Ca^{2+}-induced changes in SNAREs and synaptotagmin I correlate with triggered exocytosis from chromaffin cells: insights gleaned into the signal transduction using trypsin and botulinum toxins

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Summary

Ca^{2+}-triggered catecholamine exocytosis from chromaffin cells involves SNAP-25, synaptobrevin and syntaxin (known as SNAREs). Synaptotagmin I has been implicated as the Ca^{2+}-sensor because it binds Ca^{2+}, and this enhances its binding to syntaxin, SNAP-25 and phospholipids in vitro. However, most of these interactions are only mediated by $[Ca^{2+}]_i$ two orders of magnitude higher than that needed to elicit secretion. Thus, the Ca^{2+} sensitivities of synaptotagmin I and the other SNAREs were quantified in situ. Secretion elicited from permeabilised cells by μM Ca^{2+} was accompanied, with almost identical Ca^{2+} dependencies, by changes in synaptotagmin I, SNAP-25, syntaxin and synaptobrevin that rendered them less susceptible to trypsin. The majority of the trypsin-resistant SNAREs were not associated with SDS-resistant complexes. None of these proteins acquired trypsin resistance in cells rendered incompetent for exocytosis by run-down. Removal of nine C-terminal residues from SNAP-25 by botulinum toxin A reduced both exocytosis and the SNAREs' acquisition of trypsin resistance but did not alter the Ca^{2+} sensitivity, except for synaptotagmin I. Even after synaptobrevin had been cleaved by botulinum toxin B, all the other proteins still responded to Ca^{2+}. These data support a model whereby Ca^{2+} is sensed, probably by synaptotagmin I, and the signal passed to syntaxin and SNAP-25 before they interact with synaptobrevin.

Key words: Secretion, Large dense-core granules, SNAP-25, Synaptobrevin, *Clostridial* neurotoxins

Introduction

Membrane fusion in eukaryotic cells is essential for the transfer of lumenal contents between intracellular compartments and for excoytosis of secretory proteins, hormones and transmitters. Three membrane proteins, synaptobrevin (Sbr), synaptosome-associated protein of M_r 25,000 (SNAP-25) and syntaxin have been identified as key mediators of the exocytosis of synaptic vesicles and large dense-core granules. Each is known to be the substrate for at least one of the seven serotypes (A-G) of botulinum neurotoxin (BoNT) or tetanus toxin (TeTx), bacterial proteases that potently block neurotransmitter release (Dolly et al., 1994; Dolly et al., 2001; Niemann et al., 1994; Schiavo et al., 2000). In vitro, SNAP-25, Sbr and syntaxin spontaneously associate to form an extremely stable 7S ternary complex, which is resistant to dissociation by SDS (at up to ~80°C) and protects the individual proteins against proteolysis by BoNTs, TeTx, trypsin or proteinase K (Chen et al., 1999; Fasshauer et al., 1998a; Hayashi et al., 1994; Poirier et al., 1998). A major feature of the complex is a tight bundle of four α-helices; Sbr and syntaxin contribute one helical domain each and the remaining two are provided by the N- and C-terminal moieties of SNAP-25 (Sutton et al., 1998). The ternary complex forms a scaffold for the binding of an ubiquitous fusion-promoting protein, N-ethylmaleimide-sensitive factor (NSF), via soluble NSF-attachment proteins (SNAPs; three isoforms, α, β and γ) to form an enlarged complex with a sedimentation coefficient of 20S. Hence, Sbr, SNAP-25 and syntaxin have been termed collectively SNAREs (for SNAP receptors) (Söllner et al., 1993). These proteins alone are sufficient to promote the fusion of lipid bilayers in vitro (Weber et al., 1998).

In neurones and neuroendocrine cells, the release of neurotransmitters or hormones is tightly linked to depolarisation-induced Ca^{2+} influx. It has been suggested that Ca^{2+}-triggered SNARE complex formation mediates vesicle fusion in PC-12 cells, but complexes also form during a MgATP-dependent priming step without fusion occurring (Chen et al., 1999). Moreover, Ca^{2+} triggers secretion from semi-intact synaptosomes but has no effect on the amounts of SNAREs co-immunoprecipitated together (Leveque et al., 2000); also, neither formation nor dissociation of SNARE complexes is concomitant with the fusion of sea-urchin egg cortical vesicles (Coorssen et al., 1998; Tahara et al., 1998). Elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) may be communicated to the SNAREs by synaptotagmin I [itself a SNARE (Schiavo et al., 1995)], which changes conformation

upon binding the cation (Davletov and Sudhof, 1994), but the mechanism of such signal transduction remains speculative. In vitro, synaptotagmin I associates with syntaxin in a Ca^{2+}-dependent manner (Li et al., 1995b; Shao et al., 1997) and, also, binds to SNAP-25, but this does not require Ca^{2+}, the interaction being enhanced only weakly (Li et al., 1995b; Schiavo et al., 1997). In contrast, Ca^{2+} triggers dissociation of synaptotagmin I from SNARE complexes in permeabilised nerve endings (Leveque et al., 2000; Mehta et al., 1996). In most cases, the Ca^{2+} sensitivity of the aforementioned reactions is incompatible with the Ca^{2+} dependency of regulated exocytosis from neuroendocrine cells; such a discrepancy has given rise to conjecture that the latter may utilise a different Ca^{2+} sensor from that employed in nerve terminals (Bennett, 1997; Burgoyne and Morgan, 1995; Gerona et al., 2000; Li et al., 1995b). Indeed, a recent study of chromaffin cells from synaptotagmin-I-deficient mice showed that this protein is not essential for Ca^{2+}-triggered exocytosis but is required for an extremely fast phase, known as the exocytotic burst (Voets et al., 2001). Also, following analyses of the ternary SNARE complex structure, determined by X-ray crystallography, it has been suggested that it may bind Ca^{2+} directly (Fasshauer et al., 1998b; Sutton et al., 1998). Furthermore, the C-terminus of SNAP-25 has been implicated in Ca^{2+} sensing because the blockade of neuro-exocytosis induced by BoNT/A can be alleviated by stimulation protocols that increase $[Ca^{2+}]_i$ (Sellin, 1987; Simpson, 1989; Dolly et al., 1994). In view of these conflicting data, further investigations are needed to clarify the Ca^{2+} dependencies of SNAREs and synaptotagmin I in neuroendocrine cells and their relationship to Ca^{2+}-elicited exocytosis.

