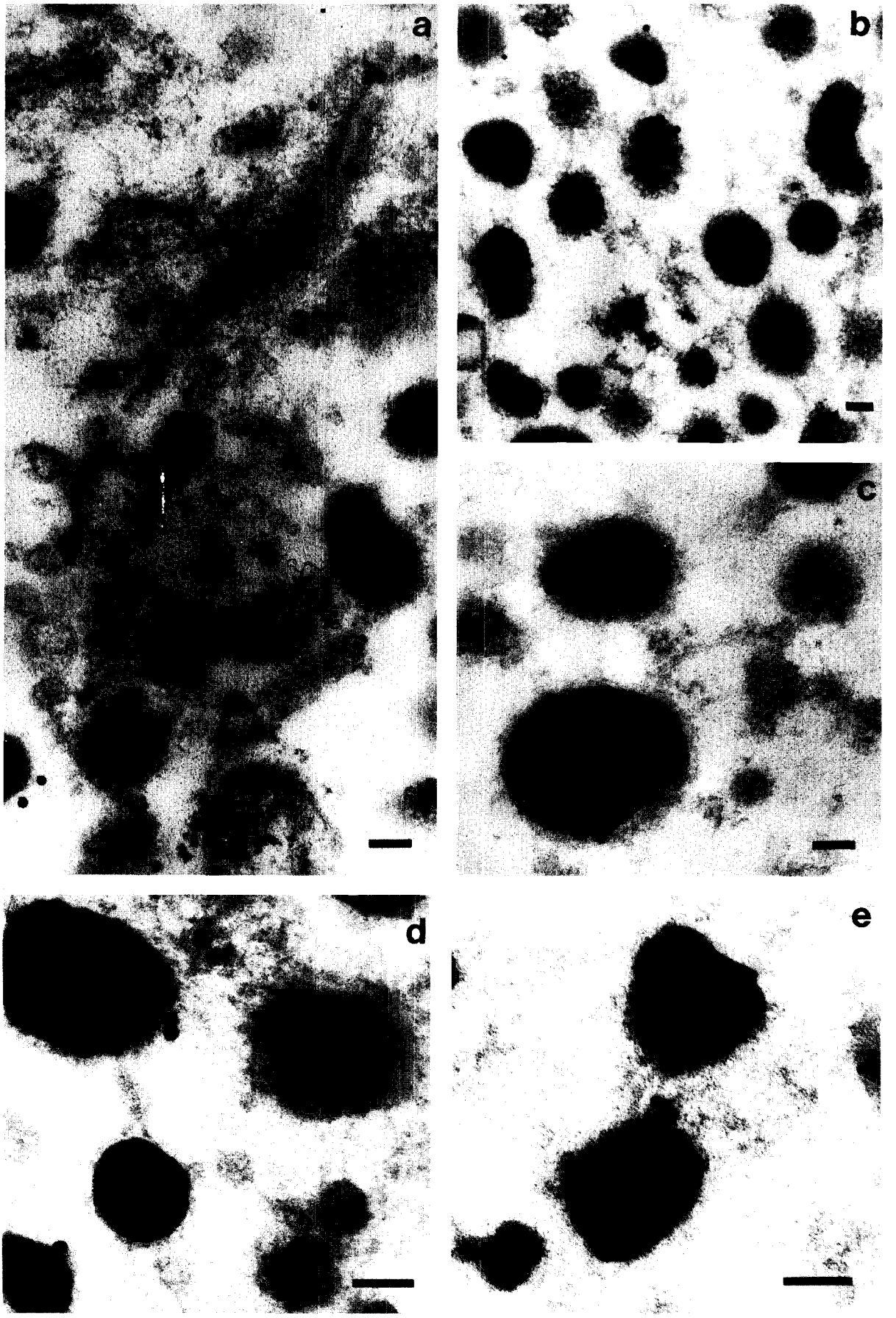
a
b
c
d
e

Fig. 3. Immunolocalization of SNAP-25, synaptobrevin and syntaxin in chromaffin cells. During immunogold localization of SNAP-25 in ultrathin sections of adrenal medulla silver-enhanced gold particles were found to be associated with the plasmalemma (a) and with chromaffin vesicle membranes (b,c). Chromaffin vesicle membranes were also decorated if antibodies directed against synaptobrevin (d) or syntaxin (e) were used. Bars, 0.1 μm.
←

