Botulinum Neurotoxin C1 Cleaves both Syntaxin and SNAP-25 in Intact and Permeabilized Chromaffin Cells: Correlation with Its Blockade of Catecholamine Release†

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ABSTRACT: The seven types (A−G) of botulinum neurotoxin (BoNT) are Zn^{2+}-dependent endoproteases that potently block neurosecretion. Syntaxin is presently thought to be the sole substrate for BoNT/C1, and synaptosomal-associated protein of $M_r = 25\,000$ (SNAP-25) is selectively proteolyzed by types A and E. In this study, the effects of C1 on Ca^{2+}-regulated exocytosis of dense core granules from adrenochromaffin cells were examined together with its underlying molecular action. Intact chromaffin cells were exposed to the toxin, and catecholamine release therefrom was then measured in conjunction with the monitoring of syntaxin cleavage by Western blotting. A good correlation was obtained between degradation of syntaxin 1A/B and reduction in Ca^{2+}- or Ba^{2+}-dependent secretion. However, blotting with antibodies against a C-terminal peptide of SNAP-25 revealed the additional disappearance of immunoreactivity, with the same toxin concentration dependency as syntaxin breakdown. Notably, the cleaved SNAP-25 product was similar in size to that produced by BoNT/A; however, contamination of BoNT/C1 by serotypes A or E was eliminated. Therefore, it is concluded that syntaxin 1A/B and SNAP-25 are cleaved in intact cells poisoned with only C1. Notably, C1 treatment of chromaffin cells abolished Ca^{2+}-evoked secretion following digitonin permeabilization, compared with partial inhibition by BoNT/A, suggesting the importance of syntaxin for catecholamine release. Unexpectedly, C1 failed to proteolyze a soluble recombinant SNAP-25, even though it served as an efficient substrate for BoNT/A. These interesting observations suggest that C1 can only efficiently cleave SNAP-25 in intact cells, possibly due to the existence therein of a unique conformation and/or the participation of accessory factors.

Seven immunologically distinct serotypes of botulinum neurotoxin (BoNT; A−G),[1] produced by *Clostridium botulinum*, and tetanus toxin (TeTx) from *C. tetani*, are potent inhibitors of neuroexocytosis. All are synthesized as a 150 kDa polypeptide and are subsequently activated, through a limited proteolytic event, to yield a light chain (LC) of 50 kDa and a heavy chain (HC) of 100 kDa which are disulfide-linked [reviewed by DasGupta (1989)]. Each BoNT serotype paralyzes peripheral cholinergic nerve endings via a triphasic mechanism initiated by binding of the HC to distinct neuronal ecto-acceptors, with subsequent internalization and translocation into the cytosol where their LCs act (Simpson, 1986; Dolly *et al.*, 1994). Although the presence of ecto-acceptors underlies this cholinergic specificity (Dolly *et al.*, 1984), exocytosis of all fast-acting transmitters from synaptic small clear vesicles (SSCVs) is blocked by these toxins (Ashton *et al.*, 1993) when administered intracellularly [reviewed in Dolly (1992, 1994)]. The LC of each BoNT acts as a Zn^{2+}-dependent endoprotease with high selectivity for one of three proteins [reviewed in Montecucco and Schiavo (1993)] proposed to constitute the core components of a SSCV docking/fusion complex (Söllner *et al.*, 1993). These are SNAP-25 (BoNT/A and -/E; Blasi *et al.*, 1993a; Schiavo *et al.*, 1993c), syntaxin (BoNT C1; Blasi *et al.*, 1993b), and synaptobrevin (Sbr, BoNT/B, -/D, -/F, -/G and TeTx; Schiavo *et al.*, 1992, 1993a,b; Yamasaki *et al.*, 1994; Link *et al.*, 1992). Notably, no evidence has been presented that any BoNT serotype cleaves more than one structurally-distinct target.

Both SNAP-25 and Sbr also seem to be essential for exocytosis of large dense-core vesicles from chromaffin cells because of the good correlations observed for the concentration dependencies for their cleavage by BoNT/A and -/B, respectively, with the levels of inhibition of catecholamine release (Lawrence *et al.*, 1996; Foran *et al.*, 1995). Accordingly, all the toxins' targets have been found in chromaffin cells in locations compatible with roles in regulated secretion [SNAP-25, Roth & Burgoyne, 1994; Sbr on chromaffin granules (CGs), Foran *et al.*, 1995; syntaxin, Hodel *et al.*, 1994]. However, neither the inhibitory action of BoNT/C1 on secretion from intact neuroendocrine cells has been examined nor have the protein(s) affected been investigated.

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[1] Abbreviations: BoNT/C1, botulinum neurotoxin type C1; LC, light chain; HC, heavy chain; SNAP-25, synaptosomal-associated protein of $M_r = 25\,000$; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; Sbr, synaptobrevin; Cbr, cellubrevin; CG, chromaffin granule; DTT, dithiothreitol; GAP-43, growth-associated protein of $M_r = 43\,000$; GST, glutathione *S*-transferase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KGEP, 139 mM potassium glutamate, 20 mM Pipes, 5 mM EGTA, and 0.5% (w/v) bovine serum albumin, pH 6.5; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Ig, immunoglobulin; MgATP, complex of Mg^{2+} and adenosine triphosphate; SSCVs, synaptic small clear vesicles; SDS, sodium dodecyl sulfate; TeTx, tetanus toxin.

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In this study, exposure of intact chromaffin cells to BoNT/C1 was found to inhibit Ca^{2+}- evoked catecholamine secretion and to result in cleavage of SNAP-25, as well as its previously identified substrate, syntaxin.