In the present study, an accepted assay of changes in protein structure - acquisition of resistance to protease digestion (Davletov and Sudhof, 1994) - was exploited to demonstrate alterations in the SNAREs in response to Ca^{2+}. For the first time, it is shown that equivalent Ca^{2+} concentrations trigger both vesicle fusion and alterations in the structure of synaptotagmin I and the other SNAREs. During Ca^{2+}-triggered exocytosis, the proteins became less susceptible to trypsin or proteinase K via a mechanism that is not related to the formation of SDS-resistant SNARE complexes. Removal by BoNTs of part of the cytoplasmic domain of Sbr (which prevents ternary SNARE complex formation) or the nine C-terminal residues of SNAP-25 did not prevent transmission of the Ca^{2+} signal to the SNAREs, although BoNT/A reduced the extent to which changes occurred.

Materials and Methods

Materials

Tissue culture media and reagents were purchased from Life Technologies (Paisley, Scotland), digitonin was from Novabiochem (Nottingham, England) and all other chemicals, including the monoclonal antibody HPC1, were obtained from Sigma Chemical Co. (Poole, England). An antibody raised against a peptide corresponding to the 18 C-terminal residues of SNAP-25 was bought from Autogen-Bioclear (Calne, England). The production of antibodies against the recombinant SNARE proteins, Sbr, SNAP-25 or syntaxin and the purification of BoNT/A has been described previously (Foran et al., 1996; Lawrence et al., 1996). Pure BoNT/B and antibodies to synaptotagmin I were gifts from C. C. Shone.

Chromaffin cell preparation and culture

Bovine adrenal chromaffin cells were prepared and maintained as monolayer cultures as described previously (Foran et al., 1996; Lawrence et al., 1996; Lawrence et al., 1994). The cells were used for experiments between three and 10 days after isolation. When required, they were pre-intoxicated with BoNT/A or B using a protocol that facilitates their uptake (see Lawrence et al., 1996), then returned to the standard culture 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 room temperature (~22°C), cells were rinsed with a HEPES-buffered saline solution (HBS; 145 mM NaCl, 4.8 mM KCl, 1.2 mM NaH_2PO_4, 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 piperazine-N,N'-bis-(2-ethanesulfonic acid), pH 6.5]. Aliquots of $CaCl_2$ were added to the KGEP to produce the desired concentrations of buffered, free Ca^{2+} and, where indicated in the figures, 2 mM ATP and 4 mM $MgCl_2$ were also included. After 15 minutes of stimulation, an aliquot was removed and the amount of catecholamines released from the cells assayed, as described elsewhere (Lawrence et al., 1996). Mean values (±s.d.) were determined from four wells of cells. Control untreated cells were solubilised with 1% Triton X-100 in HBS, and aliquots were assayed to determine the total cell content of catecholamines; release values were expressed as a percenatage of the latter. In an exceptional set of experiments, a 30 minute delay was included, between cell permeabilisation with digitonin and the addition of Ca^{2+}, so that the effect of run-down (detailed later) could be examined. In experiments using either trypsin or proteinase K (see figure legends for details), it 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 proteins

A membrane-enriched fraction was prepared as detailed previously (Foran et al., 1996; Lawrence et al., 1996), dissolved in 50 mM Tris.HCl, pH 5.8 containing 1% SDS plus 10 mM β-mercaptoethanol (without or with boiling; see Figure legends for details) and PAGE performed using the NuPAGE system (Novex, San Diego, USA). Proteins were transferred from the gels onto the PVDF membrane, and western blotting was performed using standard protocols (Lawrence et al., 1996); binding of primary antibodies was detected using horseradish-peroxidase- or alkaline-phosphatase-conjugated secondary antibodies and ECL or colorimetric development. Signals on western blots were quantified using a flatbed scanner linked to a PC running NIH Image software. The data presented in each of the figures are from a single experiment representative of results obtained consistently and on at least three separate occasions.

Results

Ca^{2+} protects synaptotagmin I against trypsinisation and stimulates catecholamine secretion from permeabilised chromaffin cells with the same dose dependence

Exocytosis was elicited from digitonin-permeabilised chromaffin cells, in the absence of MgATP, by the addition of Ca^{2+}; the amount of catecholamine released depended on the $[Ca^{2+}]$. Exocytosis peaked at ~20 μM in a typical experiment (Fig. 1A), although it should be noted that in some experiments more release was elicited by 100 μM Ca^{2+} (see below). Western blotting revealed that equivalent amounts of each of the SNAREs (synaptotagmin I, syntaxin, SNAP-25 and Sbr)