panel) was bimodal: it was found in the chromaffin vesicle fractions (which contain synaptobrevin as a marker) and, in larger amounts, in fractions 15–20 equilibrating at lower sucrose densities which contain lysosomes (Fig. 4). The low amounts of synaptobrevin and the hump in the absorbance at 280 nm (fraction 17) may indicate that also a few immature and lighter chromaffin vesicles exist in these fractions. Since these fractions contain several SNAP-25 immunoreactive bands it is likely that SNAP-25/syntaxin complexes with other proteins. The precise nature of these complexes was not further analyzed in this study. We focused on fractions 11–14 which represent a homogeneous population of chromaffin equilibrating at 1.6–2.0 M sucrose (see Section 2 and [12,26]), which, besides synaptobrevin and syntaxin, contain part of

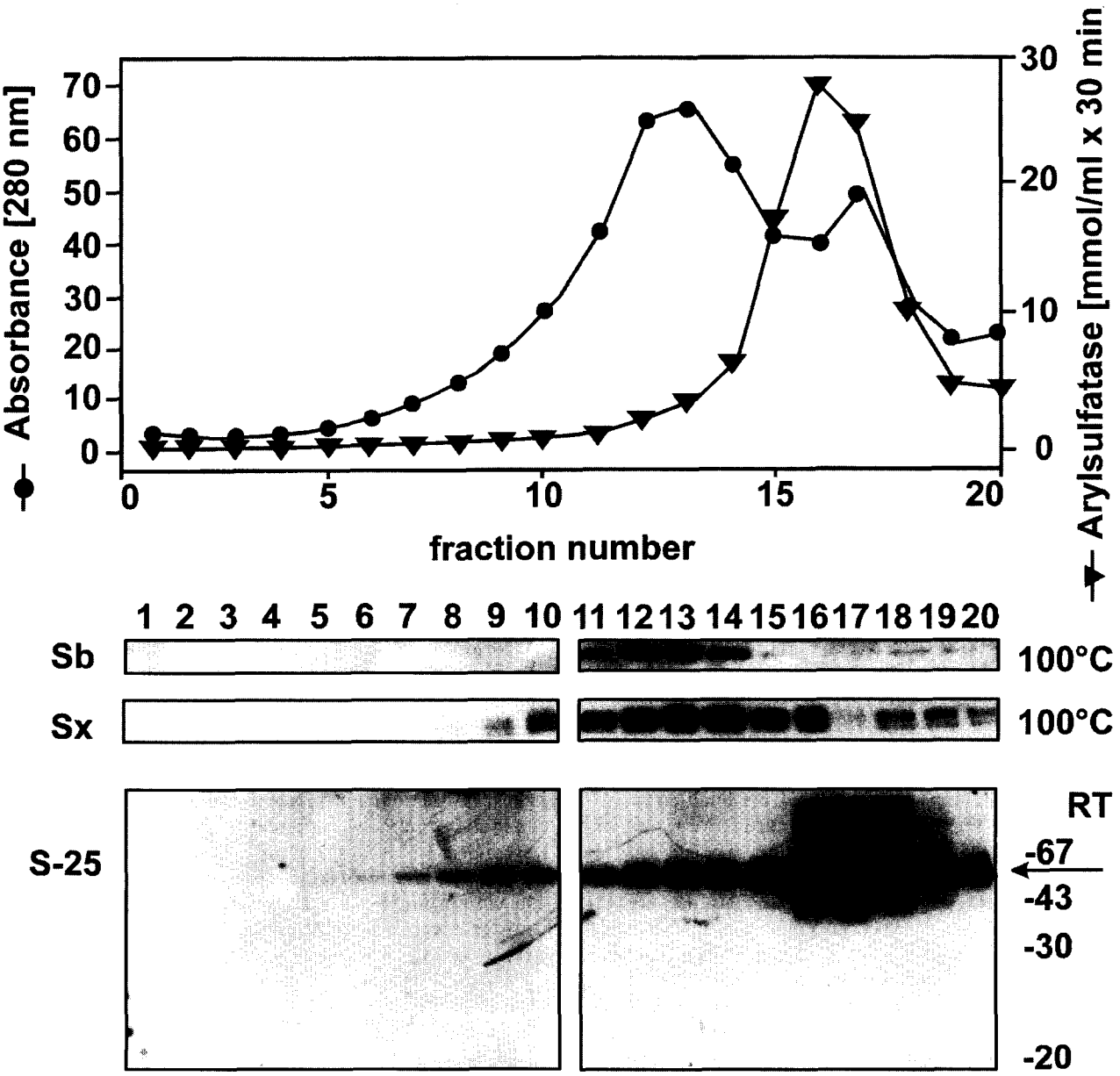

Fig. 4. Sucrose density gradient fractionation of adrenal chromaffin vesicles. Chromaffin vesicles, obtained by differential centrifugation, were further purified on a discontinuous sucrose gradient (see Section 2). In agreement with previous reports [12,38], synaptobrevin (Sb) and syntaxin (Sx) are present in fractions 11–14 containing chromaffin vesicles characterized also by the highest peak of absorbance at 280 nm determined in the TCA supernatant of the fractions (top). SNAP-25 exhibits a bimodal distribution. It is present in the chromaffin vesicle fractions and in lighter fractions containing lysosomes (see arylsulfatase activities shown at the top). The apparent molecular weight of the immunoreactive band was 60 kDa (arrow). Note that the samples used for the SNAP-25 immunoblot were not boiled but kept at room temperature (RT).