EXPERIMENTAL PROCEDURES

Materials. High-purity digitonin was purchased from Novabiochem (U.K.), tissue culture reagents were from GIBCO BRL (Paisley, Scotland, U.K.), and Immobilon-P (PVDF) membrane was from Millipore (Bedford, MA). Anti-rabbit or anti-mouse IgGs conjugated to alkaline phosphatase were purchased from Bio-Rad (Hampstead, U.K.). Lubrol PX (a nonionic detergent, Thesit) was from Boehringer Mannheim (East Sussex, U.K.). Bovine serum albumin (BSA) fraction V and monoclonal antibodies to growth-associated protein of 43 kDa (GAP-43, also known as neuromodulin) or to syntaxin isoforms 1A/B (or HPC-1) plus all other reagents were obtained from Sigma Chemical Co. Ltd. (Dorset, U.K.). A rabbit antiserum was raised against recombinant syntaxin 1A (see later) and affinity-purified on immobilized antigen. Antibodies reactive with both Sbr and Cbr were generated in rabbits against a synthetic polypeptide comprising residues 33−94 of human Sbr [a region of amino acid sequence shared by cellubrevin (Cbr); McMahon *et al*., 1993], and affinity-purified on the immobilized peptide (Foran *et al.*, 1995). Likewise, antiserum to the C-terminus of SNAP-25 was raised, using a synthetic peptide encompassing residues 195−206 covalently-linked to a carrier protein, as previously employed by Oyler *et al.* (1989), and affinity-purified on immobilized antigen prior to use. A monoclonal antibody reactive with SNAP-25 (clone SMI 81) was purchased from Affinity Research Products Ltd. (Nottingham, U.K.). Anti-synaptotagmin antibodies were raised in guinea pigs against a peptide encompassing the last 20 C-terminal residues of rat isoform 1 coupled to a carrier protein, followed by affinity purification of the immunoglobulin (Ig). The preparation of polyclonal antiserum exclusively reactive to the LC of BoNT/A is outlined in Cenci Di Bello *et al.* (1994). BoNT/A and BoNT/E were purified by modifications of the process described for BoNT/F (Wadsworth *et al.*, 1990), as specified in Shone *et al.* (1993). BoNT/E was fully nicked as described by Evans *et al.* (1986). BoNT/C1 was isolated as detailed in Schiavo *et al.* (1995) but excluding the additional metal-chelate chromatographic step. Toxins were found to be of high purity by SDS−PAGE with Coomassie staining of protein; also, all exhibited the high levels of toxicity (assessed by mouse bioassay) previously reported.

Culture of Adreno-Chromaffin Cells and Intoxication with BoNT/C1, -/A, or -/E. Bovine chromaffin cells were prepared from adrenal glands and maintained in 24-well tissue culture plates, as described previously (Lawrence *et al.*, 1994). Within 2−3 days after preparation, cells were rinsed rapidly with buffer A [5 mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM Hepes, pH 7.4, 5.6 mM glucose, 220 mM sucrose, and 0.5% (w/v) BSA] before incubation at 37 °C for 24−26 h in the same buffer in the absence or presence of BoNT/C1, -/A, or -/E at concentrations specified in the appropriate figure legends. After rinsing once with DMEM [supplemented with growth factors and antimicrobial agents as described in Lawrence *et al.* (1994)], the cells were reequilibrated with a fresh aliquot of the same medium for 24 h at 37 °C before experiments were performed.

Stimulation and Quantification of Catecholamine Secretion from Intact Chromaffin Cells. Immediately before measuring release, cells (pretreated with BoNT/C1or -/A, as outlined above) were briefly washed once with buffer B (145 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Hepes, pH 7.4) and then exposed at 22 °C for 15 min in quadruplicate to various buffers (detailed in legends to the appropriate figures). These were buffer B alone or containing either 2 mM Ba^{2+} (Ba^{2+} buffer), 2 mM $CaCl_2$ (5 mM K^+), or 55 mM K^+ buffer, which is equivalent to the 5 mM K^+ buffer except that the KCl concentration was increased by 50 mM and the NaCl concentration was reduced by the same amount (to 95 mM), thereby maintaining the ionic strength. After 15 min, aliquots of the medium bathing the cells were removed and assayed for catecholamine content by a fluorometric procedure (von Euler & Floding, 1959). The catecholamine contents of the 5 mM K^+ buffer samples were subtracted from that released into the 55 mM K^+ to calculate K^+ depolarization-evoked secretion. Likewise, the quantities of catecholamine recorded in buffer B were subtracted from those in 2 mM Ba^{2+} buffer to give Ba^{2+}-evoked release.

Permeabilization of Cells with Digitonin and Triggering of Exocytosis by Ca^{2+} or Ba^{2+}. BoNT/C1- or -/A-pretreated cells were permeabilized with 20 μM digitonin in KGEP buffer [139 mM potassium glutamate, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 6.5, 5 mM EGTA, 100 μM D600 (methoxyverapamil), and 0.5% (w/v) BSA] containing 2 mM free Mg^{2+} and 2 mM ATP, in the presence or absence of 20 μM free Ca^{2+} or 1 mM free Ba^{2+}. D600, a Ca^{2+}-channel blocker, was used to prevent Ba^{2+}-evoked secretion from any cells that might remain nonpermeabilized (TerBush & Holz, 1993). After 15 min, aliquots were removed and assayed for catecholamine content. Basal release into Ca^{2+}/Ba^{2+}-free KGEP/digitonin was subtracted from those for Ba^{2+}- or Ca^{2+}-containing buffers to give the levels of Ba^{2+}- or Ca^{2+}-dependent secretion. In another experimental protocol, toxin-free cells were exposed to 20 μM digitonin in 2 mM MgATP-containing KGEP in the absence or presence of 100 nM BoNT/C1 [which had been prereduced (at 2 μM) with 20 mM dithiothreitol (DTT) before dilution into the latter buffer]. After 15 min, the solutions were replaced with 2 mM MgATP-containing KGEP with or without 20 μM Ca^{2+}. Following a further 15 min, an aliquot of the media was removed from each well and assayed for catecholamine, as described above. Ca^{2+}-evoked secretion was calculated as above.

At the end of every experiment, the buffer remaining in some wells, treated under each condition, was removed, the cells were solubilized in 1% (v/v) Triton X-100 (in buffer B), and an aliquot was assayed for catecholamine content. These values, representing catecholamine retained within cells, were added to the respective amounts released from the same cells to calculate representative figures for total cell catecholamine (variation <10%). In every case, secretion was calculated as a percentage of the catecholamine content; for convenience, the level of release remaining after treatment with each toxin concentration was expressed as a percentage of the requisite control. Plotted data are representative of experiments carried out in quadruplicate and performed at least twice.

