Multiple forms of SNARE complexes in exocytosis from chromaffin cells: effects of Ca$^{2+}$, MgATP and botulinum toxin type A

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Summary

The changes that SNAREs undergo during exocytosis were studied in permeabilised chromaffin cells treated with Ca$^{2+}$, MgATP or botulinum neurotoxin A. High-resolution 2D SDS-PAGE revealed multiple SDS-resistant SNARE complexes having a wide range of sizes and in which SNAP-25 and syntaxin predominate over synaptobrevin. Their formation increased upon Ca$^{2+}$-stimulated exocytosis; notably, the 2D protocol proved much superior to 1D SDS-PAGE for the detection of large complexes and revealed that for forms with relative molecular mass greater than 100,000 stimulated induction was more significant than for smaller species. MgATP enhanced Ca$^{2+}$-triggered catecholamine release but reduced the content of complexes. By contrast, botulinum neurotoxin type A inhibited exocytosis and altered the stoichiometry of the SNAP-25:syntaxin binary association, without lowering its abundance. The individual SNAREs were protected against trypsin proteolysis to varying extents in binary and ternary complexes of different sizes, suggestive of distinct folding intermediates. Our data suggest that Ca$^{2+}$ triggers an early stage of SNARE complex formation causing an accumulation of partially folded intermediates, especially of binary forms, as well as their maturation into smaller, more protease resistant states. In addition, botulinum neurotoxin A inhibits exocytosis by perturbing the syntaxin:SNAP-25 ratio in binary intermediates.

Key words: Secretion, Membrane fusion, SNAP-25, Syntaxin, Synaptobrevin, Clostridial neurotoxins

Introduction

In chromaffin cells, catecholamines are stored in large dense-core granules and released by exocytosis, in response to increased concentrations of intracellular free Ca$^{2+}$ (Burgoyne and Morgan, 1995), which requires synaptosomal-associated protein of molecular weight 25×10$^3$ (Mr = 25 K) (SNAP-25)$^1$, syntaxin and synaptobrevin. This process is inhibited by botulinum neurotoxins (BoNTs), which cleave SNAP-25 (BoNT/A, /C1 and /E), synaptobrevin (BoNT/B, /D, /F and /G) or syntaxin (BoNT/C1) (Niemann et al., 1994; Schiavo et al., 2000). These three proteins spontaneously associate in vitro to form a stable complex that is resistant to denaturation by SDS at temperatures up to ~80°C (Hayashi et al., 1994) and which binds N-ethylmaleimide-sensitive factor (NSF) via soluble NSF attachment proteins (SNAPs); hence, SNAP-25, syntaxin and synaptobrevin are known collectively as SNAREs (for SNAP-receptors) (Söllner et al., 1993). The SNAREs catalyse membrane fusion following reconstitution into lipid vesicles (Weber et al., 1998). Formation of the SDS-resistant SNARE complex in vitro is prevented by cleavage with BoNT/E of SNAP-25 between Arg180-Ile181, which removes 26 residues from its C-terminus (Hayashi et al., 1994). Accordingly, in semi-intact PC-12 cells, this toxin severely reduces the amount of Ca$^{2+}$-triggered exocytosis (Banerjee et al., 1996); however, evoked secretion could be recovered by the addition of a peptide corresponding to residues 142-206 of SNAP-25 (Chen et al., 1999). This SNAP-25 C-terminal fragment was shown to be incorporated into an SDS-resistant complex of Mr=65 K together with syntaxin, synaptobrevin and the SNAP-25$_{1-180}$ cleavage product of BoNT/E. Based on these data, it was proposed that Ca$^{2+}$-induced formation of the latter drives membrane fusion. Unfortunately, no comparison was made between the amounts of the 65K entity present in Ca$^{2+}$-stimulated and Ca$^{2+}$-free cells. Thus, the induced formation of this complex during Ca$^{2+}$-triggered exocytosis has yet to be established conclusively, especially because the peptide was incorporated into complexes even in the absence of Ca$^{2+}$ (Chen et al., 1999). Moreover, if exocytosis is linked to SNARE complex formation, it should be possible to demonstrate this relationship in cells that have not been poisoned by BoNT/E, but to date this has not been reported. Such an investigation is warranted, particularly in view of the amount of SNAP-25 recombinant complex not being altered by Ca$^{2+}$-stimulated exocytosis from semi-intact nerve-endings (Leveque et al., 2000; Mehta et al., 1996). Also, several different SDS-resistant complexes were observed in the nerve terminal membranes (Hayashi et al., 1994; Otto et al., 1997), whereas only a single complex was seen in PC-12 cells (i.e. a single band of Mr=105 K for the native ternary SNARE complex or one band of Mr=65 K for the abnormal Ca$^{2+}$-induced complex containing SNAP-25$_{1-180}$ plus the exogenous C-terminal peptide) (Chen et al., 1999). The basis of these large complexes is not clear; they may be higher order oligomers of SNAP heterotrimers (Tokumaru et
Aliquots of CaCl₂ were added to the KGEP to produce the desired concentrations of buffered-free Ca²⁺ and, where indicated in the (Lawrence et al., 1994). Where used, trypsin (see figure legends for catecholamines released from the cells assayed, as described the stimulation period, an aliquot was removed and the amount of proteins and metabolites through the detergent-induced pores. After (known as 'run-down'), which occurs due to loss of soluble cytosolic figures, 2 mM ATP and 4 mM MgCl₂ were also included. In all medium and maintained for 24-72 hours before further manipulations.

