Blockade by Botulinum Neurotoxin B of Catecholamine Release from Adrenochromaffin Cells Correlates with Its Cleavage of Synaptobrevin and a Homologue Present on the Granules†

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Received November 7, 1994; Revised Manuscript Received January 20, 1995®

ABSTRACT: Botulinum neurotoxin type B blocks transmitter release via a selective endoproteolysis of the small clear vesicle membrane protein synaptobrevin that is essential for neuro-exocytosis. In view of the distinct characteristics of exocytosis of adrenochromaffin granules and considering the controversy over the presence of synaptobrevin on the latter, this study aimed to determine the molecular basis of the inhibition by this toxin of secretion from chromaffin cells. Thus, affinity-purified antibodies against a synaptobrevin synthetic peptide were used to quantify its concentrations in subcellular fractions of bovine adrenal medulla. The latter, as well as density gradient centrifugation and size-exclusion chromatography, showed that >70% of the protein copurifies with the granules and their marker, dopamine β-hydroxylase. Notably, much lower concentrations of synaptobrevin and synaptophysin were found in chromaffin granules than in synaptic small clear vesicles (~9% and ~2%, respectively); however, isolated granule membranes exhibited greater enrichments (~35% and ~9%). A second immunoreactive protein was colocalized with synaptobrevin on chromaffin granules; in view of its susceptibility to the toxin and lower M_r, it is assumed to be cellubrevin and, also, because of its high homology. Involvement of synaptobrevin and cellubrevin in Ca^{2+}-triggered granule exocytosis was established by the demonstrated correlation between the extent of botulinum neurotoxin B-induced inhibition of secretion and their selective proteolysis following introduction of the toxin into intact chromaffin cells. On the basis of these collective findings, it is concluded that these proteins occur on chromaffin granules and one or both are essential for exocytosis.

Botulinum (BoNT)[1] and tetanus (TeTx) neurotoxins, produced by *Clostridium botulinum* and *tetani*, respectively, are specific inhibitors of neuro-exocytosis. These neurotoxins are dichain proteins consisting of a heavy and light chain (LC) held together by a disulfide bond and noncovalent interactions. Their action involves a triphasic mechanism initiated by binding via the heavy chain to distinct neuronal ectoacceptors, subsequent internalization, and translocation into the cytosol where their LCs act [reviewed in Simpson (1986) and Dolly (1992)]. Recently, it was reported that the LC of TeTx or BoNT (there are seven immunologically distinct serotypes, A−G) exhibit metalloprotease activities toward one of three neuronal proteins [see below and reviewed in Dolly *et al.* (1994)], actions that account for their remarkable neurotoxicities (Li *et al.*, 1994). These toxin substrates, synaptobrevin (Sbr; also, termed vesicle-associated membrane protein), syntaxin 1A or HPC-1, and synaptosomal protein with M_r of 25 kDa (SNAP-25), have been demonstrated to interact *in vitro* and are proposed to comprise the core of the synaptic small clear vesicle (SCV) docking-fusion complex (Sollner *et al.*, 1993), an idea consistent with the toxins' blockade of neurotransmitter release. Sbr, an integral membrane protein of SCVs, is the target of TeTx and BoNT/B, -D, -F, and -G (Schiavo *et al.*, 1992, 1993a−c; Link *et al.*, 1992; Yamasaki *et al.*, 1994). It is inserted into SCVs by a single hydrophobic C-terminal region, with the remainder of the protein being exposed cytoplasmically (Trimble *et al.*, 1988; Baumert *et al.*,1989). In vertebrates, two isoforms of Sbr (1 and 2) are differentially expressed within the central and peripheral nervous systems (Elferink *et al.*, 1989). Cellubrevin (Cbr), an ubiquitous vesicle protein, with a strong homology to the hydrophilic domain of Sbr but containing a dissimilar N-terminal head region, is also cleaved by TeTx LC (McMahon *et al.*, 1993). BoNT/A and -E selectively proteolyse SNAP-25 (Blasi *et al.*, 1993a), and BoNT/C1 splits HPC-1 (Blasi *et al.*, 1993b).

The possiblity that Sbr is involved in Ca^{2+}-evoked exocytosis of catecholamine from chromaffin granules (CGs), as well as neuropeptide release from large dense core vesicles (LDCVs), has been raised by their established sensitivities to BoNT/B or TeTx (Bittner *et al.*, 1989; Lawrence *et al.*, 1994; Janicki & Habermann, 1983; McMahon *et al.*, 1992). However, Baumert *et al.* (1989) reported no significant labeling of neuropeptide-containing LDCVs with anti-Sbr antibodies, using immunogold electron microscopy. Although other subcellular organelles were not analyzed in this

† This work was supported in part by The Speywood Laboratory Ltd., USAMRDC under Grant DAMD 17-91-Z-1035, and a studentship to G.L. from BBSRC and CAMR.

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® Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: BoNT/B, botulinum neurotoxin type B; TeTx, tetanus toxin; LC, light chain; DTT, dithiothreitol; SCVs, small clear vesicles; LDCVs, large dense core vesicles; SLMVs, synaptic-like microvesicles; RP-HPLC, reverse-phase high-performance liquid chromatography; BSA, bovine serum albumin; SBTI, soybean trypsin inhibitor; CG, chromaffin granule; S2P, high-speed centrifugation pellet of S2 supernatant; p38, synaptophysin; SNAP-25, synaptosomal-associated protein with M_r of 25 kDa; HPC-1, syntaxin 1A; DβH, dopamine β-hydroxylase; Sbr, synaptobrevin; Sbr r33−94, residues 33−94 of human synaptobrevin-2; Cbr, cellubrevin; PIPES, piperazine-*N*,*N*′-[bis(2-ethanesulfonic acid)]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TBS, 25 mM Tris·HCl, pH 7.5, containing 150 mM NaCl; Ig, immunoglobin.