Quantification of the Proteolytic Activities of BoNT/C1, -/A, or -/E in Chromaffin Cell Cultures. A differential centrifugation method was employed to enrich plasmalemma

and granule membranes (P2 fraction) from small quantities of intoxicated and control chromaffin cell cultures. A minimum of 8 wells ($\sim 1 \times 10^6$ cells/well), treated without or with various concentrations of toxin (as described above), were washed twice with buffer B (to remove proteinaceous growth medium). Cells were scraped from the wells with a rubber-tipped spatula into a 1 mL volume of homogenization buffer (50 mM $NaHCO_3$, pH 8.5, containing 2 mM PMSF, 5 mM iodoacetamide, 2.5 mM EDTA, 2 mM dipicolinic acid, 1 mM 1,10-phenanthroline, 1 mM benzamidine, 25 μg/mL pepstatin A, 20 μg/mL leupeptin, and 20 μg/mL soybean trypsin inhibitor). The harvested cells were lysed by 2 cycles of freeze/thawing followed by 10 passes through a 26 gauge needle. All subsequent centrifugation steps were performed at 4 °C. The homogenate was centrifuged at $1000g_{max}$ for 10 min (yielding pellet P1), and the resultant supernatant (S1) was recentrifuged at $260000g_{av}$ for 1 h to sediment all remaining membranes (P2) which included both granule and plasmalemma markers. The P2 pellets were resuspended in 80 μl of 0.1 M Tris·HCl, pH 6.8, containing 2% (w/v) SDS and 1 mM EDTA and solubilized with heating at 90 °C for 10 min. Insoluble material was removed by centrifugation at $10000g_{max}$ (at room temperature) for 10 min, and protein concentrations of the supernatants were determined from the absorbancies at 280 nm. Equal amounts of protein from the SDS-containing supernatants (from cells treated with each toxin concentration) were subjected to SDS−PAGE, electrophoretically transfered to PVDF membrane, and immunoblotted overnight at 4 °C with primary antibodies (see legend to Figure 1). In each case, the choice of two available antibodies used to detect either syntaxin or SNAP-25 are specified in the figure legends. The membranes were then rinsed 3 times with 50 mM Tris·HCl, pH 7.4, containing 0.15 M NaCl and 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween-20) followed by four 10 min washes in the latter before a 2 h incubation at room temperature with blocking solution [3% skimmed milk powder and 1% (w/v) BSA] containing a 1:1000 (v/v) dilution of either anti-rabbit or anti-mouse Ig alkaline phosphatase conjugated secondary antibody. Primary Ig bound on Western blots was visualized using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Immunoblots were quantified by scanning with a Hirshmann 400 densitometer, and background readings were subtracted. Additional controls were run to ensure that the developed bands yielded values in the linear range by relating them to the signals recorded for known quantities of non-toxin-treated chromaffin cell membrane protein.

Toxin Incubation with Recombinant SNAP-25. For use as a potential substrate, glutathione *S*-transferase (GST)−SNAP-25 fusion protein (containing a cleavage site for thrombin) was expressed and affinity-purified on glutathione−agarose, as outlined in Zhou *et al.* (1995), and cleaved with thrombin to release the SNAP-25. Toxins were activated by prereduction with 20 mM DTT in 0.1 M Hepes·NaOH (pH 7.0) for 30 min at 37 °C, the DTT concentration being subsequently diluted to 2 mM in the final reaction mixture. Incubations of the prereduced toxins with recombinant SNAP-25 were performed as outlined in the legend to Figure 4. Where specified, recombinant SNAP-25 was preincubated for 30 min at 37 °C with a 3-fold molar excess of recombinant His^6-tagged syntaxin 1A, a soluble truncated protein lacking the last 27 C-terminal residues (kindly provided by D. Liu). The extents of cleavage of all the substrates were assessed by Western blotting, as outlined above.

RESULTS

BoNT/C1-Mediated Blockade of Ca^{2+}-Evoked Catecholamine Release in Intact and Digitonin-Permeabilized Cells Correlates with the Proteolyses of Syntaxin 1A/B and SNAP-25. The presence of syntaxin on plasma membranes of chromaffin cells (Hodel *et al.*, 1994), as well as the demonstration that it occurs complexed to SNAP-25 and Sbr in a detergent extract of the latter (Roth & Burgoyne, 1994), warranted an examination of whether the extent of its cleavage in BoNT/C1-intoxicated cells corresponded to the inhibition of secretion attained. Chromaffin cells can be poisoned by prolonged exposure to either BoNT/A or -/B in a low ionic strength buffer (Marxen *et al.*, 1989; Foran *et al.*, 1995). As anticipated, this method revealed a dose-dependent blockade by BoNT/C1 of catecholamine secretion evoked by depolarization of intact cells or, following digitonin permeabilization of samples of the same cells, that elicited by 20 μM Ca^{2+} or 1 mM Ba^{2+} (Figure 1A). A meaningful comparison of the latter values could, thus, be made with the degrees of cleavage of syntaxin, monitored by Western blotting. Initial difficulties, experienced in the immunodetection of the toxins' targets in whole cells, were overcome by using differential centrifugation to enrich the relevant plasmalemma and granule membranes (see Experimental Procedures), as reflected in the contents of relevant marker proteins (Figure 1B). Syntaxin was recognized using a monoclonal antibody which is reported to react with isoforms 1A and 1B (Inoue *et al*., 1992). The P2 membrane fractions were found to contain 82% of the total syntaxin 1A/B immunoreactivity, despite retaining only ~25% of the total cell protein (not shown); this enrichment facilitated its quantitation by densitometric scanning of Western blots (see Experimental Procedures). The highest toxin concentration used gave a complete block of exocytosis elicited from intact cells by either 55 mM K^+ or 2 mM Ba^{2+}, or from digitonin-permeabilized cells by 20 μM Ca^{2+} or 1 mM Ba^{2+} (Figure 1A); this was accompanied by a corresponding loss of intact syntaxin 1A/B to 13.3 ± 2.5% of the content of toxin-free controls, quantified by densitometric scanning of blots (Figure 1B,D). Note the near-identical dose dependencies for blockade by BoNT/C1 of secretion triggered by each of the stimuli, with the exception of 55 mM K^+ which appears to be slightly more susceptible. For the first time, a good correlation between the diminished levels of secretion and the amounts of syntaxin 1A/B remaining was demonstrated in BoNT/C1-poisoned cells (Figure 1A,D). Immunoblotting for established BoNT/C1-insensitive markers [e.g., Sbr/Cbr (Hodel *et al.*, 1994; Foran *et al.*, 1995) or synaptotagmin (Fournier *et al.*, 1989) on CGs and GAP-43 (Vitale *et al.*, 1994) in the plasma membrane] demonstrated equivalent enrichments of plasmalemma and granule membranes in the P2 pellets derived from cells treated with different toxin concentrations (Figure 1B). BoNT/C1 intoxication of cells did not cause any significant change in staining patterns for other detectable proteins on SDS−PAGE (results not shown), consistent with the toxin's apparent selective proteolytic action (Blasi *et al.*, 1993b). However, blotting of the same membranes using Ig, raised against a synthetic peptide encompassing the last 12 C-terminal residues of SNAP-25, revealed an additional dose-dependent decrease in its reactivity (Figure 1B). The concentration dependence for clea-