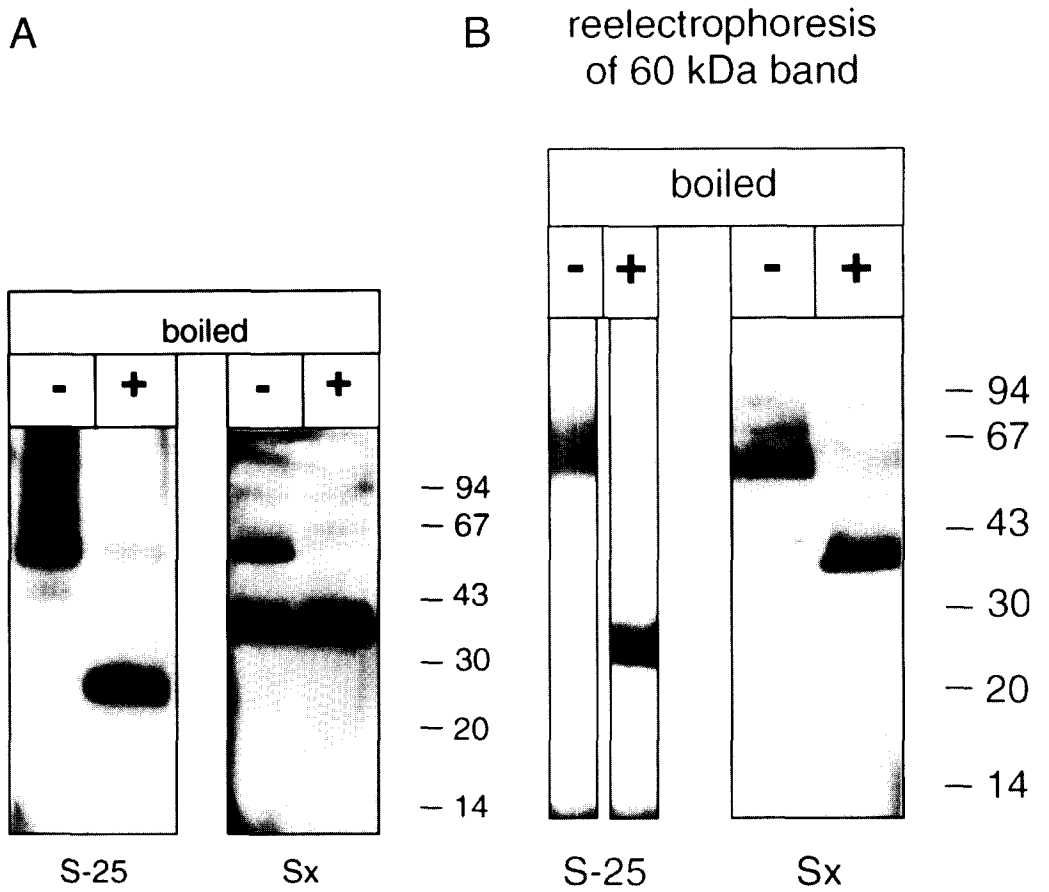

Fig. 5. Heterodimers of SNAP-25 and syntaxin in chromaffin vesicle membranes. In unboiled samples of chromaffin vesicle membranes a SNAP-25 (S-25) form of 60 kDa predominates. In contrast in boiled samples SNAP-25 exhibited a molecular weight of 25 kDa (A). The intensity of the syntaxin (Sx) band of 35 kDa increases upon boiling of the samples. The immunoreactive band of 60 kDa observed in unboiled samples was identified by reelectrophoresis of the material as a complex of SNAP-25 and syntaxin (B).

the cellular SNAP-25 (Fig. 4). It should be noted that the samples analyzed for SNAP-25 were not boiled prior to SDS-PAGE. Interestingly the molecular weight of the SNAP-25 immunoreactive band in the blots was about 60 kDa, that is about 35 kDa larger than that of authentic SNAP-25.

We harvested the chromaffin vesicle fractions (11–14) from the sucrose gradients, isolated their membranes (see Section 2), and analyzed them further. Again, in unboiled samples the anti-SNAP-25 antibody labelled a band of 60 kDa (Fig. 5A). Boiling of the samples caused a shift of the immunoreactive band to 25 kDa (Fig. 5A). The difference in electrophoretic mobility during SDS-PAGE of boiled and unboiled samples suggested the presence of a complex of SNAP-25 and syntaxin in chromaffin vesicle membranes. Moreover an antibody directed against syntaxin revealed an immunoreactive band