0006-2960/95/0434-5494$09.00/0

way, significant Sbr immunoreactivity was revealed by light microscopy in whole chromaffin cells; it was, thus, suggested that Sbr is likely to reside on the membranes of synaptic-like microvesicles (SLMVs) in endocrine cells. Furthermore, studies carried out by Hohne-Zell *et al.* (1993) concluded that Sbr cleavage is not the mechanism by which TeTx (identical in its proteolytic activity to BoNT/B, employed in this study) inhibit catecholamine release from chromaffin cells. Moreover, there are considerable differences between the regulated neurosecretion pathways for SCVs and CGs. Notably, neurotransmitter release occurs very rapidly upon depolarization, at active zone regions where vesicle docking and fusion take place; also, membrane retrieval and vesicle recycling follow quickly (von Gersdorff & Matthew, 1994). In contrast, the speed of CG fusion is much slower; in addition, the Ca^{2+} trigger involved in catecholamine release is more sensitive because it responds to the significantly lower bulk cytoplasmic Ca^{2+} concentrations, in areas distant from the point of Ca^{2+} entry (Augustine & Neher, 1992).

In order to decipher the molecular basis of these differences, it is imperative to identify the proteins responsible for exocytosis in neuroendocrine cells so that a comparison can be made with their neuronal counterparts. In this study, strong evidence is presented for Sbr and Cbr being components of CG membranes; additionally, it is established that their presence is not due to contamination by SLMVs. Furthermore, we show that the inhibition of Ca^{2+}-evoked catecholamine release by BoNT/B in intact chromaffin cells is associated with a selective proteolytic cleavage of Sbr, and its homologue Cbr, demonstrating that one or both are essential for exocytosis of neuroendocrine CGs. Consistent with this conclusion, Hodel *et al.* (1994) reported, during the preparation of this article, that Sbr immunoreactivity was detectable in adrenal medullary CGs, though a contribution to this staining by contaminating vesicles was not excluded.

EXPERIMENTAL PROCEDURES

Materials. Sephacryl S-1000 HR resin and Immobilon-P (PVDF) membrane were from Pharmacia and Millipore, respectively; CNBr-activated Sepharose 4B, bovine serum albumin (BSA) fraction V grade, and protein A were supplied by Sigma. Sucrose, dithiothreitol (DTT), organic solvents, Hepes, and all other buffer components were obtained in Analar grade from either Sigma or BDH. BoNT/B was purified by modification of the process described for BoNT/F (Wadsworth *et al.*, 1990), as specified in Shone *et al.* (1993); trace contaminating proteases, monitored using endoprotease chromogenic substrates, were removed by ion exchange chromatography on a Pharmacia Mono-S column, as outlined in Foran *et al.* (1994). BoNT/B was fully nicked as outlined in Evans *et al.* (1986). Monoclonal antibodies to synaptophysin (p38) and HPC-1 were purchased from Sigma. A rabbit polyclonal serum to dopamine β-hydroxylase (DβH) was kindly provided by Prof. D. K. Apps, University of Edinburgh. Anti-rabbit or -mouse IgG conjugated to alkaline phosphatase was purchased from Bio-Rad.

Subcellular Fractionation of Bovine Adrenal Medullae and Cerebral Cortex. CGs were prepared essentially as described by Smith and Winkler (1967). Briefly, bovine adrenal medullae were homogenized 1/10 (w/v) with a Potter-Elvehjem homogenizer in 0.3 M sucrose buffered with 10 mM Hepes·NaOH, pH 7.4, containing protease inhibitors (EDTA, 5 mM; *o*-phenanthroline, 1 mM; PMSF, 2 mM; iodoacetamide, 5 mM; soybean trypsin inhibitor, 20 μg/mL; pepstatin A, 20 μg/mL; benzamidine, 1.5 mM). All subsequent centrifugation steps were performed using a Beckmann 45 Ti rotor at 4 °C. The homogenate was centrifuged at 2100 rpm for 10 min, and the resultant supernatant (S1) was recentrifuged at 13 000 rpm for 25 min to sediment the large granules (P2) from the S2 supernatant (see below). P2 was resuspended [1:10 (w/v)], using the aforementioned buffered sucrose solution (including protease inhibitors) but omitting the upper fluffy layer and the denser erythrocytes, overlayed (~5 mL) on 50 mL of 1.8 M sucrose in the above buffer medium, and spun at 40 000 rpm for 1 h. The pellet (P3), purified CGs, was resuspended (to 2 mg/mL protein) in 0.3 M buffered sucrose (without protease inhibitors) and used directly or rapidly frozen in aliquots and stored at −60 °C. Some lysis of CGs was evident after freezing; otherwise, fresh and frozen CGs exhibited the same levels of the antigens under study and behaved identically on continuous sucrose gradients and size-exclusion chromatography. Recentrifugation of the P3 pellet through another 1.8 M sucrose pad did not significantly alter the relative enrichments of these antigens but lowered the yields. The S2 supernatant obtained above was centrifuged at 43 000 rpm for 1 h to sediment the S2P membranes.