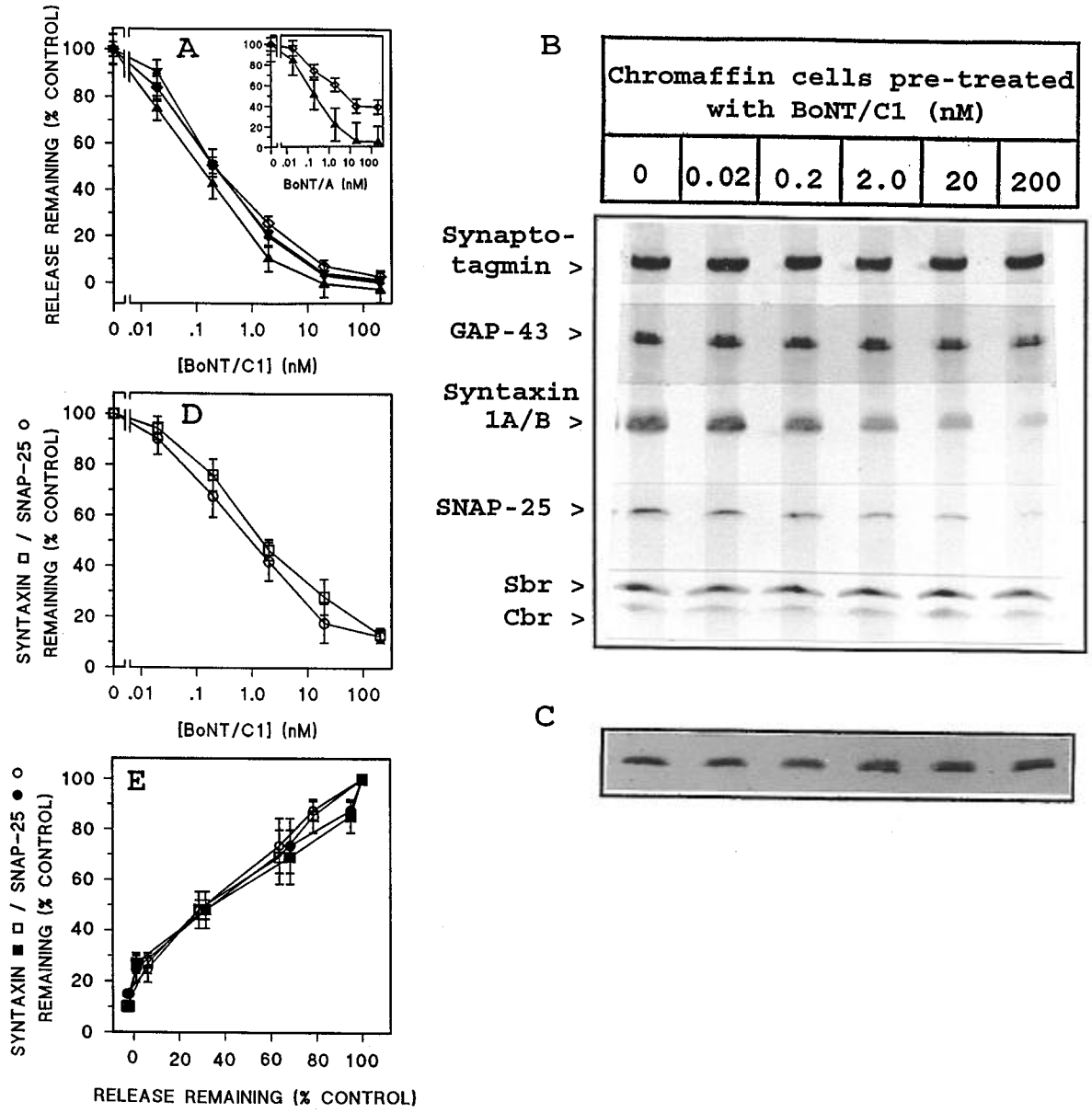

FIGURE 1: BoNT/C1-induced inhibition of catecholamine release correlates with the proteolyses of syntaxin 1A/B and SNAP-25. Intact chromaffin cells, preexposed to increasing concentrations of BoNT/C1 (A) or -/A (A, inset panel), as detailed under Experimental Procedures, were rinsed with buffer B before exposure to either the same buffer, 5 mM K^+, 55 mM K^+, or 2 mM Ba^{2+} buffer. Release evoked from intact cells by 2 mM Ba^{2+} (♦) or 55 mM K^+ (▲) was calculated by subtraction of the values obtained for the catecholamine content of buffer B or 5 mM K^+ medium from the amounts in the Ba^{2+} or 55 mM K^+ buffers, respectively. Alternatively, the BoNT/C1 pretreated cells were subsequently permeabilized with 20 μM digitonin in toxin-free KGEP buffer, containing MgATP without or with the inclusion of 20 μM Ca^{2+} (◊) or 1 mM Ba^{2+} (▼). After 15 min, aliquots were removed, and their catecholamine contents were quantified fluorometrically (±SD; $n = 4$). Values for basal release into the appropriate control media were subtracted from the requisite totals obtained in the presence of Ca^{2+} or Ba^{2+} to yield figures for the various types of stimulus-dependent secretion. Data in the inset (A) are the average of two independently-performed determinations in which intact cells were treated as above but with BoNT/A before the levels of secretion subsequently evoked by 55 mM K^+ (▲) or, after permeabilization, by 20 μM Ca^{2+} (◊) were quantified. The cells exposed to buffer B were solubilized in Triton X-100 and assayed for catecholamine in order to obtain a representative value for total cell content; secretion was calculated as a percent of the latter value. Secretion evoked from cells treated with toxin was expressed as a percent of the appropriate toxin-free control (thus, termed release remaining; A, E); this enabled direct comparison with losses of syntaxin and SNAP-25 (see below). Immediately after measuring secretion, equal amounts of SDS-solubilized P2 membrane fractions were prepared from cells preexposed to each toxin at the concentrations shown, and identical amounts of protein (40 μg) were subjected to SDS−PAGE, transferred to PVDF membranes, and blotted overnight with the requisite antibodies at the following dilutions: (B) anti-synaptotagmin (1:500), anti-GAP-43 (1:500), anti-syntaxin 1A/B (monoclonal; 1:500), affinity-purified anti-SNAP-25 raised against the C-terminal peptide (1:100), anti-Sbr/Cbr (1:100); and (C) anti-SNAP-25 (clone SMI 81, 1:1000). Each primary antibody was blotted individually and detected using alkaline phosphatase-conjugated secondary antibodies (see Experimental Procedures); only the relevant track positions are shown in panels B and C. The levels of immunoreactivities on blots were quantified by densitometric scanning, and the resultant values for SNAP-25 (○) or syntaxin (□) in toxin-treated cells were expressed (D) relative to toxin-free control cells (±SEM; $n = 3$). In panel E, equivalent data obtained from an independent set of experiments in which release was evoked from intact cells by 55 mM K^+ (■, syntaxin; ●, SNAP-25) or, after digitonin permeabilization, by 20 μM Ca^{2+} (□, syntaxin; ○, SNAP-25) are plotted. Note that some of the symbols overlap.