of 60 kDa in addition to syntaxin of 35 kDa in blots of unboiled samples (Fig. 5A) and only a syntaxin immunoreactive band of 35 kDa after boiling. Additional experiments provided evidence that the immunoreactive band of 60 kDa represents a heterodimer of syntaxin and SNAP-25. Cutting out the 60 kDa band from the acrylamide gels followed by boiling and reelectrophoresis resulted in a band of 35 kDa immunoreactive with the antibody directed against syntaxin and a band of 25 kDa immunoreactive with the antibody directed against SNAP-25 (Fig. 5B). Thus it is evident that SNAP-25 localized ultrastructurally to chromaffin vesicles forms a complex with syntaxin that is stable in SDS at room temperature but disassembles upon boiling. Synaptobrevin was not found in a complex stable in SDS which is consistent with the observation that synaptobrevin of chromaffin vesicle membranes is readily cleaved by tetanus toxin [12], while complexes of synaptobrevin with syntaxin and SNAP-25 formed in solubilized brain are resistant to tetanus toxin [39]. SNAP-25 monomers, detected during the analysis of whole chromaffin cells (see Fig. 2), were absent from chromaffin vesicle membranes but syntaxin exists also as a monomer within the membranes (Fig. 5A). Thus chromaffin vesicle membranes differ in the types of monomers present in addition to the SNAP-25/syntaxin heterodimers when compared to total cellular membranes.