In two series of experiments, the P3 (CGs) fraction as well as partially lysed CGs (see below) and S2P membranes was subjected to analytical sucrose gradients (continuous) ranging from 0.4 to 2.0 M (in 10 mM Hepes, pH 7.4, including 1 mM EDTA plus a 2 mL 2.5 M sucrose pad). The gradients (10 mL) were poured with an automated Densiflow-II gradient maker/fractionator. Samples (0.4 mL), resuspended at 1−2 mg/mL protein, were overlayed on the gradients in SW40Ti tubes and spun at 40 000 rpm for 3 h followed by fractionation into 11 equal fractions. The latter were diluted 4-fold with H_2O to reduce their initial densities and concentrated by chloroform/methanol precipitation (Wessel & Flugge, 1984) prior to SDS−PAGE and immunoblotting. Lysis of CGs was achieved by suspending an aliquot of P3 in 5 mM Hepes·NaOH, pH 6 (2 mg of protein/mL), and subjecting this to three freeze−thaw cycles over 30 min followed by the addition of NaCl to 0.2 M. After 10 min, the sample was mixed with an equal volume of 0.6 M sucrose with 10 mM Hepes·NaOH, pH 7.4, containing the protease inhibitors and centrifuged as above on a 0.4−2.0 M sucrose gradient. All samples to be employed in electrophoresis and immunoblotting for measuring relative antigen enrichments were pelleted in a 50 Ti rotor at 48 000 rpm for 1 h to remove soluble protein.

Bovine SCVs were prepared by the method of Hell *et al.* (1988), using the modifications of Matsuoka *et al.* (1990) for their isolation from frozen cortex; in this protocol, the final size-exclusion chromatography was on Sephacryl S-1000 HR rather than controlled pore glass beads, both of which share a similar pore size. For examination of the distributions of DβH, Sbr, and p38 immunoreactivities in vesicles of different sizes, P3 or SCVs were subjected to size-exclusion chromatography onto a column of Sephacryl S-1000 HR (dimensions 1 cm × 45 cm) pre-equilibrated in 0.3 M sucrose (buffered with 10 mM Hepes, pH 7.0, containing 1 mM EDTA) and run at a flow rate of 4−5 mL/h. Eluted fractions, monitored at 280 nm and weighed to allow accurate comparison of elution position to be made

between runs, were concentrated by chloroform/methanol precipitation prior to electrophoresis and immunoblotting.

Immunological Techniques. Polyclonal antibodies were generated in rabbits against a synthetic peptide encompassing residues 33−94 of human Sbr-2 (Sbr r33−94), using standard immunization protocols. This and related Sbr peptides (r45−94, r55−94, r65−94, and r53−68) were synthesized and purified, as detailed previously (Foran *et al.*, 1994). Immunization of rabbits involved injection with 100 μg of purified Sbr r33−94 peptide in Freunds complete adjuvant at four sites (two each, subcutaneous and intramuscular). Boosters were performed similarly at 14, 28, 56, and 72 days but using Freunds incomplete adjuvant; such a long peptide was chosen to obtain antibodies capable of detecting Sbr and homologues. One month after the first injection, adequate titers were obtained, as determined from the dilution required to visualize Sbr of SCVs in Western blots (detailed below). Affinity purification of Sbr-reactive immunoglobins (Ig) was achieved by a double pass of 4 mL of antiserum [diluted to 8 mL with 25 mM Tris·HCl, pH 7.4, buffer containing 150 mM NaCl (TBS)] at 1 mL min^{-1} through a 2 mL column of Sepharose CNBr coupled to 2 mg of Sbr r33−94 peptide. Following collection of breakthrough, the column was washed with four column volumes of TBS and, similarly, with 50 mM Tris·HCl, pH 7.0, containing 0.5 M NaCl. The antibodies were eluted using successive 1 mL aliquots of 0.1 M glycine·HCl, pH 2.5, containing 0.2 M NaCl and collected in tubes containing 0.2 mL of a neutralizing buffer (1 M Tris·HCl, pH 8.0). Quantitation of Ig was by absorbance at 280 nm; their purity was ascertained by SDS−PAGE (Laemmli, 1970). For blotting, purified antibodies were used at a 1:100 dilution relative to the original antiserum; this was equivalent to a final Ig concentration of 1−2 μg mL^{-1}.

Qualitative and Quantitative Detection of Antigens. All protein samples were subjected to SDS−PAGE and electrophoretically transferred to PVDF membranes. After blocking the membrane, initially using 3% (w/v) BSA in TBS followed by 5% (w/v) skimmed milk powder in TBS, the samples were incubated with primary antibodies in the latter solution overnight at room temperature. Qualitative detection of antigens on Western blots was performed using anti-rabbit or -mouse immunoglobin alkaline phosphatase-conjugated secondary antibodies, in conjunction with the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (de Paiva *et al.*, 1993). The PVDF membranes labeled with primary antibodies were washed briefly twice and then four times for 10 min each with TBS containing 0.1% (v/v) poly(oxyethylene) sorbitan monolaurate (Tween-20) followed by a 2 h incubation at room temperature with blocking solution containing a 1:1000 (v/v) dilution of alkaline phosphatase-conjugated secondary antibody. Alternatively, primary antibody binding was quantitatively detected using ^{125}I-labeled protein A, as detailed by Burnette (1981). Protein A was radiolabeled to ~300 Ci/mmol by the chloramine-T method (Dorval *et al.*, 1975), and ~1 × 10^7 cpm was applied in blocking solution for 2 h at room temperature to the PVDF membrane on which the primary antibodies had been bound. Quantitative measurements of the relative p38 and Sbr contents in samples were performed by excising the [^{125}I]protein A immunoblotted bands from the PVDF membrane (after visualization by autoradiography) and counting in a γ-spectrophotometer; comparisons were only made between samples on the same blotted membrane. In order to avoid errors that could arise when comparing subcellular fractions that contain very different antigen contents (and, thus, [^{125}I]protein A binding), a range find was performed for each sample and a dilution was chosen that gave equivalent cpm values to those of known amounts of SCV protein and was within the linear part of the standard curve for [^{125}I]protein A binding. The antigen contents of samples were then expressed relative to the values detected for SCVs (for equal amount of protein). Additional steps employed for accurate assessment of the combined Sbr/Cbr contents of chromaffin cells after toxin treatment involved extrapolation from a linear standard curve relating the cpm values (radiolabeled bands) to known quantities of non-toxin-treated chromaffin cells. Careful evaluation showed that all of the transferred antigen (Sbr or p38) was retained by the first of two PVDF membranes. Background cpm values on the blotted membranes were subtracted from samples. [^{125}I]protein A label was visualized on X-ray films by intensifying screen-enhanced autoradiography of the Western blots at −60 °C for appropriate periods.