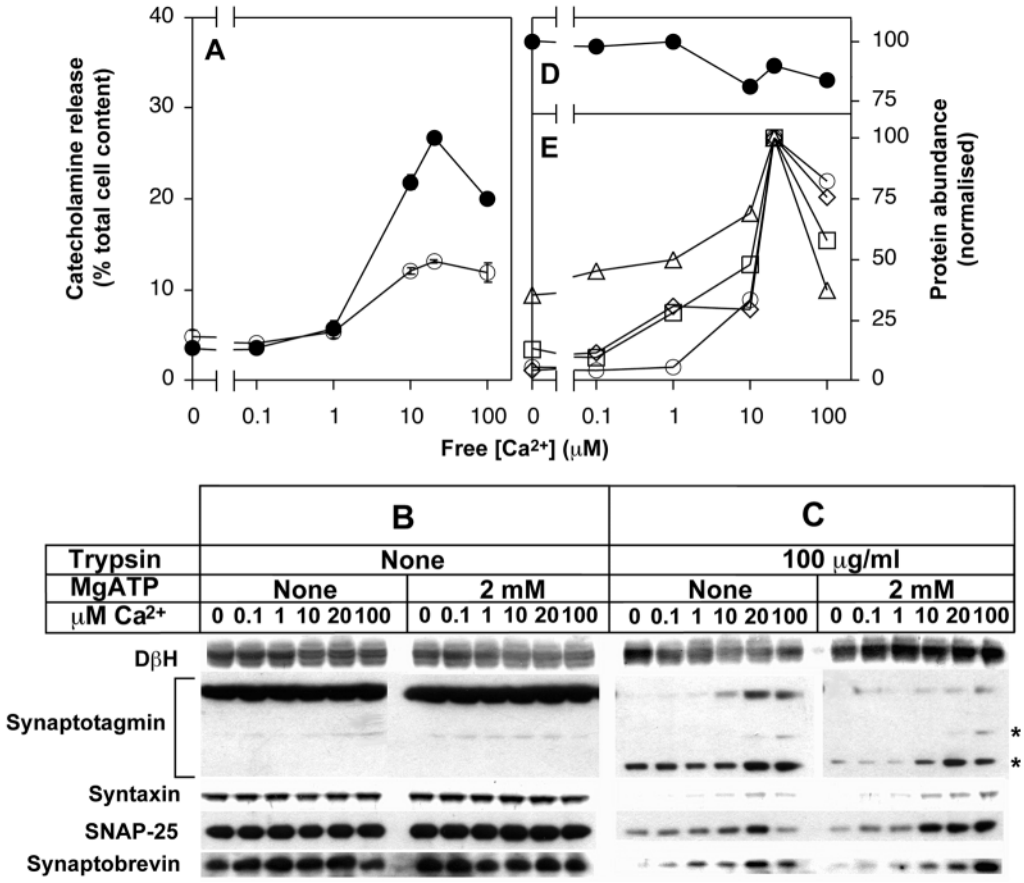

Fig. 1. Ca^{2+} induces changes in the conformation of SNAREs in permeabilised chromaffin cells: acquisition of resistance to trypsin correlates with the extent of exocytosis. Chromaffin cells permeabilised using 20 μM digitonin in KGEP without (○) or with 2 mM MgATP (●) and the indicated buffered, free [Ca^{2+}]. After 15 minutes, aliquots were assayed (±s.d, *n*=4; some error bars are obscured by symbols) for released catecholamine (A). The cells were maintained for a further 30 minutes, 2 mM PMSF was added and a membrane fraction prepared from four wells; after boiling for 2 minutes in 1% SDS, the samples were subjected to SDS-PAGE and western blotting, with antibodies reactive with the specified proteins (B). Only the relevant track positions are shown. (C) Western blots of membranes from cells treated as above, except that 100 μg/ml trypsin was added to the KGEP immediately after the removal of aliquots for the catecholamine assay; thus, the cells were exposed to the protease for 30 minutes. (D,E) The intensity of all the immuno-signals, in cells exposed to trypsin in the absence of MgATP, were computed from digitised images (see Materials and Methods). For each protein, the values were normalised as a percentage of the highest intensity signal, then plotted against [Ca^{2+}]; values for DβH are plotted in (D) while synaptotagmin I (○), syntaxin (□), SNAP-25 (△) and synaptobrevin (◇) are shown in (E). Plotted data are representative of results obtained on at least three separate occasions.

were recovered in the cells' membrane fraction irrespective of exposure to Ca^{2+} (Fig. 1B); the quantity of dopamine-β-hydroxylase (DβH), which was used as a control, also remained constant. As all of the SNAREs have been implicated in membrane fusion, it seems probable that each undergoes conformational changes during this process. Demonstration of altered sensitivity to proteolytic enzymes is widely employed as an indicator of structural alterations in proteins (Fasshauer et al., 1998a; Hayashi et al., 1994; Poirier et al., 1998). Pertinently, acquisition of resistance to trypsinisation has been used to demonstrate a Ca^{2+}-induced change in recombinant synaptotagmin I in vitro and for the native protein in lysed synaptosome membranes (Davletov and Sudhof, 1994). In this study, these findings were confirmed in situ and extended by applying the assay to semi-intact chromaffin cells that retain Ca^{2+}-stimulated exocytosis, as demonstrated above, and advantageously allow correlation of the two sets of measurements. Membranes from cells exposed to increasing [Ca^{2+}] plus 100 μg/ml trypsin were subjected to SDS-PAGE followed by western blotting (Fig. 1C); equivalent amounts were loaded as indicated by the signals obtained for DβH (Fig. 1C,D) which remained largely unaltered because it is protected against proteolysis by its intravesicular localisation. In contrast, the amounts of intact synaptotagmin I detected in the trypsinised membranes were increased by raising the [Ca^{2+}] (Fig. 1C) (but, under the conditions selected, only a fraction remained intact; see below). This increased recovery can be ascribed to a Ca^{2+}-induced protection of synaptotagmin I against trypsinisation, as the cation did not affect the protein content from non-trypsinised cells. Moreover, two proteolytic fragments of synaptotagmin I were observed (marked with asterisks) - their abundance also being raised by elevating the [Ca^{2+}]. Increasing the trypsin concentration resulted in a reduction in the amount of synaptotagmin I recovered; 100 μg/ml trypsin was used throughout as it consistently revealed the protective effect of Ca^{2+} on synaptotagmin I (and the other SNAREs; see below). Thus, the relative, but not absolute, amounts of synaptotagmin I could be related with the level of exocytosis. To determine the Ca^{2+} dependence of this protective effect, the synaptotagmin I signals in cells exposed to incremental amounts of the cation were quantified, normalised as a percentage of the peak value (which was obtained at 20 μM Ca^{2+}) and plotted against [Ca^{2+}] (Fig. 1E). Importantly, this demonstrated that the Ca^{2+} dependence of the apparent structural changes in synaptotagmin I, induced during exocytosis, is very similar to that for catecholamine release (compare Fig. 1A with E). There is a good correlation between the extent of protection of synaptotagmin I and the amount of exocytosis; both are only induced by >1 μM Ca^{2+} and peak at

the same concentration. Thus, synaptotagmin I fulfils one of the key criteria for acceptance as a Ca^{2+} sensor that triggers membrane fusion.

Ca^{2+} also makes the other SNAREs less susceptible to trypsinisation

The same assay of protection against protease digestion was then applied to determine whether Ca^{2+} induces changes in other SNAREs. In the absence of Ca^{2+}, syntaxin, SNAP-25 and Sbr all proved to be susceptible to trypsin; increasing amounts of each protein were retained upon the incremental addition of Ca^{2+}, peaking at 20 μM (Fig. 1C). Note that Ca^{2+} maintains each SNARE protein intact because, with the occasional exception of a minor proteolytic fragment of SNAP-25, no other signals were observed (not shown). The Ca^{2+} dose dependency for acquisition of resistance to trypsin was ascertained for each of the SNAREs (Fig. 1E), as described for synaptotagmin I. Importantly, these proved to be equivalent to that for both the Ca^{2+} sensitivity of synaptotagmin I protection and exocytosis.