We also investigated the sensitivity to BoNT/A of SNAP-25 in chromaffin vesicle membranes. We found that SNAP-25 was resistant to BoNT/A under all circumstances tested with the exception of the addition of OG where a small fraction of SNAP-25 was cleaved (Fig. 6). This detergent caused the solubilization of SNAP-25 from the membranes (not shown). Preincubation at room temperature or boiling in 1% OG (see Section 2) did not change the electrophoretic mobility of SNAP-25 suggesting that OG, unlike SDS, does not cause the dissociation of the heterodimers but alters the conformation of SNAP-25, which apparently results in a slow attack of BoNT/A. In summary it can be concluded that vesicular SNAP-25 bound to syntaxin is resistant to BoNT/A. Moreover immunocytochemistry of SNAP-25 at the ultrastructural

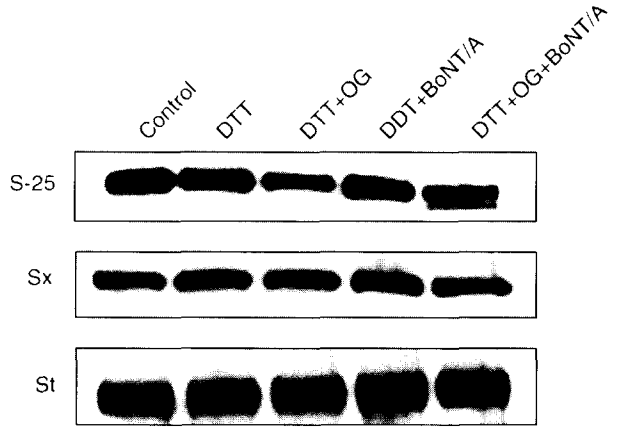

Fig. 6. SNAP-25 of chromaffin vesicles is resistant to botulinum neurotoxin A. Chromaffin vesicle membranes prepared from chromaffin granules recovered from the sucrose density gradients (see Section 2) were incubated for 30 min with 100 nM BoNT/A. The different additions (20 mM DTT, 1% OG, BoNT/A) indicated at the top did not change syntaxin (Sx)- or synaptotagmin (St)-immunoreactive bands in the boiled samples. Only SNAP-25 (S-25) was partly attacked by BoNT/A if the detergent OG was present.

level, subfractionation studies and the analysis of isolated chromaffin vesicles show that stable SNAP-25 heterodimers, resistant to SDS at room temperature, exist in the chromaffin vesicle membrane.

4. Discussion

In the present study, we have shown that SNAP-25 exists in adrenal chromaffin cells not only at the plasmalemma but also at the membranes of catecholamine storing chromaffin vesicles. In chromaffin vesicles SNAP-25 and syntaxin form a binary complex, stable at room temperature even in the presence of SDS. SNAP-25 and syntaxin are regarded as essential components of the exocytosis apparatus, because botulinum neurotoxins exert their inhibitory action on exocytosis by selective cleavage of these proteins. We propose that preexisting vesicular SNAP-25/syntaxin heterodimers, which have previously been shown to associate with soluble SNAPs [4,7], participate in exocytotic vesicle docking and fusion as vesicular SNAP receptors.

In previous investigations resistance to SDS as a signature of high stability has only been observed in complexes of higher order, that is in SNAP-25/syntaxin/synaptobrevin heterotrimers [9,40]. Heterotrimeric complexes have been found in solubilized adrenal chromaffin and pheochromocytoma cells [13,36,41], in detergent extracts of brain and upon incubation of the recombinant proteins in media containing nonionic detergents [3,5,9,39,40]. These complexes, formed in the presence of detergents in the aforementioned studies, are assumed to represent intermediates generated during docking of synaptic vesicles and chromaffin vesicles to the plasmalemmal target membrane, a process predicted by the SNARE hypothesis [2,3,22]. In adrenal chromaffin cells most secretory vesicles are undocked. Thus the stable heterodimers of SNAP-25 and syntaxin found in the membranes of intact vesicles are likely precursors of heterotrimers which may be formed upon vesicle docking.