Low Ionic Strength Protocol for Intoxication of Cell Cultures with BoNT/B. Chromaffin cells were prepared and cultured in 24-well plates (~2 × 10^6 cells/well) as previously described (Lawrence *et al.*, 1994); 2−3 days after plating, culture medium was removed, and following rapid rinsing with low-ionic strength buffer [mM: NaCl, 5; KCl, 4.8; $CaCl_2$, 2.2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; Hepes, 20; glucose, 5.6; sucrose, 220 (pH 7.4); and 0.5% (w/v) BSA], cells were incubated in the same buffer at 37 °C for 24 h in the absence or presence of BoNT/B (60 nM fixed concentration or 0.02−200 nM for dose dependency measurements), as described in the figure legend. Cells were then rinsed with, and incubated in, fresh culture medium, and experiments, as described in the figure legend, were performed 24 h later. Immediatly before experiments, cells were washed in buffer (mM: NaCl, 145; KCl, 5; NaH_2PO_4, 1.2; glucose, 10; Hepes, 20, pH 7.4). Buffers used for incubation of intact cells were 5 mM K^+ buffer (mM: NaCl, 145; KCl, 5; NaH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 10; Hepes, 20, pH 7.4) and 55 mM K^+ buffer (mM: NaCl, 95; KCl, 55; NaH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 10; Hepes, 20, pH 7.4). After 15 min, the catecholamine content of each was measured by a fluorometric procedure (von Euler & Floding, 1959); values for the 5 mM K^+ samples were subtracted from those for the 55 mM K^+ buffer to determine depolarization-dependent secretion. Alternatively, cells were permeabilized in digitonin (20 μM) buffer [mM: K^+glutamate, 139; piperazine-*N*,*N*′-[bis(2-ethanesulfonic acid)] (PIPES, pH 6.5), 20; ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5; D-600, 0.1; Mg·ATP, 2 μM; and 0.5% (w/v) BSA] omitting or containing either 20 μM Ca^{2+} or 1 mM Ba^{2+}. Catecholamine content of the digitonin buffer sample was subtracted from those containing Ba^{2+} or Ca^{2+} to calculate cation-dependent release. Six seperate wells from the same plate, identically-treated with toxin, were washed twice with Krebs buffer (to remove proteinaceous growth medium) and then scraped from the wells with a plastic pipette tip and solubilized in 150 μL/well of a toxin-inactivating buffer [0.1 M glycine·HCl, pH 2.5, containing 0.2 M NaCl, 1% (w/v) SDS, and 5 mM EDTA]. The pooled solubilized extracts were vortexed and allowed to stand on ice for 10 min prior to centrifugation (10000*g* for 5 min) to