vage of SNAP-25 by BoNT/Cl was similar to that for its truncation of syntaxin; thus, the almost complete block of exocytosis elicited by 200 nM BoNT/C1 (see earlier) was accompanied by a corresponding loss of intact SNAP-25 to 12.5 ± 2.5% of the content of toxin-free controls (Figure 1D). Additional blotting of these P2 membrane fractions

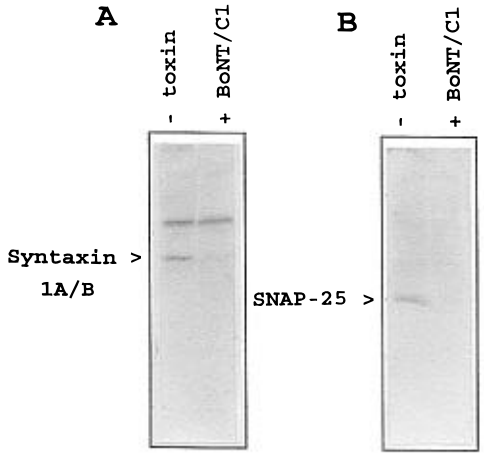

FIGURE 2: Rapid and near-complete proteolyses of syntaxin and SNAP-25 upon application of BoNT/C1 to permeabilized chromaffin cells. Cells were prerinsed with buffer B and permeabilized by exposure to 20 μM digitonin in 2 mM MgATP containing KGEP for 15 min, in the absence or presence of 100 nM prereduced BoNT/C1 (see Experimental Procedures). The latter buffer was replaced with a new aliquot of 2 mM MgATP-containing KGEP buffer, with or without 20 μM Ca^{2+}, and secretion over a further 15 min was quantified. Aliquots were removed, and the mean ($\pm$SD; $n = 4$) stimulus-dependent catecholamine secretion was calculated (as specified in Figure 1). For the measurement of syntaxin and SNAP-25 contents, P2 membrane fractions were prepared from digitonin-permeabilized cells treated identically with BoNT/C1 (as above) for 15 min, before immediate inactivation of toxin using homogenization buffer (see Experimental Procedures). Equal amounts of SDS-solubilized P2 membrane fractions (30 μg of protein) from permeabilized cells that had been incubated with or without toxin were subjected to SDS−PAGE (12.5% acrylamide), transferred to PVDF membranes, and blotted overnight with the requisite antibodies at the following dilutions: (A) affinity-purified anti-recombinant syntaxin 1A [see Experimental Procedures; 1:200; please note that this Ig preparation also recognizes isoform 1B, as well as an additional protein of ~50 kDa in a specific manner, reactivity being diminished by preincubation with immunogen (results not shown)]; (B) affinity-purified anti-SNAP-25 raised against the C-terminal peptide (1:200). Primary antibody binding was detected using species-specific alkaline phosphatase-conjugated secondary antibodies, as detailed in Figure 1.

with a monoclonal antibody reactive to SNAP-25 (clone: SMI 81) revealed that the portion of the SNAP-25 molecule retained in the membrane after BoNT/C1 truncation was stable and had not redistributed to another cellular location, as immunoreactivity was undiminished (Figure 1C). The close correlation between the extents of cleavage of SNAP-25 plus syntaxin 1A/B and the levels of exocytosis remaining in BoNT/C1-treated intact cells is highlighted in Figure 1E; two separate sets of experiments gave very similar results.