Stimulation and assay of catecholamine release
Immediately prior to experiments, which were all performed at ~22°C, cells were rinsed with a Hepes-buffered saline solution (145 mM NaCl, 4.8 mM KCl, 1.2 mM NaH₂PO₄, 20 mM Hepes, pH 7.4) before permeabilisation by exposure to 20 μM digitonin in KGEP (139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES, pH 6.5). Aliquots of CaCl₂ were added to the KGEP to produce the desired concentrations of buffered-free Ca²⁺ and, where indicated in the figures, 2 mM ATP and 4 mM MgCl₂ were also included. In all experiments, Ca²⁺ and MgATP (if included) were co-applied to the cells with digitonin to avoid deterioration of the exocytotic response (known as ‘run-down’), which occurs due to loss of soluble cytosolic proteins and metabolites through the detergent-induced pores. After the stimulation period, an aliquot was removed and the amount of catecholamines released from the cells assayed, as described (Lawrence et al., 1994). Where used, trypsin (see figure legends for details) was added directly to the digitonin-containing KGEP from a 10 mg/ml stock in the same buffer.

Enrichment, SDS-PAGE and western blotting of membrane-bound proteins
At the end of the experiments, a membrane-enriched fraction was prepared by scraping the cells from the plates with a rubber policeman, followed by trituration through a 26 G needle. Large debris was removed from the lysed cells by centrifugation at 1000 g for 5 minutes and the membranes in the supernatant were pelleted at 360,000 g for 20 minutes. The sediment was dissolved in 50 mM Tris.HCl, pH 5.8 containing 1% SDS and subjected to PAGE using the NuPAGE system (Novex, San Diego), according to the manufacturer's instructions. Relative molecular mass values were calculated by reference to the migration of protein standards (Multimark, Novex). Proteins were transferred from the gels onto PVDF membrane at 50 mV for 12-16 hours while fully immersed in 25 mM Tris, 192 mM glycine containing 20% (v/v) methanol. Western blotting was performed using standard protocols (Lawrence et al., 1996), binding of primary antibodies being detected using horseradish peroxidase-conjugated secondaries and visualised by the ECL system (Amersham-Pharmacia, UK). Digital images were captured using a flat-bed scanner and signal intensities were determined using NIH Image. The data presented in each of the figures are representative of results obtained consistently and on at least three separate occasions.

Materials and Methods
Materials
Tissue culture media and reagents were purchased from Life Technologies (Paisley, UK), digitonin was from Novabiochem (Nottingham, UK) and all other chemicals, including the monoclonal antibody HPC1, were obtained from Sigma Chemical Co. (Poole, UK). The production of antibodies against the recombinant SNAP-25, or a synthetic synaptobrevin peptide, has been described previously (Foran et al., 1996; Lawrence et al., 1996). BoNT/A was purified to homogeneity according to a procedure described previously (Shone and Tranter, 1995).