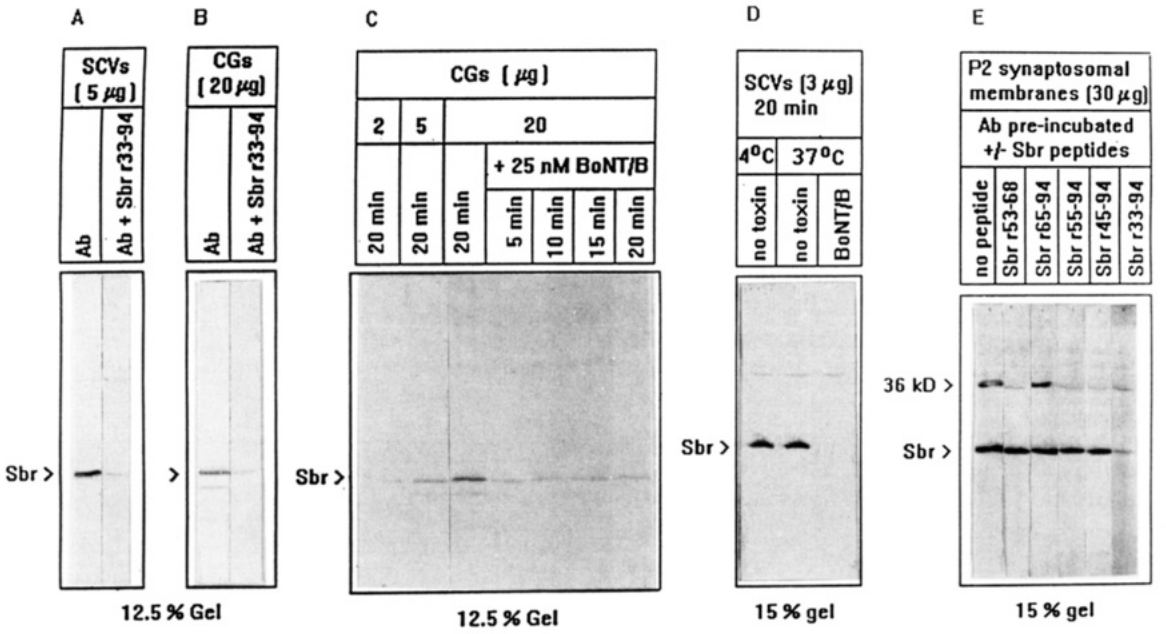

FIGURE 1: Characterization of the affinity-purified anti-Sbr antibody by immunoblotting of CGs and SCVs. The amounts of each protein sample specified were subjected to SDS−PAGE, on the percent gel indicated, and transferred to PVDF membranes before overnight incubation at 22 °C with affinity-purified anti-Sbr peptide antibody (dilution 1:100, equivalent to 2 μg/mL IgG). Where stated, the antibody was preincubated at 22 °C for 15 min with 2 μg/mL Sbr r33−94 (the immunogen) or the smaller synthetic Sbr peptides (the numbers describe its position in the sequence of human Sbr-2) before addition to the blotted membrane. In some cases, prereduced BoNT/B (25 nM) was incubated at 37 °C for the specified times (C) with 1 mg of protein/mL CGs or (D) 0.2 mg/mL SCVs (purified without the final Sephacryl S-1000 HR step) prior to SDS−PAGE. Toxin-free incubations were performed for 20 min at 37 °C (unless otherwise specified) to eliminate losses of immunoreactivity contributed by endogenous proteases in the vesicular fractions. Dilutions of the toxin-free CG incubations are included to aid assessment of the extent of toxin cleavage. Bound Sbr antibody was detected using alkaline phosphatase-conjugated secondary antibodies. Different developing times were used for each gel panel. The P2 synaptosomal membranes (E) were prepared from rat cortex as outlined in Ashton and Dolly (1988).

remove insoluble material; all of the Sbr reactivity was retained in the detergent supernatants (results not shown). The latter was concentrated by chloroform/methanol precipitation, as specified above, and the resultant pellet was redissolved overnight in 100 μL of buffer [0.1 M Tris·HCl, pH 6.8, containing 2% (w/v) SDS, and 1 mM EDTA]. Protein concentrations of the supernatants were measured by the colorometric method of Bradford (1976); equal amounts of protein were subjected to SDS−PAGE, electrophoretically transferred to PVDF membrane, immunoblotted for Sbr, and detected by [^{125}I]protein A binding (see above).

Measurement of BoNT/B-Mediated Cleavage of Sbr in Isolated CGs and SCVs. BoNT/B was prereduced with 20 mM DTT for 30 min at 37 °C before addition to purified CGs (P3 fraction, 1 mg of protein/mL) or bovine SCVs (P3 fraction, 0.2 mg of protein/mL). Incubations were performed in the absence or presence of 25 nM toxin in 50 mM Hepes·NaOH, pH 7.0, containing 0.3 M sucrose, 1 mM DTT, and 0.1 mM zinc acetate. At appropriate intervals, incubations were terminated by the addition of SDS−PAGE sample buffer [50 mM Tris·HCl, pH 6.8, 1.2% (w/v) SDS, and 0.005% (w/v) bromophenol blue containing 1% (v/v) β-mercaptoethanol and 1 mM EDTA] followed by immediate boiling. The samples were then analyzed by quantitative Western blotting, as detailed above. It should be noted that the antibody preparation used in the latter method recognizes intact Sbr or Cbr (see below) but is unreactive with their products after cleavage by BoNT/B; thus, decreases in the intensities of Sbr and Cbr bands after treatment of the samples with toxin provided a measure of the extents of cleavage. Lack of antibody reactivity with the cleavage products could reflect the loss of an immunodominant epitope and/or the degradation of the fragments by proteases in the membrane preparations used.

RESULTS

Preparation and Characterization of Antibodies Directed against the Hydrophilic Domain of Sbr. Rabbit polyclonal antibodies specific for Sbr were affinity purified from serum by specific adsorption to the immunogen (Sbr r33−94) immobilized on Sepharose 4B. Purity of the resultant antibodies was established by the sole presence of IgG bands on SDS−PAGE gels. A yield of 0.2 mg of IgG/mL of serum was obtained. On immunoblots of SCVs, a major band of apparent M_r = 20 kDa (on a 12.5% polyacrylamide gel) was obtained (Figure 1A) having an identical mobility to that visualized using an antibody raised against intact Sbr (de Paiva *et al.*, 1993), whereas CGs yielded two bands of similar mobility (Figure 1B,C); one had the same apparent M_r as Sbr, but the other was smaller ($M_r \sim 17$ kDa) and much less abundant. In each case, immunolabeling of the bands was diminished by inclusion of the immunogen (Figure 1A,B). Confirmation that this antibody reacts with Sbr or homologous proteins was provided by the reduced level of labeling seen following selective cleavage (see later) by BoNT/B of Sbr in bovine SCVs (Figure 1D) or CGs, as well as that of the lower M_r species present in the latter (Figure 1C). A time course of Sbr and Cbr cleavage by BoNT/B (Figure 1C) indicated a resistant fraction (see later). The 17-kDa band is unlikely to be a degradation product of Sbr because its intensity did not increase with incubation time in the absence of BoNT/B (data not shown). Instead, it probably represents the closely-related protein Cbr of this same apparent M_r, which is known to be cleaved by TeTx,

as observed here with BoNT/B; also, it has been shown to occur in adrenal medulla (McMahon *et al.,* 1993). The antibody would be expected to label Cbr because 60 out of 62 residues in the immunogen are identical between rat Cbr and Sbr-2 from human or rat. Thus, this smaller Sbr-like band has, reasonably, been regarded as Cbr in this study; in fact it has very recently been identified as Cbr using antibodies reactive exclusively with the latter (K. Foster, personal communication).