MgATP enhances secretion but does not alter SNARE susceptibility to trypsin

Inclusion of 2 mM MgATP increased the amount of hormone released at each Ca^{2+} concentration (Fig. 1A). In the presence of the nucleotide, the level of exocytosis clearly peaked at 20 μM Ca^{2+} and is significantly lower at 100 μM Ca^{2+}. By contrast, 20 and 100 μM Ca^{2+} elicit similar amounts of MgATP-independent secretion. These findings accord with a previous study on the Ca^{2+} dependency of both MgATP-independent and -requiring stages of exocytosis from chromaffin cells (Bittner and Holz, 1992); in the presence of MgATP, secretion peaks at ~20 μM Ca^{2+} because higher concentrations inhibit priming. MgATP failed to alter significantly the Ca^{2+} dependence for SNAP-25, Sbr and syntaxin acquiring resistance to trypsin (Fig. 1C). Despite its enhancement of secretion, the amount of SNAREs surviving tryptic digestion was only marginally augmented by the nucleotide; in fact, the Ca^{2+}-induced protection of synaptotagmin I against proteolysis was attenuated by MgATP. For the other SNAREs, there was a good agreement overall between extent of protection and the level of Ca^{2+}-induced secretion; at 100 μM Ca^{2+} there were some anomalous points, the reason for which is not clear.

Ca^{2+} fails to induce SNARE resistance to trypsin in cells that are rendered incompetent for exocytosis owing to run-down

To gain further evidence that the Ca^{2+}-induced changes in the SNAREs are caused by their involvement in the exocytotic process, the experimental conditions were manipulated such that the cells could be exposed to the cation without catecholamine secretion being elicited. Delaying the addition of Ca^{2+} to permeabilised cells by ≥15 minutes following their permeabilisation abolishes their Ca^{2+}-triggered secretory response (Hay and Martin, 1992; Holz et al., 1989; Lawrence et al., 1994). This phenomenon, termed 'run-down', is caused by the loss of proteins and metabolites (e.g. MgATP) required for exocytotic reactions (priming and fusion), and it can be attenuated if the latter factors are added exogenously (Hay and Martin, 1992; Holz et al., 1989; Sarafian et al., 1987). Thus, susceptibility to trypsin was compared in cells treated with or without 30 minutes of run-down before application of the cation. As expected, Ca^{2+} elicited only a minimal level of exocytosis after run-down, in contrast to the robust secretion seen when it was co-applied with digitonin (Fig. 2A). As noted previously, samples that had exhibited exocytosis showed protection of synaptotagmin I against tryptic proteolysis,

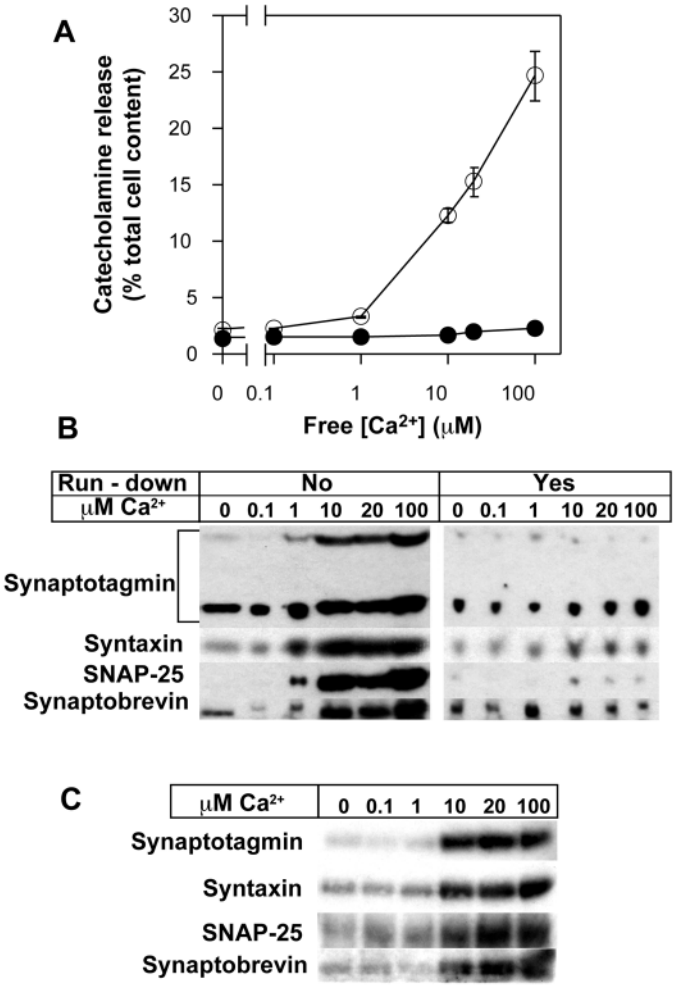

Fig. 2. SNAREs in cells subjected to run-down do not acquire resistance to trypsin. (A) Cells were permeabilised with digitonin in KGEP containing Ca^{2+} at the indicated concentrations (○) or with digitonin in KGEP lacking Ca^{2+} and maintained for 30 minutes before the addition of the cation (●). In both cases, aliquots were removed for catecholamine assay 15 minutes after the application of Ca^{2+} and released catecholamine was calculated (±s.d., n=4; some error bars are obscured by symbols). Trypsin was immediately added (100 μg/ml final concentration) and incubated for 30 minutes before adding 2 mM PMSF, harvesting the cells and isolating a membrane-enriched fraction. The latter samples were boiled for 2 minutes before being subjected to SDS-PAGE and immunoblotting (B), as described in Fig. 1. In (C), cells were permeabilised and exposed for 15 minutes to incremental [Ca^{2+}] before the addition of 10 μg/ml proteinase K. After a further 30 minutes, 2 mM PMSF was added, membranes were prepared and analysed by western blotting, as above.

whereas virtually no full-length synaptotagmin I was detected in the cells that had been subjected to run-down, despite extensive development of the western blot (Fig. 2B). Likewise, the Ca^{2+}-induced acquisition of resistance to trypsin by syntaxin, SNAP-25 and Sbr was severely attenuated when the secretory response was diminished by run-down (Fig. 2B). Thus, it appears that the trypsin resistance of each of the SNAREs can be induced only in cells that are competent for regulated exocytosis (but see below).

Ca^{2+} induces SNARE resistance to proteinase K

The induction by Ca^{2+} of protease-resistant SNAREs was confirmed using proteinase K, which targets distinct peptide bonds from those broken by trypsin. The latter cleaves between the carboxylic side of the basic amino acids lysine and arginine and any residue, whereas proteinase K cuts bonds on the carboxylic side of aliphatic, aromatic or hydrophobic amino acids linked with any other residue. In general, proteinase K cleaves each SNARE slightly closer to their coiled-coil domains than trypsin when pre-formed complexes are exposed to either protease in vitro, thereby, producing a slightly smaller 'minimal core SNARE complex' (Fasshauer et al., 1998a). In permeabilised chromaffin cells, increasing [Ca^{2+}] induced incremental levels of resistance to proteinase K in all of the four SNAREs (Fig. 2C). As noted for trypsin, major signals were observed for full-length SNAREs, with truncated forms being less abundant, presumably because of rapid proteolysis or severance from the membrane.