Synaptobrevin, although present in the chromaffin vesicle membrane [12,37], does not associate with stable vesicular SNAP-25/syntaxin heterodimers to form stable heterotrimers. Several reasons may account for this inability. First, synaptobrevin and SNAP-25/syntaxin heterodimers present in the same vesicle may have an inappropriate orientation for the formation of a heterotrimer as opposed to synaptobrevin in a docked vesicle facing plasmalemmal syntaxin and SNAP-25. Second, protein-protein (or protein-lipid) interactions, for example binding of synaptotagmin [42,43], complexins [44] or sec1 homologues [45–47] to syntaxin, may prevent the formation of the heterotrimers in the vesicular membrane. Specifically, n-sec1 inhibits the interaction of other proteins with syntaxin, including synaptobrevin and SNAP-25 [5]. A sec1 homologue associated with chromaffin vesicles [37] may possess this function. Third, synaptobrevin binding to the heterodimer may require conformational changes in the components of the exocytosis apparatus which can be elicited in vitro by addition of detergent (see above) but may occur in vivo during vesicle docking or fusion.

Syntaxin, SNAP-25/syntaxin heterodimers, SNAP-25/syntaxin/synaptobrevin heterotrimers bind SNAPs but synaptobrevin alone does not [4,7,11]. Thus in adrenal chromaffin vesicles the observed stable SNAP-25/syntaxin heterodimers and syntaxin monomers are the only known SNAP receptors present. The complexes of SNAPs with recombinant syntaxin, heterodimers, and heterotrimers disassemble after binding of NSF in a ATP-dependent manner [3,4,6]. Thus it is possible that vesicular and/or cellular complexes between SNAPs, syntaxin or the stable heterodimers can disassemble with ATP. Recently also synaptic vesicles have been shown to contain both SNAP-25 and syntaxin [48,49]. It is unknown whether SNAP-25 and syntaxin form heterodimers within the membranes of synaptic vesicles but it is interesting to note that syntaxin of synaptic vesicles was found to be susceptible to botulinum neurotoxin C1 [49]. This observation suggests that most syntaxin present in synaptic vesicle, similar to syntaxin in chromaffin vesicles (see above) is not bound to SNAP-25. However, vesicular complexes or monomers of SNAP-25 and syntaxin may serve as SNAP receptors operating during exocytosis. Interaction with soluble SNAPs and NSF may also occur in docked vesicles in which vesicular and plasmalemmal SNAP-25 and syntaxin are coupled via SNAPs and multimeric NSF. In this scenario disassembly of this initial symmetric complex could be followed by the formation of a heterotrimer composed of vesicular synaptobrevin and plasmalemmal SNAP-25 and syntaxin.

Recent functional studies revealed that SNAP-mediated pathways enhance exocytosis of synaptic vesicles and chromaffin vesicles [50,51] implying that soluble SNAPs and NSF, detected first to be essential for constitutive fusion events (reviewed in [22]), also operate in regulated exocytosis. Clostridial neurotoxins made it possible to identify synaptobrevin, syntaxin and SNAP-25 as essential proteins of the exocytosis apparatus of neurons and endocrine cells. According to the SNARE hypothesis specificity of targeting during exocytosis is assured by complex formation between the v-SNARE synaptobrevin and syntaxin as well as SNAP-25, previously located exclusively to the plasmalemma. However, recent observations (this study and [38,48,49]) have shown that SNAP-25 and syntaxin, as monomers or as heterodimers, are present in addition in the vesicular membrane. Thus these proteins may serve during exocytosis both as v- and t-SNAREs. For example during docking, vesicular and plasma-

lemmal SNAP-25 and syntaxin first coupled via SNAPs/NSF but then disassembled by ATP could be replaced by a complex between synaptobrevin and SNAP-25/syntaxin in the t-SNARE position. The resulting heterodimeric complexes bind again the soluble components NSF and SNAPs, in competition with the vesicular calcium sensor synaptotagmin [52,53]. Subsequent steps may be followed by membrane fusion and neurotransmitter or hormone release.

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