The Sbr-reactive antibody preparation additionally recognized a 36-kDa protein in rat P2 synaptosomal membranes (Figure 1E) and chromaffin cells (see later) in a specific manner, reactivity being diminished by preincubation with the immunogen. This 36-kDa protein differs from Sbr not only in size but also in being absent from purified CGs (Figure 1B) and SCVs that had been gel filtered on Sephacryl S-1000 HR (data not shown); only minimal amounts were detected in incompletely-purified SCVs (Figure 1D). Unlike Sbr/Cbr, the 36-kDa M_r protein was not proteolysed by BoNT/B in SCVs (Figure 1D) or chromaffin cells (see later). Accordingly, it was found in competitive Sbr-labeling studies to share only some Sbr epitopes (Figure 1E). Preincubating the antibody preparation with a number of smaller Sbr synthetic peptides revealed that immunoreactivity toward the 36-kDa band was reduced by peptides containing residues 53−68, whereas the complete structure was required to antagonize labeling of Sbr, pinpointing that residues 33−45 contribute, at least in part, to the reactivity.

Subcellular Localization of Sbr within the Adrenal Medulla. Using the procedure illustrated in Figure 2A, a substantial purification of CGs was achieved, as apparent from the most significant enrichment of DβH—their characteristic marker [reviewed in Winkler (1976) and see later]—in the P3 fraction relative to the crude homogenate or other fractions obtained (note that P3 is prepared from the P2 fraction) (Figure 2B). Fractionation of 100 mg of adrenal medullary homogenate protein yielded ~5 mg of CGs (P3) which represents ~30% of the total content, presuming that they constitute ~15% of the total protein (Winkler, 1976); higher recovery would probably be achieved if lysis of CGs could have been avoided. The significant amount of DβH in the S2P fraction is due to lysed CGs because following physical disruption or hypotonic lysis of CGs their membranes become much less dense and, thus, remain in the S2 supernatant [see later and Viveros *et al.* (1971) and Obendorf *et al.* (1988)] following pelleting of the intact CGs (Figure 2A). Compared to fresh adrenal medulla, no significant changes were noted in the relative amounts of Sbr antigens in chromaffin cells or their subcellular fractions 4 days after plating and culturing (data not shown).

Further immunoblotting experiments revealed that immunoreactivity toward Sbr and Cbr was present in bovine adrenal medullary homogenates, with a notable enrichment of both antigens in the P3 fraction which contains CGs (Figure 2B). Quantitation of the immunoblotting patterns was achieved by means of direct measurement of [^{125}I]protein A binding to IgG attached to the antigens. This revealed a 4.8 (± 0.6)-fold enrichment (±SEM; n = 4) of both Sbr and Cbr antigens (relative to the homogenate) in the P3 fraction (Table 1) which qualitatively appears to match the enrichment of DβH (detection by alkaline phosphatase method, Figure 2B). If it is presumed that the proteins of CGs comprise ~15% of total adrenal medullary protein (see above), then it can be estimated that the bulk (>72%) of the total Sbr and Cbr coisolated with CGs and their marker, DβH (a contribution from SLMVs was excluded, see later). S2P also exhibited a significant amount of Sbr/Cbr labeling; however, the presence of DβH in the latter fraction (Figure 2B), and its constant ratio to Sbr/Cbr in S2P and P3, suggests that lysed CGs therein could contribute a large proportion of this reactivity. Thus, the amount of Sbr/Cbr in the P3 fraction, together with that accounted for by the lysed CGs in the S2P fraction, would summate to an even higher total value than that cited above. Consistent with reports (Meyer & Burger, 1979) on the presence of plasma membranes in the S2P fraction, it was seen to be enriched in the marker HPC-1 (Figure 2B), a protein recently detected in chromaffin cells (Hodel *et al.,* 1994). Notably, low levels of HPC-1 were visible in the purified P3 fraction, reaffirming the purity of the CGs.

Table 1: Relative Proportions of Sbr/Cbr and p38 in Subcellular Fractions from Adrenal Medulla

fraction	% relative antigen content[a] p38	Sbr/Cbr	p38/Sbr ratio
homogenate		1.8 ± 0.3	
CGs/P3	2.4 ± 1.1	8.9 ± 1.5	0.27
S2P	7.6 ± 1.5	6.2 ± 1.4	1.2
CG membranes	8.9 ± 1.8	34.9 ± 5.9	
SCVs	100	100	1.0

[a] Subcellular fractions from adrenal medulla or cerebrocortical SCVs were subjected to SDS−PAGE, electrophoretically transferred to PVDF membranes, and immunoblotted with either anti-Sbr or -p38 antibodies. The relative amounts of primary antibody binding were quantified using the [^{125}I]protein A detection procedure detailed in methods. The average values (±SEM; n = 4) calculated are relative to those obtained for equivalent amounts of SCV protein.

Immunoblots of equivalent antigens in rat P2 synaptosomes (Figure 2C), run on the same gels as those in Figure 2B, established that their mobilities correspond to those from adrenal medullae; DβH is below the detectable level, and the Cbr band is absent, as shown above for bovine SCVs.