BoNT/A reduces the amounts of Ca^{2+}-induced SNAP-25, Sbr and syntaxin resistant to trypsin, but their concentration dependence remains unaltered

BoNTs cause neuromuscular paralysis via the blockade of acetylcholine release from motor nerve terminals (Dolly et al., 2001). BoNT/A differs from the other BoNTs in that its effect can be overcome transiently by high-frequency nerve stimulation, the addition of agents that facilitate Ca^{2+} entry into the presynaptic neurone (e.g. 4-aminopyridines) or by increasing the extracellular Ca^{2+} concentration (Simpson, 1989). Likewise, blockade by BoNT/A of evoked transmitter release from brain isolated nerve terminals can be reversed by Ca^{2+}-specific ionophores that render the presynaptic membrane permeable to the cation (Dolly et al., 1994). Thus, it has been proposed that BoNT/A inhibits evoked exocytosis by lowering the Ca^{2+} sensitivity of the membrane fusion apparatus (Sellin, 1987). Notably, the removal of nine amino acids from the C-terminus of SNAP-25 does not stop this protein participating in SNARE complexes in vitro or in vivo, although their stability (i.e. resistance to SDS denaturation) is reduced (Hayashi et al., 1995; Pellegrini et al., 1995; Lawrence and Dolly, 2002). If formation of these complexes drives membrane fusion, and BoNT/A does not block this, it might be that the toxin inhibits exocytosis by preventing transmission of the Ca^{2+} signal to the SNAREs. To test this hypothesis, chromaffin cells were exposed to BoNT/A using a protocol that results in internalisation of the toxin, with consequent cleavage of virtually all the cells' complement of SNAP-25 and near-complete blockade of catecholamine exocytosis in response to depolarising stimuli such as nicotine, 2 mM Ba^{2+} or 55 mM K^+ (Lawrence et al., 1996). Nevertheless, when these cells are permeabilised, a fraction of the secretory response can be elicited by the addition of Ca^{2+} (Lawrence et al., 1996). To ascertain whether the Ca^{2+} sensitivity of the exocytotic trigger had been altered by BoNT/A, pre-poisoned cells were permeabilised and exposed to the cation at various concentrations, as described previously for toxin-free cells (see Fig. 1). Notably, the BoNT/A-poisoned cells secreted much less catecholamine than the toxin-free controls (Fig. 3A), but the Ca^{2+}-concentration dependence for the residual secretion remained unaltered. Membranes were prepared from these BoNT/A-poisoned and control cells and boiled before being subjected to analysis for each SNARE by western blotting (Fig. 3B,C). BoNT/A cleaved SNAP-25, revealed by the exclusive disappearance of the signal for IgG reactive with its C-terminal residues (Fig. 3B), with no change in the other SNAREs as expected from the toxin's absolute specificity. Next, the influence of toxin treatment on the susceptibilities of the SNAREs to trypsin was investigated. The levels protected against trypsinisation were attenuated for SNAP-25, syntaxin and Sbr, but not synaptotagmin, following treatment with BoNT/A (Fig. 3C; note that equivalent amounts of protein from BoNT/A-treated and control cells were used and the samples analysed together by identical procedures). This experiment was repeated several times with no consistent BoNT/A-induced change in the amounts of trypsin-resistant synaptotagmin being observed. Notably, despite a reduction in the amount of SNAREs (except synaptotagmin) being protected, the [Ca^{2+}] dependencies of their protection were unchanged (Fig. 3D-G); the reasons for the somewhat anomalous values observed at 100 μM Ca^{2+} are unclear.

Ca^{2+} protects SNARE monomers against trypsinisation

A plausible explanation for the SNAREs' acquisition of resistance to trypsin is that during the triggering of exocytosis by the cation, monomeric SNAREs associate to form a heterotrimeric complex. Several in vitro experimental findings lend support to this hypothesis. (i) Recombinant syntaxin, SNAP-25 and Sbr spontaneously form a complex when mixed (Hayashi et al., 1994); (ii) the SNARE complex protects its individual components against cleavage by trypsin or BoNTs (Fasshauer et al., 1998a; Hayashi et al., 1994; Poirier et al., 1998); (iii) homotypic yeast vacuole fusion only proceeds between vesicles proffering 'monomeric' (i.e. can be solubilised by SDS without boiling) SNAREs rather than cis complexes of SNAREs (Ungermann et al., 1998); and (iv) in BoNT/E-poisoned PC-12 cells, a SNAP-25 C-terminal peptide that restored Ca^{2+}-triggered exocytosis was incorporated into a SNARE complex in the cell membrane (Chen et al., 1999).

To determine whether SDS-resistant complex formation underlies the acquisition by SNAREs of resistance to trypsin, cells were exposed to various concentrations of Ca^{2+}; some samples were subsequently treated with 100 μg/ml trypsin where indicated, then membranes were prepared, solubilised in SDS sample buffer and divided into two. One half of each sample was boiled, the other not; both were subjected to SDS-PAGE followed by western blotting for the SNAREs. Notably, boiling of non-trypsin-treated samples clearly increased the intensity of signals for each of the SNAREs at the expected position for their respective monomers (Fig. 4A). Thus, it