In addition to the above studies on intact cells poisoned by extracellular exposure to BoNT/C1, some cells were treated with toxins for the first time following permeabilization to yield access to their intracellular substrates (Figure 2). Brief application (15 min at 25 °C) of prereduced BoNT/C1 (100 nM) resulted in rapid and near-complete proteolyses of both syntaxin and SNAP-25, relative to the contents of toxin-free controls (Figure 2A,B). This was accompanied by a corresponding blockade of Ca^{2+}-evoked catecholamine release to 11.9 $\pm$ 3.2% of that for non-toxin-treated samples. Prereduction of BoNT/C1 was found to be necessary for its inhibitory effect, as nonreduced toxin lowered secretion by only a minimal amount (11.2 $\pm$ 5.3%) with respect to controls. This finding is in agreement with previous studies using BoNT/B and TeTx where disulfide reduction was found to be prerequisite for their LCs to express activity and cleave Sbr (Schiavo *et al.*, 1992). In accordance with the Zn^{2+}-dependent proteolytic action of BoNT serotypes (Schiavo *et al.*, 1992), preincubation of prereduced BoNT/C1 with 1 mM *o*-phenanthroline (a divalent cation chelator possessing a much higher affinity for Zn^{2+} relative to other divalent cations, e.g., Ca^{2+} or Mg^{2+}) for 30 min at 37 °C reduced by ~50% the level of toxin-induced inhibition of catecholamine release (results not shown); such a partial antagonism concurs with its incomplete inhibition of the enzymatic activity of BoNT/C1 that was reported by Blasi *et al.* (1993b).

SNAP-25 Is Proteolyzed at Its C-Terminus in Intact Cells Poisoned by BoNT/C1. In order to confirm the validity of our demonstration that SNAP-25 is proteolyzed by BoNT/C1 in poisoned cells, it was deemed necessary to eliminate the possibility of contamination of the C1 preparation by either /E or /A. The presence of the latter was excluded by the total absence in the BoNT/C1 preparation of any reactivity toward an anti-BoNT/A-LC antiserum, even though a 20-fold excess of C1 was blotted relative to the amount of BoNT/A that was readily detectable (results not shown); thus, type A could not have been responsible for the SNAP-25 cleavage observed in the dose−response studies in Figure 1. Next, the size of the SNAP-25 fragment detected in BoNT/C1-poisoned cells was examined using a monoclonal antibody (clone: SMI 81) that recognizes the different toxin-truncated fragments of SNAP-25 (Figure 3A); electrophoresis conditions were optimized to achieve the best possible separation of intact and truncated fragments. Notably, BoNT/E yielded a cleaved SNAP-25 product significantly smaller than those produced by BoNT/A or -/C1, thereby providing affirmative evidence that the cleavage in C1-poisoned cells was not mediated by contaminating BoNT/E (Figure 3A); the partial cleavage seen with BoNT/E correlates with the similarly low level of blockade of secretion observed (not shown) and is consistent with the minimal reduction in reactivity observed upon blotting with the SNAP-25 C-terminal antibody (Figure 3B). BoNT/C1 gave a shift in the electrophoretic mobility of the immunoreactivity approximately equal to that seen with type A (Figure 3A), and both toxins removed reactivity on blots toward the SNAP-25 C-terminal antibody, confirming their proteolytic actions (Figure 3B). This indicates that BoNT/C1 cleaves SNAP-25 at a site near the C-terminus, as established for BoNT/A (Blasi *et al.*, 1993a).

Even though BoNT/C1 treatment removes a similarly small sized fragment from the C-terminus of SNAP-25 as does type A, Ca^{2+} failed to elicit significant catecholamine secretion from permeabilized cells that had been poisoned with C1 before exposure to digitonin (detailed above). In contrast, although preintoxication of intact cells with BoNT/A abolished K^+ depolarization evoked release, an appreciable response (equivalent to ~40% of the level secreted by toxin-free control) could be evoked by Ca^{2+} from the cells after digitonin permeabilization (Figure 1A insert). The distinct inhibition pattern of C1 could arise from its additional cleavage of syntaxin.

BoNT/C1 Is Unable To Cleave a Soluble Recombinant Form of SNAP-25. Having demonstrated that BoNT/C1 poisoning of chromaffin cells results in the cleavage of both syntaxin and SNAP-25, attempts were made to ascertain whether it could proteolyze a recombinant SNAP-25 generated following thrombin cleavage of its GST-fusion protein (see Experimental Procedures). The largely-free recombinant SNAP-25 served as an efficient substrate for BoNT/A (Figure

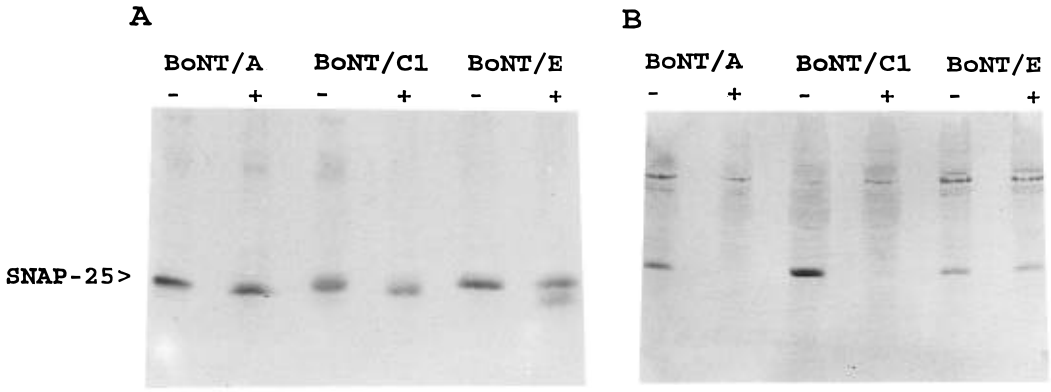

FIGURE 3: SNAP-25 is proteolyzed at its C-terminus in cells poisioned with BoNT/C1: comparision of the relative size of the truncated protein with that produced by either type A or type E. Intact chromaffin cells were preexposed to buffer A for 24 h in the absence or presence of either 200 nM BoNT/A, 200 nM BoNT/C1, or 200 nM BoNT/E. Equal amounts of SDS-solubilized P2 membrane fractions were prepared from cells treated with or without the specified toxins, and these were subjected to SDS−PAGE (10% acrylamide), transferred to PVDF membranes, and blotted overnight with the requisite antibodies at the following dilutions: (A) anti-SNAP-25 (monoclonal SMI 81; 1:1000); (B) affinity-purified anti-SNAP-25 raised against the C-terminal peptide (1:100). Identical amounts of protein (20 μg) were blotted in all cases except that 60 μg was loaded for samples in panel B treated with and without BoNT/C1. Detection of antibodies and their visualization were as in Figure 1.