Chromaffin cell preparation and culture
Bovine adrenal chromaffin cells were isolated as described previously (Lawrence et al., 1994), and maintained as monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate, 50 μg/ml gentamycin, 10 μM cytine arabinofuranoside, 8 μM fluorodeoxyuridine, 2.5 μg/ml fungizone, 25 IU penicillin, 25 μg/ml streptomycin, 25 mM N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES) pH 7.4. The cells were used for experiments between 3 and 10 days after isolation and where stated pre-intoxicated with BoNT/A, using a protocol that facilitates its uptake (Lawrence et al., 1996). Briefly, the cells were exposed for 24 hours at 37°C to BoNT/A in 5 mM NaCl, 4.8 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 20 mM Hepes pH 7.4, 5.6 mM glucose, 220 mM sucrose and 0.5% [w/v] bovine serum albumin. After washing, the cells were returned to the standard culture medium and maintained for 24-72 hours before further manipulations.

Results
Multiple forms of SDS-resistant SNARE complexes occur in unstimulated permeabilised chromaffin cells: their abundance is increased by Ca²⁺
Chromaffin cells were permeabilised and exposed for 15 minutes to KGEP in the absence or presence of 20 μM Ca²⁺; the cation stimulated a large increase in catecholamine release above the basal value (to 2.7-times the basal value; Fig. 1A). To determine whether the amounts of SDS-resistant SNARE complexes in the cells were altered during Ca²⁺-triggered exocytosis, a membrane-enriched fraction was subjected to SDS-PAGE without boiling, transferred to PVDF and analysed by western blotting with SNAP-25 antibodies. This revealed a number of SDS-resistant complexes in the membranes of nonstimulated cells (Fig. 1B; two major bands of Mr=195 K and 120 K plus several less intense signals), concurring with findings for nerve terminals (Hayashi et al., 1995; Otto et al., 1997). Following Ca²⁺-stimulation the largest band was found to diminish, the Mr=120 K signal intensified and two new faster migrating bands (Mr=83 K and 63 K) appeared. These findings are dissimilar to those reported for PC-12 cells (Chen et al., 1999), in which Ca²⁺ caused a diminution of a Mr=105 K band and the appearance of a Mr=65 K complex. Unfortunately, the SDS-resistant SNARE complexes did not transfer efficiently and consistently from the gel to PVDF (the abundance of the Mr=83 K and 63 K bands, in particular, were subject to experimental variation); thus, an improved method (Otto et al., 1997) was adopted for their separation and detection. As before, the samples were not heated before being subjected to SDS-PAGE but, instead of direct transfer of the proteins from the gel, the sample lanes were excised and cut into fragments. These were boiled in 1% SDS/sample buffer to dissociate the SNARE complexes and solubilise their constituents. The proteins recovered from the
The amount of SNAP-25 in SDS-resistant complexes is reduced by MgATP

Exposure of permeabilised chromaffin cells to MgATP had minimal effect on basal level of exocytosis, but significantly enhanced Ca2+-triggered release (2.2-times; Fig. 1A). By contrast, cells exposed to the nucleotide in the absence of Ca2+ showed a reduced amount of SDS-resistant complexes compared with the level in MgATP-free controls (Fig. 1B,C; in C, the signal intensity of SNAP-25 in complexes in cells treated with MgATP is 80% of the control level). The amount of complexes in cells exposed to both the nucleotide and Ca2+ (Fig. 1B,C) was lower than in cells treated with the cation alone (again, 80%); this is most likely due to SNARE complex disassembly mediated by the ATPase, NSF (Hayashi et al., 1995; Söllner et al., 1993). Thus, the amount of complexes present within the cells is not proportional to the extent of exocytosis elicited: secretion is greatest from cells exposed to Ca2+ and MgATP together, but SNARE association is most abundant in cells stimulated with the cation alone.

Syntaxin and synaptobrevin are also found in the SDS-resistant complexes in situ

Second dimension gels of the samples used in Fig. 1C were probed for syntaxin (Fig. 2A) and synaptobrevin (Fig. 2B). As expected, both proteins were also found in SDS-resistant complexes and the amounts recovered were increased (1.6-times for Sbr and 1.5-times for syntaxin) by Ca2+; however, decreased (to 20% [Sbr] and 70% [syntaxin]) by MgATP (data not shown). Additionally, two Ca2+-induced bands of smaller size (Mr = 83 K and 63 K, respectively), observed in the 1D blot, seemed much less abundant when analysed by the 2D protocol; in fact, the Mr = 63 K signal was rarely detectable in 2D gel blots (Fig. 1C; and data not shown). There are several possible reasons for this. First, as has been noted already, the large complexes are not easy to electro-blot to PVDF; thus, it is likely that smaller complexes, which migrate easier through the gel matrix, more efficiently transfer and, hence, appear anomalously abundant compared with larger forms. Second, the Mr = 83 K complex is resistant to SDS/heat denaturation, being observed intact in the gel after boiling the sample (Fig. 1C).