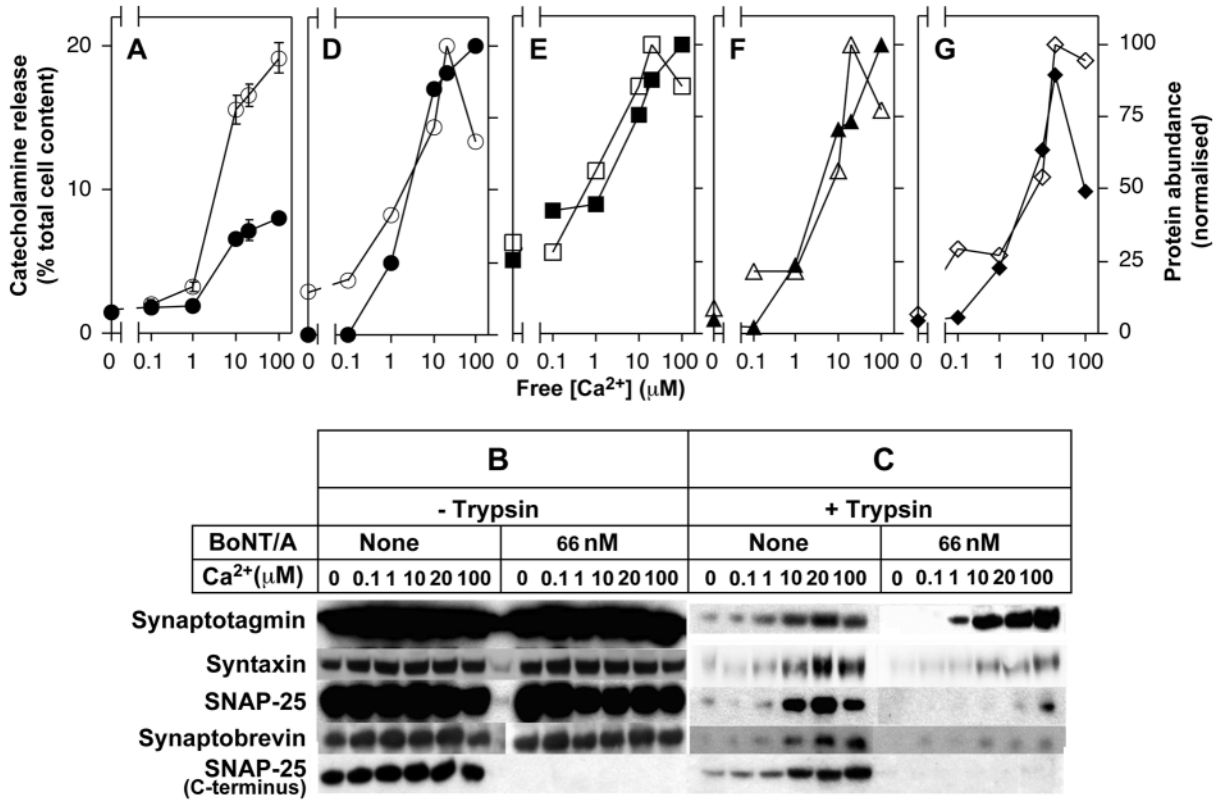

Fig. 3. The Ca^{2+} dependencies of exoytosis and for the acquisition by SNAREs of trypsin resistance are unaffected following SNAP-25 truncation by BoNT/A. Chromaffin cells, in the absence (○) or presence (●) of 66 nM BoNT/A (see Materials and Methods), were permeabilised and exposed for 15 minutes to a range of free [Ca^{2+}] (MgATP was not included), then catecholamine release was assayed as described in Fig. 1; the results are plotted in (A). Cells were incubated without (B) or with (C) trypsin (100 μg/ml final concentration) for a further 30 minutes, and 2 mM PMSF added before a membrane fraction was prepared. The latter samples were boiled for 10 minutes, subjected to SDS-PAGE and analysed by western blotting under identical conditions. The amount of SNAREs remaining in the trypsin-treated cells was quantified (see Materials and Methods) using photographic exposures optimised to show the Ca^{2+} dependence of trypsin resistance (i.e. using photographs in which the signals from BoNT/A-treated cells had been developed for longer than shown in C). The signal intensities were quantified as in Fig. 1 for control (open symbols) and BoNT/A-poisoned cells (closed symbols) and plotted for synaptotagmin I (D), syntaxin (E), SNAP-25 (F) and synaptobrevin (G). Plotted data are representative of results obtained on at least three separate occasions.

appears that a sizeable fraction of each protein is resistant to solubilisation by SDS at ambient temperatures. Nevertheless, the majority of the trypsin-resistant SNAREs in cells exposed to the protease were not in complexes that are stable in 1% SDS sample buffer containing β-mercaptoethanol, because boiling of the samples resulted in only a slight increase in band intensity for each of them (Fig. 4B). These data imply that the Ca^{2+}-induced protection against trypsin observed for the SNAREs is not, or only partly, caused by their increased incorporation into SDS-resistant complexes. In contrast to control samples, after BoNT/A intoxication, minimal levels of trypsin-resistant SNAREs were observed in non-boiled samples (Fig. 4B), but some was recovered after boiling, suggesting that only SNAREs in complexes were protected under these conditions. This would explain why much lower amounts of the SNAREs are protected in the BoNT/A-poisoned cells.

Synaptotagmin I, SNAP-25 and syntaxin respond to Ca^{2+} in the absence of Sbr

BoNT/B specifically proteolyses Sbr, removing a large portion of its cytoplasmic domain from the membrane-associated moiety. As with BoNT/A, chromaffin cells can be extensively poisoned with BoNT/B such that >90% of the cells' Sbr is degraded (Fig. 5A) and evoked transmitter release almost abolished (Foran et al., 1995; Lawrence et al., 1996). In contrast to BoNT/A, little recovery of Ca^{2+}-triggered exocytosis was obtained after permeabilisation of chromaffin cells intoxicated with BoNT/B (Fig. 5B), as expected (Lawrence et al., 1996). In the BoNT/B-treated chromaffin cells lacking most of the intact Sbr, it might seem reasonable to postulate that ternary SNARE complexes could not form and, therefore, SNAP-25 and syntaxin should not be protected against trypsinisation. However, western analysis of the membranes from cells pre-incubated with BoNT/B that had been permeabilised and exposed to trypsin in the presence of incremental [Ca^{2+}] revealed that, as well as synaptotagmin, SNAP-25 and syntaxin remained responsive to the cation and still became resistant to trypsin (Fig. 5C). In general, the Ca^{2+} sensitivity and amounts of synaptotagmin, syntaxin and SNAP-25 protected against trypsin were similar for toxin-free and BoNT/B-poisoned cells (Fig. 5C-F), although at lower [Ca^{2+}] slightly more trypsin-resistant SNAREs were observed in