Sbr Is a Component of CG Membranes. To further demonstrate that the Sbr/Cbr immunoreactivity present in the CG fraction does not arise from a minor contamination with SLMVs (diameter ~60 nm; Navone *et al.,* 1986; De Camilli & Jahn, 1990), the P3 fraction was subjected to size-exclusion chromatography on a column of Sephacryl S-1000 HR (see methods). The majority of the Sbr/Cbr reactivities appeared in the void volume, coeluting with the CG marker DβH together with virtually all of the protein (Figure 3A); as expected, the large diameter of CGs (average size ~300 nm) excluded them from the gel pores. DβH observed in the inclusion fractions is attributable to a soluble form of the enzyme released upon lysis of some CGs that occurs during the fractionation; DβH is found in the vesicle lumen where ~60% is membrane-bound with the remainder being soluble [reviewed in Winkler *et al.* (1986)]. In contrast, the p38 antigen characteristic of SCVs [isolated by a modification of the method of Hell *et al.* (1988)] partitioned into the S-1000 resin (Figure 3B), consistent with their smaller size; the elution position coincided with a large protein peak. An equivalent shaped peak of protein or immunoreactivity toward Sbr or p38, within the inclusion volume, was not observed with the CG/P3 fraction (Figure 3A); relative to

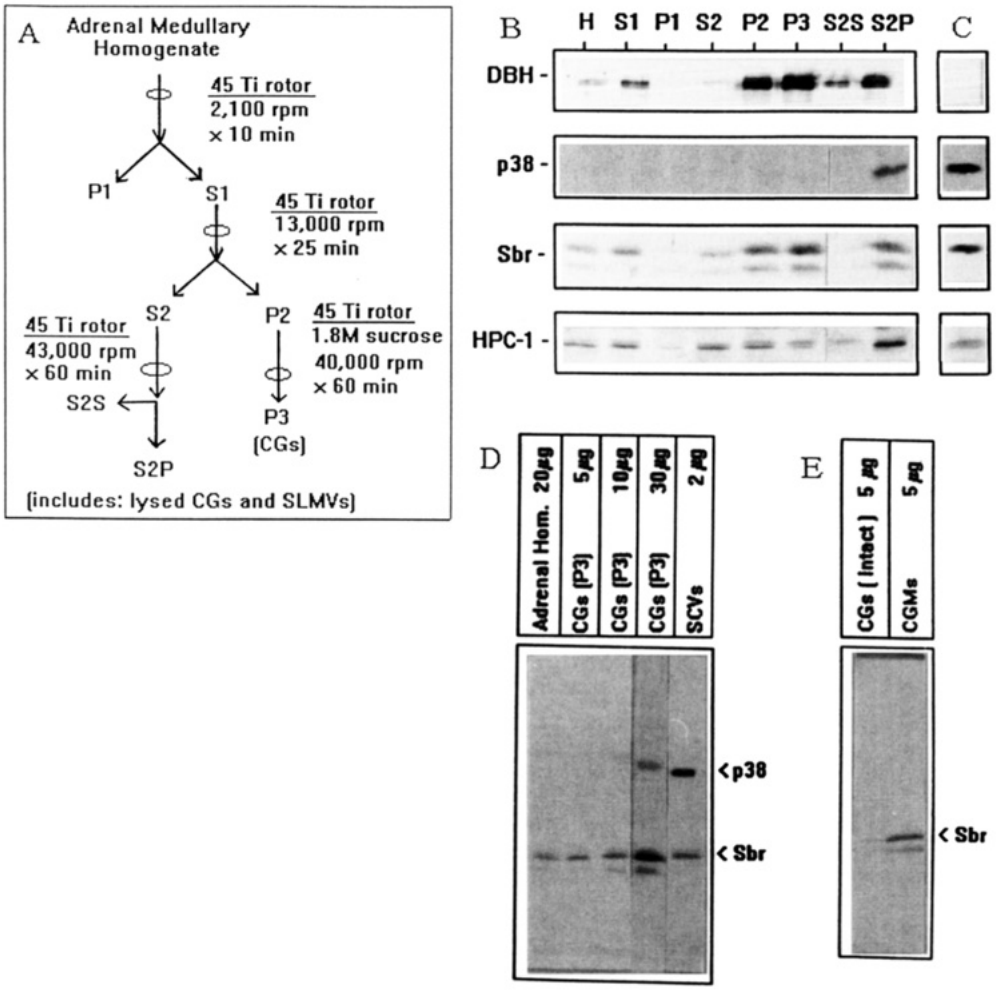

FIGURE 2: Preparation and characterization of subcellular fractions from bovine adrenal medulla. The specified amounts of fractions prepared by the method of Smith and Winkler (1967), with the modifications shown in panel A, were separated by SDS−PAGE and transfered onto PVDF membranes. The samples were incubated overnight at 22 °C with the requisite antibodies at the following dilutions: anti-DβH, 1:4000; anti-p38, 1:500; affinity-purified anti-Sbr,1:100; and anti-HPC-1, 1:500. Each primary antibody was blotted individually and visualized as in Figure 1; only the relevant track positions are shown in panels B and C. In the case of DβH blotting, 10-fold less protein was used per lane; otherwise, identical amounts of protein (20 μg) were loaded in each gel lane in panel B. In panel C, rat P2 synaptosomes (10 μg protein/lane) were treated similarly in order to compare the electrophoretic mobilities of the synaptic and adrenal antigens. Using the same immunoblotting method, the relative proportions of Sbr and p38 (D) in purified CGs (P3 fraction) and adrenal medullary homogenates were compared to SCVs purified on Sephacryl S-1000 HR. Panel E demonstrates the relative enrichment of Sbr and Cbr in CG membranes (CGMs) following hypotonic lysis of intact CGs to remove their large soluble protein content. Note that development times varied widely for the different antibodies and also between panels. These results are typical of at least two or three other CG fractionations.

the void peak, a very minor level of Sbr staining detected there is probably a result of tailing of the initial peak as p38 was totally absent.