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* The intensity of signals in blots from the second gel, originating from slices of the Mr = 55-220 K region of the primary gel, were quantified using NIH-IMAGE (see Materials and Methods for details) and these values were summed to obtain a figure for the total amount of the SNAP-25 present in complexes. In the case of cells exposed to neither Ca2+ nor MgATP, the figure was normalised to 1.0 and values for cells treated with the other conditions were expressed relative to this. The same semi-quantitative procedure was performed to examine qualitatively the effects of Ca2+ and MgATP on the amounts of synaptobrevin and syntaxin present in complexes in situ.

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**Fig. 1.** Ca2+ induces the formation of multiple MgATP-dissociatable complexes in situ. Chromaffin cells were permeabilised with 20 μM digitonin in KGEP and maintained in the absence or presence of 20 μM Ca2+, with or without the inclusion of 2 mM MgATP. After 15 minutes, aliquots were removed and assayed for catecholamines. Cells in some wells were solubilised in Triton X-100 and used to estimate the total amount of catecholamine remaining and the amounts released (A) were expressed (±s.d. n=4) as a percentage of the total cell content, calculated by summing the amounts released with the level remaining. The remaining cells were lysed in KGEP, 2 mM PMSF was added and membrane-enriched fractions were isolated, as detailed in the Materials and Methods. The latter were dissolved in 50 mM Tris-HCl, pH 5.8 containing 1% SDS before adding 4× sample buffer and being subjected to SDS-PAGE. The separated proteins were either transferred directly to PVDF (B) or extracted from the gel by boiling in fresh sample buffer and re-electrophoresed before transfer (C). The PVDF membranes were blotted with antibodies raised against recombinant SNAP-25. Immunodetection of primary antibody binding was performed as described in Materials and Methods. Note that the left and right panels show signals for two different bands (Mr = 25 K and 83 K, respectively).
increase in the level of synaptobrevin within complexes was blocked by MgATP, which had minimal effect upon the increased incorporation of syntaxin. However, synaptobrevin was only just detectable in the complexes, despite extensive development of the blot such that the signal for monomer (at \( M_r = 18 \) K) was over-exposed. This suggests that a large fraction of the SDS-resistant complexes contain SNAP-25 and syntaxin but lack synaptobrevin.

BoNT/A alters the composition of SNARE complexes in situ

Pre-treatment of chromaffin cells with BoNT/A potently blocks agonist-evoked release of catecholamines (Foran et al., 1996; Lawrence et al., 1996). This toxin cleaves SNAP-25 between Gln197-Arg198, thereby, removing nine amino acids from its C-terminus (Niemann et al., 1994; Schiavo et al., 2000). This region is important for stability of the ternary SNARE complex in vitro (Hayashi et al., 1994); complexes formed with BoNT/A-truncated SNAP-25 are 50% less likely to become SDS-resistant than are those incorporating full-length SNARE. Thus, it was suspected that cell poisoning with BoNT/A would reduce the amounts of SDS-resistant SNARE complexes in both resting and Ca\(^{2+}\)-stimulated chromaffin cells. Exposure of cells to the toxin, using a protocol that results in cleavage of >95% of their SNAP-25, is accompanied by a similar reduction in depolarisation-induced exocytosis (Lawrence et al., 1996). Control and BoNT/A-poisoned chromaffin cells were permeabilised and exposed for 45 minutes with or without Ca\(^{2+}\), before isolating a membrane-enriched fraction for analysis by 2D SDS-PAGE and western blotting. Unexpectedly, in the absence of Ca\(^{2+}\), the level of syntaxin associated with SDS-resistant complexes was increased (5.7-times) following BoNT/A (Fig. 3A). By contrast, the amount of SNAP-25 associated with complexes was reduced (to 70% of the control amount). As noted for toxin-free cells (Fig. 2; Fig. 3A), there was virtually no detectable synaptobrevin associated with these complexes. The

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**Fig. 2.** Detection of synaptobrevin and syntaxin in the multiple SNARE complexes in situ. Aliquots of the samples used in Fig. 1C were subjected to SDS-PAGE and western blotting with antibodies raised against a synthetic synaptobrevin peptide (A) or monoclonal HPC1, which is specific for syntaxin (B).