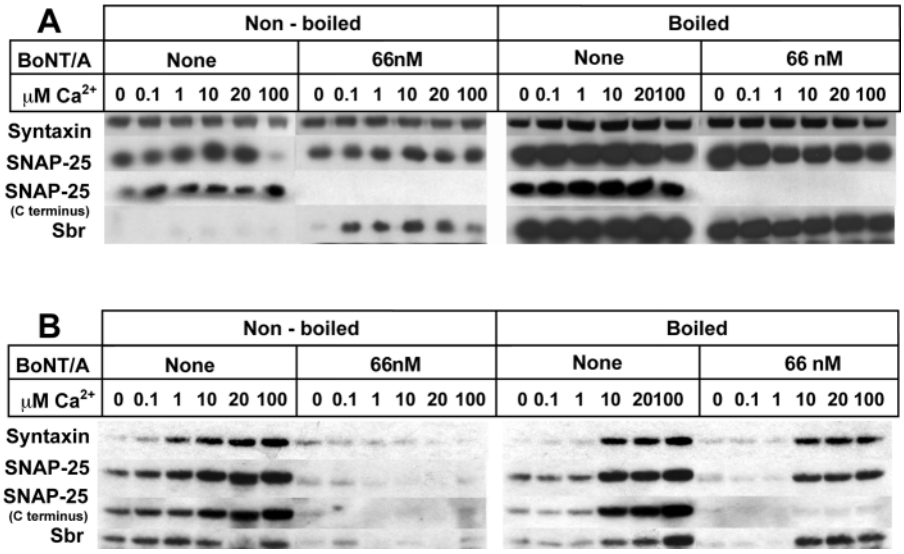

Fig. 4. The majority of the trypsin-resistant SNAREs are not in SDS-resistant complexes. Chromaffin cells were permeabilised and exposed for 15 minutes to various [Ca^{2+}], then maintained for a further 30 minutes in the absence (A) or presence (B) of 100 μg/ml trypsin, before being harvested in the presence of 2 mM PMSF and their membranes isolated. The samples were solubilised in SDS-PAGE sample buffer and split into two; one of each was boiled for 2 minutes. Boiled and non-boiled samples were subjected to SDS-PAGE and western blotting.

BoNT-poisoned cells than in control cells. Of course, only a faint immuno-signal for trypsin-resistant Sbr was observed in the BoNT/B-poisoned cells (Fig. 5C, single asterisk), but extended photographic exposure (double asterisk) showed that Ca^{2+} protected this residual level against proteolysis.

Discussion

The Ca^{2+} dependence of synaptotagmin 1 in situ is consistent with it being involved in Ca^{2+}-elicited exocytosis in chromaffin cells

Ca^{2+} stimulates a large amount of catecholamine exocytosis from chromaffin cells in a short period; thus, they provide an ideal model for the study of what happens to the SNAREs during membrane fusion, and its regulation by Ca^{2+}. The results presented here clearly show that synaptotagmin I and the other three SNAREs respond to relatively low [Ca^{2+}], in fact, with a dependence identical to that for evoked catecholamine release. Synaptotagmin I, which contains two Ca^{2+}-binding domains, C2A and C2B (Bennett, 1997; Burgoyne and Morgan, 1995; Li et al., 1995b), is a putative Ca^{2+} sensor. Following observations that exocytosis from chromaffin cells can be elicited by lower [Ca^{2+}] than required for neurotransmitter release from certain neurons, it was suggested that granule exocytosis may involve a synaptotagmin isoform with higher Ca^{2+} affinity than synaptotagmin I (Bennett, 1997; Burgoyne and Morgan, 1995; Li et al., 1995b). However, this study implicates synaptotagmin I in granule exocytosis because the affinity-purified antibody used was raised against its 20 C-terminal residues, a region that is poorly conserved in the other isoforms (Li et al., 1995b). Synaptotagmin I binds to acidic phospholipids with an EC_{50}

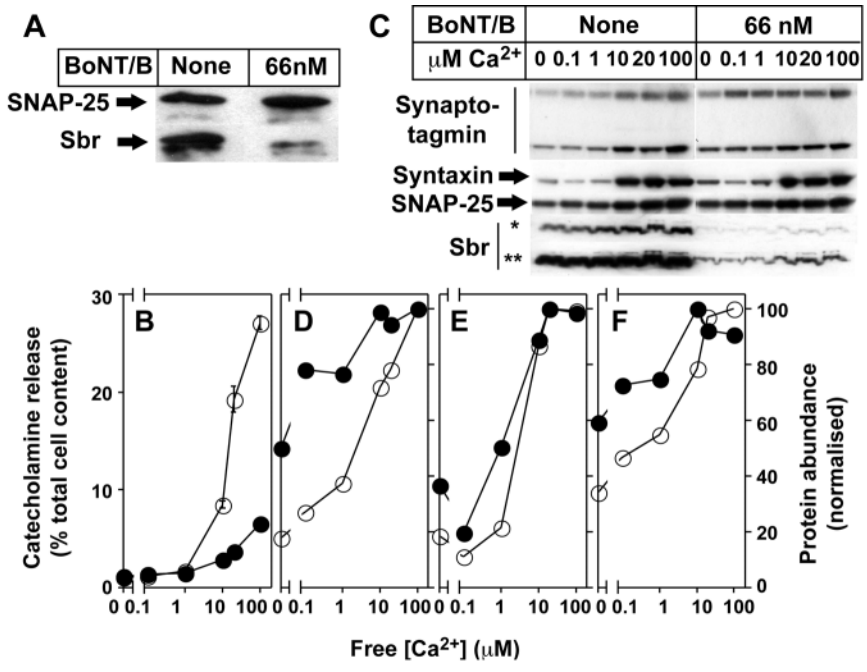

Fig. 5. Sbr is not required for Ca^{2+} to trigger changes in synaptotagmin I, SNAP-25 and syntaxin. (A) Membranes, prepared from intact control and BoNT/B-treated chromaffin cells, were subjected to SDS-PAGE followed by western blotting. (B-F) Toxin-free (○) and BoNT/B-treated (●) chromaffin cells were permeabilised by exposure to digitonin in KGEP, with the inclusion of incremental free [Ca^{2+}]. Following a 15 minute interval, aliquots were removed and assayed (±s.d.; n=4) for catecholamine (B); then 100 μg/ml trypsin was added and 30 minutes later 2 mM PMSF was included and a membrane fraction prepared. The samples were boiled for 2 minutes prior to SDS-PAGE and western blotting (C); the amounts of synaptotagmin (D), syntaxin (E) and SNAP-25 (F) present in each were quantified and normalised as a percentage of the largest value in each [Ca^{2+}] series. Plotted data are representative of results obtained on at least three separate occasions.

for Ca^{2+}=6 μM (Li et al., 1995a), very close to the value observed herein for half-maximal stimulation of exocytosis from permeabilised cells [also, see (Burgoyne and Morgan, 1995)] and for its acquisition of resistance to tryptic digestion in situ (shown herein) and in vitro (Davletov and Sudhof, 1994). Moreover, though not abolished, exocytosis is perturbed in chromaffin cells of synaptotagmin-I-null mutant mice (Voets et al., 2001). Thus, synaptotagmin 1 is not the only Ca^{2+} sensor for exocytosis in these cells but cannot be disqualified from a role in the slow, sustained release measured herein, because the patch-clamp capacitance assays in the latter study monitored only the first few seconds of exocytotic activity; this often highlights deficiencies in the initial fast phase that are not reflected in a significant reduction in the amount of hormone released during a more prolonged burst of secretion. For example, direct quantification of catecholamines showed that SNAP-25 mutants substituted at Q174 support exocytosis from neuroendocrine cells as effectively as wild-type (Chen et al., 1999; Gil et al., 2002), even though capacitance measurements had suggested a severe deficiency in membrane fusion rates in cells expressing this mutant (Wei et al., 2000).