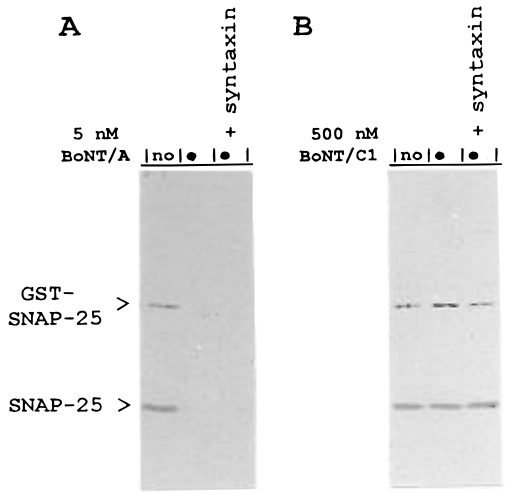

FIGURE 4: Recombinant SNAP-25 is not cleaved by BoNT/C1 but is a good substrate for type A. Prereduced toxins, at the specified concentrations, were incubated at 37 °C for 60 min in 50 mM Hepes·NaOH (pH 7.4) containing 150 mM NaCl and 0.01% (w/v) Lubrol PX (to eliminate protein losses) together with largely-free recombinant SNAP-25 (0.4 μM), for either 30 min with BoNT/A (A) or 60 min with BoNT/C1 (B). Where indicated, SNAP-25 was preincubated for 30 min at 37 °C with a 3-fold molar excess of a soluble recombinant syntaxin 1A (lacking the last 27 C-terminal residues) prior to the addition of toxins. Reactions were terminated by the addition of sample buffer and immediate boiling, prior to SDS−PAGE, electrophoretic transfer to PVDF membranes, and immunoblotting with affinity-purified anti-SNAP-25 C-terminal peptide Ig (as in Figure 1). Equal amounts of protein from control and toxin-treated samples were blotted.

4A); incubation with 5 nM prereduced toxin for 30 min at 37 °C achieved near-complete proteolysis, in agreement with previous reports (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993c). In contrast, BoNT/C1 did not exhibit detectable cleavage of the recombinant, even when incubations were performed for 60 min using 500 nM toxin (Figure 4B). Preincubation of SNAP-25 with a soluble recombinant syntaxin 1A (lacking the last 27 C-terminal residues; see Experimental Procedures), prior to the addition of toxins, resulted in the formation of a binary complex *in vitro* (results not shown); it was suspected that this association might convert SNAP-25 into a more favorable conformation for cleavage by BoNT/C1. However, no improvement in BoNT/C1-mediated proteolysis was seen, compared to the control incubation (Figure 4B), and neither was cleavage by BoNT/A perturbed (Figure 4A).

DISCUSSION

The novel finding, herein reported, that a *Clostridial* neurotoxin (BoNT/C1) can proteolyze two structurally-distinct proteins is of great importance because, hitherto, all these toxins had been found to exhibit strict substrate selectivity (see the introduction) with activities apparently limited to the scission of single peptide bonds in sole substrates (or very closely-related homologues). However, it is notable that the newly-discovered additional target of BoNT/C1 (SNAP-25) associates with syntaxin (also cleaved by BoNT/C1) and Sbr [proteolyzed by BoNT/B, -/D, -/F, -/G, and TeTx; see introduction) (Hayashi *et al.*, 1994)] and, moreover, is the documented substrate for BoNT/A and -/E (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993c). There is no evidence that any of these toxins has a physiologically relevant action on additional proteins other than the three aforementioned constituents of a tripartite complex which has been proposed to play an essential role in the docking and/or fusion of secretory vesicles (Söllner *et al.*, 1993; Niemann *et al.*, 1994). The ability of BoNT/C1 to proteolyze two members of the latter complex, despite them having no significant homology, may be due to the presence in both proteins of a short (five residues in length) motif that exhibits similar charge distribution and might act as a substrate recognition domain (Rossetto *et al.*, 1994). Efficient proteolysis of SNAP-25 resulted from poisoning of intact chromaffin cells with BoNT/C1, and the protein was rapidly cleaved when control cells were permeabilized and then exposed to the toxin. Thus, the inability of BoNT/C1 to cleave SNAP-25 in purified membrane fractions (results not shown), or recombinant SNAP-25, *in vitro* suggests some conformational requirement of the substrate for recognition and/or cleavage by the toxin. This could possibly involve intricate protein complexes or additional accessory factors that are present only in whole or semi-intact (i.e., digitonin-permeabilized) cell preparations. In contrast, BoNT/A and -/E cleave native, detergent-solubilized (Schiavo *et al.*, 1993a), and recombinant SNAP-25 (Schiavo *et al.*, 1993c) and, therefore, appear to have a much less stringent structural requirement of their substrate. Notably, membrane-anchored

syntaxin is efficiently cleaved by BoNT/C1, but it is a poor substrate after detergent solubilization (Blasi *et al.*, 1993b); i.e., both targets of BoNT/C1 must be in specific conformations for their proteolysis to occur. It is also noteworthy that BoNT/C1 cleaves SNAP-25, but not syntaxin, in detergent-solubilized cell membranes (albeit at a rate much slower than observed with permeabilized cells); thus, proteolysis of the latter protein is not a prerequisite for the toxin's action on SNAP-25 (data not shown).

The observed dual substrate specificity of BoNT/C1 complicates its use for investigating the individual roles of syntaxin and SNAP-25 in exocytosis. Nevertheless, it is clearly apparent that syntaxin is essential for this process because anti-syntaxin antibodies inhibit Ca^{2+}-evoked catecholamine release from permeabilized chromaffin cells (Gutierrez *et al*., 1995) or after injection into PC 12 cells (Bennett *et al*., 1993). Moreover, BoNT/A and -/C1 appear to cleave SNAP-25 at nearby peptide bond(s), but the latter toxin abolished the secretion triggered by Ca^{2+} after membrane permeabilization whereas BoNT/A gave only a partial reduction (despite having completely blocked these same cells' secretory response to K^+ depolarization before membrane disruption). This implies that syntaxin is indispensable for Ca^{2+}-evoked secretion from digitonin-permeabilized chromaffin cells but, conversely, that appreciable exocytosis can occur under these conditions when most of the SNAP-25 has been truncated (Lawrence *et al.*, 1996). To validate the importance of syntaxin, it will be necessary to demonstrate that BoNT/C1 removes no more amino acid residues from SNAP-25 than BoNT/A; the low content of SNAP-25 in chromaffin cells precluded these experiments.