**Fig. 3.** BoNT/A alters the ratio of SNAREs in complexes. (A) Control and BoNT/A-pre-treated chromaffin cells (see Materials and Methods) were permeabilised with 20 \( \mu \)M digitonin in KGEP, in the absence or presence of 20 \( \mu \)M Ca\(^{2+}\). After 45 minutes, 2 mM PMSF was added, the cells were lysed and a membrane-enriched fraction prepared. Two-dimensional SDS-PAGE and western blotting was performed, as described in Figs 1 and 2. (B) Cells were treated as in (A), except that 15 minutes after permeabilisation the cells were exposed to 100 \( \mu \)g/ml trypsin for 30 minutes.
same pattern was observed for cells exposed to Ca\(^{2+}\); in the presence of the cation, the amounts of SNAP-25 and syntaxin in complexes were increased in BoNT/A-treated cells, as noted previously for control cells. Synaptobrevin remained below the limit of detection.

Several SDS-resistant SNARE complexes exist

The observed separation of multiple SNARE species may indicate their multi-merisation or, possibly, occurrence in several folded states. These possibilities were evaluated by examining their susceptibility in situ to degradation by trypsin. This was performed in tandem with the experiment documented in Fig. 3A. The cells were treated identically, except that trypsin was added 15 minutes after permeabilisation and maintained for the remaining 30 minutes before preparing membranes for biochemical analysis. In the absence of Ca\(^{2+}\), virtually all the syntaxin was proteolysed but a significant amount of SNAP-25 remained (Fig. 3B). In BoNT/A-treated cells, a lower level of SNAP-25 was recovered (Fig. 3B), which is not surprising as less SNAP-25 is associated with the complexes following intoxication (Fig. 3A). The notable appearance of trypsin-resistant synaptobrevin was unexpected (Fig. 3B) in view of it being absent from complexes in cells not exposed to trypsin (Fig. 3A); perhaps the protease blocked turnover of ternary complexes. Less synaptobrevin was found in complexes from BoNT/A-treated cells (Fig. 3A). After Ca\(^{2+}\) stimulation, the amount of trypsin-resistant SNAREs recovered increased in all cases (Fig. 3B); this accords with the fact that the cation stimulates complex formation (Figs 1, 2, Fig. 3A). Likewise, the induction by Ca\(^{2+}\) of protease-resistant SNAP-25 complexes was not blocked by BoNT/A (Fig. 3B); SNAP-25 and synaptobrevin increased 2.4- and 7.8-times, respectively, compared with 2.1- and 1.1-times in control. The fold-induction of trypsin-resistant syntaxin is not quantifiable, but clearly it was not inhibited, as expected, because this toxin does not inhibit complex formation in trypsin-free cells (Fig. 3A). Notably, different sized complexes offered varying degrees of protection to each of the SNAREs. It was the highest mobility complexes that best protected synaptobrevin (M\(_r\)=100 K), intermediate complexes for syntaxin (M\(_r\)=100-155 K) and SNAP-25 was protected over a broad range (M\(_r\)=55-155 K). This suggests that the relative accessibility of each SNARE to trypsin is not the same for complexes of different molecular mass which, in turn, may imply that these represent distinct folded states rather than multimers of SNARE complexes (see Discussion).