The SNARE complex itself has been proposed as a candidate Ca^{2+} sensor (Fasshauer et al., 1998b; Sutton et al., 1998), but Ca^{2+} binding at physiologically relevant concentrations remains to be shown. Furthermore, the structure solved for the cis SNARE complex is likely to be an end product of fusion (Chen et al., 1999) and, therefore, could not sense Ca^{2+} before fusion. Also, the bulk of the catecholamine released from permeabilised cells exposed to >10 μM Ca^{2+} derives from undocked granules distal to the plasmalemma, which have to be mobilised to reach the cell membrane before fusing; these granules are enriched for synaptotagmin and Sbr but not SNAP-25 nor syntaxin, thus, SNARE complexes are unlikely to be involved in their Ca^{2+}-triggered mobilisation.

SDS-resistant complex formation is not the major means by which SNAREs acquire resistance to trypsin

In non-toxin-treated cells, the majority of the SNAREs protected by Ca^{2+} against trypsin were solubilised by SDS as monomers without boiling, indicating that they were not in SDS-resistant complexes. Although the increased trypsin resistance could be induced within each protein individually, their identical Ca^{2+} sensitivity suggests that they acquire this property simultaneously. The data do not preclude a role for SDS-resistant SNARE complex formation during exocytosis, as proposed by Chen et al. (Chen et al., 1999), but shows that Ca^{2+} induces other persistent changes in the SNAREs. Unfortunately, it is impractical to use milder detergents to preserve and assay weak complexes formed in situ because they do not prevent the generation of artefactual SNARE interactions during sample preparation for PAGE (Otto et al., 1997).

Priming is essential for synaptotagmin and the other SNAREs to remain responsive to Ca^{2+}

In cells that were rendered incompetent for exocytosis owing to 'run-down', synaptotagmin and the other SNAREs did not acquire increased resistance to trypsin in the presence of Ca^{2+} (Fig. 2). The diminution in secretory activity is caused by loss from the cells of proteins and metabolites (e.g. MgATP) that are essential for priming reactions that precede Ca^{2+}-elicited fusion (Hay and Martin, 1992; Holz et al., 1989; Sarafian et al., 1987). Thus, it appears that the role of priming is to maintain synaptotagmin and the other SNAREs in a Ca^{2+}-responsive state, whereas fusion entails Ca^{2+} induction of conformational changes in these 'primed' SNAREs. Importantly, these data clearly indicate that acquisition of trypsin resistance is gained via synaptotagmin and SNARE function and is not some artefact of the assay.

Distinct effects of *Clostridial* toxins provide clues to the mechanism of Ca^{2+} signal transduction to the SNAREs

As noted above, in BoNT/B-poisoned cells, the Ca^{2+} signal can still be communicated to SNAP-25 and syntaxin despite their lack of Sbr; this supports the hypothesis that SDS-resistant ternary complex formation is not the only way in which the SNAREs can become less susceptible to trypsin. Binary SNAP-25–syntaxin complexes may be induced by Ca^{2+}, either to form or to adopt a novel 'activated' conformation (Lawrence and Dolly, 2002) before association with Sbr. The data does not rule out Ca^{2+} signal transmission to SNAP-25–syntaxin already associated with Sbr in toxin-free cells, but mitigates against hypotheses that propose an Sbr–SNAP-25 interaction preceding the binding of syntaxin (Chen et al., 2001). In cells exposed to BoNT/A, SNAP-25 is C-terminally truncated, and the amounts of SNAP-25, syntaxin and Sbr that resist trypsin degradation are severely reduced; by contrast, the abundance of trypsin-resistant synaptotagmin I was not lowered. Thus, the toxin does not perturb Ca^{2+} sensing by the latter, but attenuates transmission of the Ca^{2+} signal to the other SNAREs. The data dispel the popular hypothesis that BoNT/A simply lowers the Ca^{2+} affinity of the exocytotic apparatus [(Gerona et al., 2000; Sellin, 1987; Simpson, 1989) and see above]. The toxin reduces the amount of each SNARE being protected against trypsin, but the Ca^{2+} sensitivity for this and (most importantly) exocytosis is not shifted. Moreover, the above-noted hypothesis cannot explain the lack of response from BoNT/A-treated intact chromaffin cells to depolarising stimuli such as elevated $[K^+]$ or nicotine (Lawrence et al., 1996), because these treatments raise the free $[Ca^{2+}]$ at sub-plasmalemmal exocytotic release sites to >100 μM (Burgoyne, 1991). Rather, the severe inhibition of responses to the latter could be a consequence of the transient Ca^{2+} signal they induce [<1 minute (Burgoyne, 1991)]; in this context, it is noteworthy that the stoichiometry of SNAP-25–syntaxin in complexes, which may be vital for the fast exocytotic response, is altered by BoNT/A (Lawrence and Dolly, 2002). In permeabilised cells, the Ca^{2+} stimulus is maintained for an extended period (15 minutes), and this may be why exocytosis can still proceed, albeit at an attenuated rate, after inhibition with BoNT/A; the toxin selectively inhibits fast phases of exocytosis more strongly than slow responses (Xu et al., 1998).

Finally, a sequence for putative reactions that occur in response to Ca^{2+} can be proposed from the differing effects of BoNT/A and B on acquisition of resistance to trypsin. Firstly, synaptotagmin may be the mediator for recruitment of undocked granules to release sites. Upon docking, the C-terminus of SNAP-25 is implicated in Ca^{2+} sensing by Sbr and syntaxin, as BoNT/A reduces the amounts of all three proteins,

but not synaptotagmin, that respond to the signal. In contrast, Sbr seems not to be essential for synaptotagmin I, SNAP-25 and syntaxin to respond to Ca^{2+}. Synaptotagmin I is the only one of the four proteins that responds to the elevated [Ca^{2+}] irrespective of the participation of the other three SNAREs; all the latter require the C-terminus of SNAP-25, but not Sbr, for optimal sensitivity. Thus, synaptotagmin 1 responds to elevated $[Ca^{2+}]_i$ and, with the possible aid of additional Ca^{2+} sensors, transfers the signal to SNAP-25/syntaxin before the involvement of Sbr.

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