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REFERENCES

Ashton, A. C., de Paiva, A. M., Poulain, B., Tauc, L., & Dolly, J. O. (1993) in *Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects* (DasGupta, B. R., Ed.) pp 191−213, Plenum Publishing Corp, New York.

Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D., & Scheller, R. H. (1993) *Cell 74*, 863−873.

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., Camilli, P. D., Südhof, T. C., Niemann, H., & Jahn, R. (1993a) *Nature 365*, 160−163.

Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H., & Jahn, R. (1993b) *EMBO J. 12*, 4821−4828.

Cenci di Bello, I., Poulain, B., Shone, C. C., Tauc, L., & Dolly, J. O. (1994) *Eur. J. Biochem. 219*, 161−169.

DasGupta, B. R. (1989) in *Botulinum Neurotoxins and Tetanus Toxin* (Simpson, L. L., Ed.) pp 53−67, Academic Press, New York.

Dolly, J. O. (1992) *Handb. Exp. Pharmacol. 102*, 681−717.

Dolly, J. O., Black, J. D., Williams, R. S., & Melling, J. (1984) *Nature 307*, 457−460.

Dolly, J. O., de Paiva, A., Foran, P., Lawrence, G., Daniels-Holgate, P., & Ashton, A. C. (1994) *Semin. Neurosci. 6*, 149−158.

Evans, D. M., Williams, R. S., Shone, C. C., Hambleton, P., Melling, J., & Dolly, J. O. (1986) *Eur. J. Biochem. 154*, 409−416.

Foran, P., Lawrence, G., & Dolly, J. O. (1995) *Biochemistry 34*, 5494−5503.

Fournier, S., Novas, M. L., & Trifaro, J.-M. (1989) *J. Neurochem. 53*, 1043−1049.

Gutierrez, L. M., Quintanar, J. L., Viniegra, S., Salinas, E., Moya, F., & Reig, J. A. (1995) *Biochem. Biophys. Res. Commun. 206*, 1−7.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C., & Niemann, H. (1994) *EMBO J. 13*, 5051−5061.

Hodel, A., Schäfer, T., Gerosa, D., & Burger, M. M. (1994) *J. Biol. Chem. 269*, 8623−8626.

Inoue, A., Obata, K., Akagawa, K. (1992) *J. Biol. Chem. 267*, 10613−10619.

Lawrence, G. W., Weller, U., & Dolly, J. O. (1994) *Eur. J. Biochem. 222*, 325−330.

Lawrence, G. W., Foran, P., & Dolly, J. O. (1996) *Eur. J. Biochem.* (in press).

Link, E., Edelmann, L., Chou, J. H., Binz, T., Yamasaki, S., Eisel, U., Baumert, M., Sudhof, T. C., Niemann, H., & Jahn, R. (1992) *Biochem. Biophys. Res. Commun. 189*, 1017−1023.

Marxen, P., Fuhrmann, U., & Bigalke, H. (1989) *Toxicon 27*, 849−859.

McMahon, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., & Sudhof, T. C. (1993) *Nature 364*, 346−349.

Montecucco, C., & Schiavo, G. (1993) *Trends Biochem. Sci. 18*, 324−327.

Niemann, H., Blasi, J., & Jahn, R. (1994) *Trends Cell Biol. 4*, 179−185.

Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsly, M., Bloom, F. E., & Wilson, M. C. (1989) *J. Cell Biol. 109*, 3039−3052.

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Doloye, F., Lozzi, L., & Shone, C. C. (1994) *Nature 372*, 415−416.

Roth, D., & Burgoyne, R. D. (1994) *FEBS Lett. 351*, 207−210.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R., & Montecucco, C. (1992) *Nature 359*, 832−835.

Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F., & Montecucco, C. (1993a) *J. Biol. Chem. 268*, 23784−23787.

Schiavo, G., Shone, C. C., Rossetto, O., Alexander, F. C. G., & Montecucco, C. (1993b) *J. Biol. Chem. 268*, 11516−11519.

Schiavo, G., Santucci, A., DasGupta, B. R., Mehta, P. P., Jontes, J., Benfenati, F., Wilson, M. C., & Montecucco, C. (1993c) *FEBS Lett. 335*, 99−103.

Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H., & Montecucco, C. (1995) *J. Biol. Chem. 270*, 10566−10570.

Shone, C. C., Quinn, C. P., Wait, R., Hallis, B., Fooks, S. G., & Hambleton, P. (1993) *Eur. J. Biochem. 217*, 965−971.

Simpson, L. L. (1986) *Infect. Immun. 52*, 858−862.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., & Rothman, J. (1993) *Nature 362*, 318−324.

TerBush, D. R., & Holz, R. W. (1993) *J. Neurochem. 58*, 680−687.

Vitale, N., Deloulme, J.-C., Theirse, D., Aunis, D., & Bader, M.-F. (1994) *J. Biol. Chem. 269*, 30293−30298.

von Euler, U. S., & Floding, I. (1959) *Acta. Physiol. Scand. (Suppl). 118*, 45−56.

Wadsworth, J. D. F., Desai, M. S., Tranter, H., King, H. J., Hambleton, P., Melling, J., Dolly, J. O., & Shone, C. C. (1990) *Biochem. J. 268*, 123−128.

Yamasaki, S., Binz, T., Hayashi, T., Szabo, E., Yamasaki, N., Eklund, M., Jahn, R., & Niemann, H. (1994) *Biochem. Biophys. Res. Commun. 200*, 829−835.

Zhou, L., de Paiva, A., Liu, D., Aoki, R., & Dolly, J. O. (1995) *Biochemistry 34*, 15175−15181.

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