Discussion

Multiple forms of SNARE complex are induced during exocytosis

Permeabilised chromaffin cells were used to investigate the effect on SNARE complexes of agents that either trigger (Ca\(^{2+}\)), augment (MgATP) or inhibit (BoNT/A) catecholamine release. Although it has been proposed that Ca\(^{2+}\) induces SNARE complex formation during exocytosis in neuroendocrine cells (Chen et al., 1999), a direct comparison of the amounts of SDS-resistant SNAP-25 complexes in nonstimulated and Ca\(^{2+}\)-treated cells has not been made until now. These complexes were found to exist in several forms, separable by SDS-PAGE over a wide size-range, in permeabilised chromaffin cells lacking Ca\(^{2+}\) and, thus, not stimulated to elicit exocytosis. These findings concur with prior studies on synaptosomes (Hayashi et al., 1994; Otto et al., 1997) but are at variance with the existence of a single complex in PC-12 cells (Chen et al., 1999). However, the procedure used in the latter study was shown herein to be problematic, because SDS-resistant complexes are not transferred efficiently from the gel to PVDF and/or they react sub-optimally with antibodies. Two bands were observed in western blots of 1D SDS-PAGE gels of resting chromaffin cells, at M\(_r\)=195 K and 120 K; the latter may correspond to the single M\(_r\)=105 K band observed in Ca\(^{2+}\)-stimulated PC-12 cells (Chen et al., 1999) (unfortunately, it is not known if this complex is present in nonstimulated PC-12 cells as this data was not shown). However, an improved 2D SDS-PAGE method revealed that these two are not the only forms of SDS-resistant complexes; in fact, they do not even represent the most abundant species. Nevertheless, the central proposal of the latter study – that Ca\(^{2+}\) induces the formation of SDS-resistant SNARE complexes during triggered exocytosis – is confirmed. However, it has now become clear that the cation triggers the formation of a wide range of complexes and not just a single entity; possibly, explaining the noted inconsistencies between complex formation and stimulation of exocytosis (Chen et al., 1999). Moreover, complexes did apparently form, albeit at a reduced rate, in the absence of Ca\(^{2+}\) and triggered exocytosis; this was most obvious when MgATP was omitted, presumably precluding their disassembly by NSF (Söllner et al., 1993). The relationship between exocytosis and complex formation is qualitative; no clear correlation between the amount of complexes and the extent of exocytosis was evident (this study and data not shown). Moreover, MgATP reduced the complexes but enhanced exocytosis. The amount of SNARE complexes present in permeabilised nerve terminals was found to remain unchanged during neurotransmitter release (Leveque et al., 2000; Mehta et al., 1996); however, Triton X-100 was used to solubilise and isolate the synaptosomal membrane proteins, despite the known inability of this detergent to prevent the formation of complexes (Otto et al., 1997).

Binary SNAP-25:syntaxin complexes predominate over ternary SNARE forms

Another notable finding from our investigation is that the majority of SDS-resistant complexes in chromaffin cells lacks synaptobrevin. There have been prior reports of association of SNAP-25 with the cytosolic domain of syntaxin, but this was deemed not SDS-resistant (Fasshauer et al., 1997; Hayashi et al., 1994), although they could gain added stability from the presence of the transmembrane anchor of syntaxin (Poirier et al., 1998). Thus, the composition of these complexes might be important for fast secretory responses, as BoNT/A slows down the rate of the exocytotic burst and the C-terminus of SNAP-25 has been implicated in a late step of exocytosis (Xu et al., 1999). Curiously, trypsin increased the amount of synaptobrevin recovered in the SDS-resistant complexes, possibly due to a dynamic association of this protein that is upset in trypsin-treated cells.
Different sized complexes could be distinct folded states that may facilitate fast exocytosis.

The spread of SDS-resistant complexes over a wide size-range suggests that they occur in a variety of states (Hayashi et al., 1994; Otto et al., 1997; Brunger, 2001). This would explain the non-overlapping trypsin-sensitivity profiles for the individual components. It is tempting to speculate that complexes of varied mobility on SDS-PAGE represent distinct folded intermediates. Fully-folded complexes would be more compact and, hence, likely to migrate faster on SDS-PAGE and be more resistant to protease attack. An alternative hypothesis, that the less mobile bands could be oligomers of SNARE heterotrimers (Tokumaru et al., 2001), is difficult to reconcile with the greater protease resistance observed for smaller complexes. Site-directed mutagenesis of residues in one of the four helices of the SNARE bundle creates complexes exhibiting different degrees of thermal stability (Chen et al., 1999), indicating that stable, SDS-resistant abnormally folded states can be created in vitro. Likewise, stable folded complexes can be formed between SNAP-25, syntaxin and C-terminally-truncated forms of synaptobrevin; these appear to be folded completely N-terminus to the truncation site, but are unstructured at the C-terminal end (Margittai et al., 2001).

Ca\(^{2+}\) triggered the formation of complexes across the entire size range, which could be achieved by stimulation of an early stage of a SNARE association and folding reactions that can then proceed in its absence. Alternatively, there could be various intermediate steps that are also accelerated by Ca\(^{2+}\); indeed, several kinetically distinct phases of secretion can be distinguished by their Ca\(^{2+}\)-sensitivities (Bittner and Holz, 1992). Moreover, in an attempt to reconcile biochemical and electrophysiological data, it has been proposed that the fastest phase of exocytosis may involve maturation of a partially folded SNARE complex intermediate. Accordingly, an antibody that binds to SNAP-25 in partially, but not fully, folded complexes delays the exocytotic burst, whereas another that recognises only free (i.e. non-complexed) SNAP-25 retarded only the slower phases of secretion (Xu et al., 1999). Low [Ca\(^{2+}\)] (\(< 1 \mu M\) may stimulate the early stages of SNARE association and higher levels (\(> 3 \mu M\)) could trigger exocytosis by inducing maturation of partially folded complexes (Voets, 2000). Therefore, it is notable that the putative Ca\(^{2+}\)-sensor, synaptotagmin, exhibits a dual Ca\(^{2+}\) affinity for promotion of its binding to syntaxin, with EC\(_{50}\)=0.7 \(\mu M\) and 180 \(\mu M\), respectively (Chapman et al., 1995). In permeabilised neuroendocrine cells, secretion is triggered by \(> 1 \mu M\) [Ca\(^{2+}\)], and is optimal at \(\sim 20 \mu M\) [although higher levels accelerate the fastest phase (Bittner and Holz, 1992); therefore, high [Ca\(^{2+}\)] is not essential for complex maturation, but only for acceleration of the final steps (Xu et al., 1998)]. This is almost exactly equivalent to the Ca\(^{2+}\)-dependence for stimulation of conformational changes in synaptotagmin (Davletov and Sudhof, 1994) and for inducing it to bind acidic phospholipids (Davletov and Sudhof, 1993). Thus, the latter may be critical interactions that, by bringing complexes in the process of maturation into close proximity with the cell membrane (Davis et al., 1999), drive the fusion reaction. Accumulation of partially folded SNARE complex intermediates could be an important mechanism for synaptic plasticity; indeed, enhancement of the content of SNAP-25-syntaxin complexes in synaptosomes caused an increase in the ready releasable pool of neurotransmitter (Lonart and Sudhof, 2000).

BoNT/A inhibits secretion by perturbing binary complexes that may be vital for fast exocytotic response.

In chromaffin cells poisoned with BoNT/A, the composition and state (trypsin sensitivity profile) of the SNARE complexes were perturbed. These findings support the proposal (Xu et al., 1999) that the partially folded complexes are important for the fastest phase of secretion, as BoNT/A slows down the exocytotic burst (Xu et al., 1998). The toxin blocks secretion in response to depolarising stimuli almost completely; this is in accordance with the hypothesis that such responses require a pool of partially folded SNARE complexes to drive secretion rapidly, before the [Ca\(^{2+}\)] signal fades. By contrast, following permeabilisation, a lower but persistent increase of [Ca\(^{2+}\)] does elicit secretion from BoNT/A-poisoned cells. This would suggest that BoNT/A does not prevent the slower formation of complexes: exactly as observed in situ in the cells. Hence, BoNT/A blocks the accelerated phase of exocytosis stimulated by high [Ca\(^{2+}\)], but not slow release mediated by lower [Ca\(^{2+}\)] (Xu et al., 1998). This proposal does not support a prior hypothesis that BoNT/A reduces the Ca\(^{2+}\) sensitivity of exocytosis due to a reduction of the Ca\(^{2+}\)-affinity for the promotion of synaptotagmin binding to SNAP-25 (Gerona et al., 2000; Schiavo et al., 1997). It is possible that the latter interaction could be involved in the most rapid exocytotic burst elicited by high [Ca\(^{2+}\); (Xu et al., 1998). However, the interaction is not likely to mediate the much slower Ca\(^{2+}\)-triggered exocytosis that ensues from permeabilised neuroendocrine cells; the latter response requires less Ca\(^{2+}\) and the EC\(_{50}\) is not (or only modestly, at most) altered by poisoning of the cells with BoNT/A (Gerona et al., 2000; Lawrence et al., 1996). In summary, high-resolution 2D SDS-PAGE has demonstrated that the relationship between Ca\(^{2+}\)-induced SNARE complex formation and triggered exocytosis is more complicated than previously reported. Specifically, large SNAP-25:syntaxin complexes are present before secretion has occurred and more are created during the reaction; ternary complexes and smaller forms are relatively rare. However, this may be due to the rapid turnover of the latter, which can be blocked by trypsin. Our interpretation of the data is that Ca\(^{2+}\) stimulates an early step of syntaxin association with SNAP-25 and that the resultant components go through a number of folded states before fusion is triggered by the binding of synaptobrevin and formation of the SDS and trypsin-resistant ternary complex; it is envisaged that Ca\(^{2+}\) would regulate this series of reactions at several points.

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