When the CG-containing fraction (P3) was subjected to density gradient centrifugation (0.4−2.0 M sucrose), a majority of the loaded protein sedimented to the denser layers (Figure 4A), typical of CGs (Smith & Winkler, 1967); again, DβH detected at the top of the gradient arose from release of its soluble form when lysis of some CGs occurred. Figure 4A shows typical immunoblots of the gradient fractions with antibodies against Sbr, p38, or DβH. Immunoreactivities toward Sbr and DβH were highest for the dense CG fraction, while a lower level of both was found in a more diffuse lighter band. Lysed CG membranes which are known to be very much less dense most probably account for the latter. To confirm the identity of the broad lighter layer of Sbr and DβH, lysed CGs (see methods) were centrifuged on an identical sucrose gradient. As expected, this shifted a large proportion of the protein, Sbr, and DβH from the denser to lighter fractions (Figure 4B), exactly where the lower levels of Sbr staining were noted with the P3 fraction prior to lysis of CGs (Figure 4A). It is notable that after hypotonic lysis and freeze−thawing treatments, a portion of the CG protein remained at the original position near the bottom of the gradient; this is due to incomplete lysis. In a separate study using [^{125}I]protein A binding to the IgG labeled antigen, the lysed CG membranes were shown to give a 3.9 ($\pm$ 0.9)-fold enrichment ($\pm$SEM; $n = 4$) in Sbr/Cbr immunoreactivities over intact CGs (Table 1). This finding is also reflected in both the immunoblots of intact CGs and their membranes (Figure 2E) and by the observation that the bulk of the protein in CGs is lost upon lysis, with only 20−25% of total granule fraction protein being recovered in the membrane pellet (*i.e.*, membrane-bound) (data not shown).

The S2P fraction was also subjected to density gradient centrifugation. The presence of SLMVs in the latter fraction was indicated by its distinctly higher p38 to Sbr ratio relative to that of the CGs/P3 fraction (see Figure 2B and Table 1) which was 1.27 and 0.27, respectively. In this case (Figure 4C) a broad peak of Sbr, p38, and DβH was recorded,

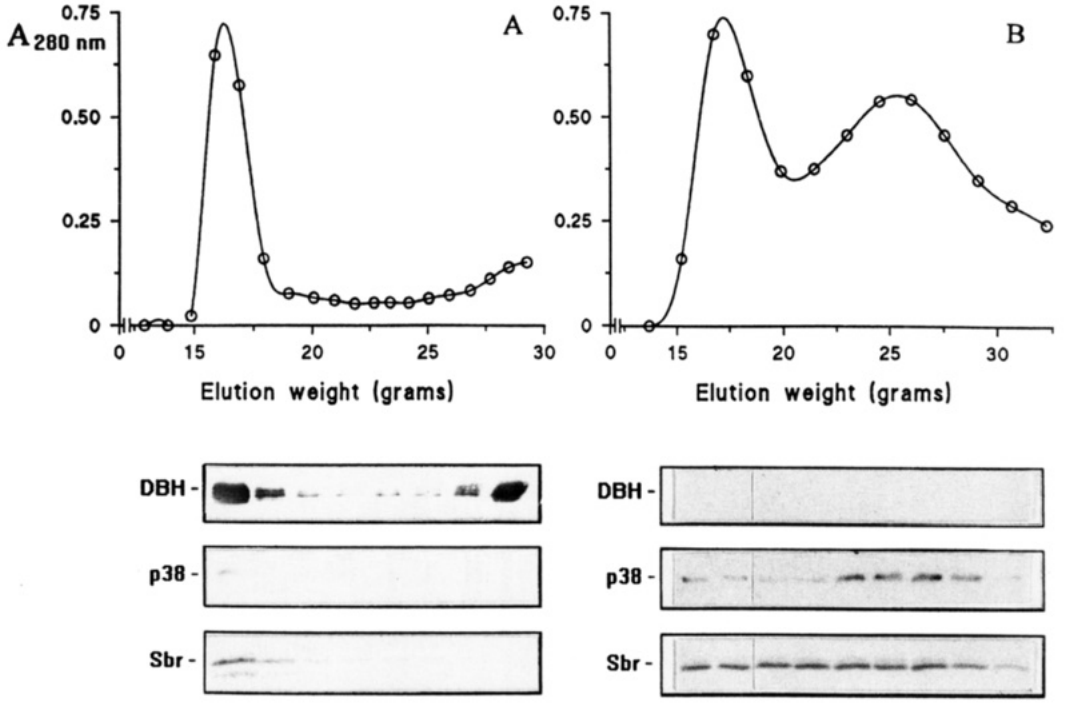

FIGURE 3: Size-exclusion chromatography of CGs and SCVs. CGs/P3 fraction (A; 0.6 mg of protein) or SVCs [B; 0.7 mg of protein, P3 fraction; see Hell *et al.* (1988)] were chromatographed in 0.3 M sucrose buffered with 10 mM Hepes·NaOH, pH 7.0, and containing 1 mM EDTA on a column (1 cm × 45 cm) of Sephacryl S-1000 HR. The elute was monitored for A_{280}, and each of the fractions in panel A was concentrated by chloroform/methanol precipitation prior to SDS−PAGE and Western blotting as detailed in Figure 2. A constant proportion of eight pairs of consecutive fractions from panel A (16 and 17 onwards) were blotted and nine paired samples from panel B (beginning at ~16.5 g) were blotted. Eluted fractions were measured by weight rather than volume in order to improve the accuracy and reproducibility. The results shown, obtained using very similar amounts of protein, are typical of two independent experiments for each vesicle type.

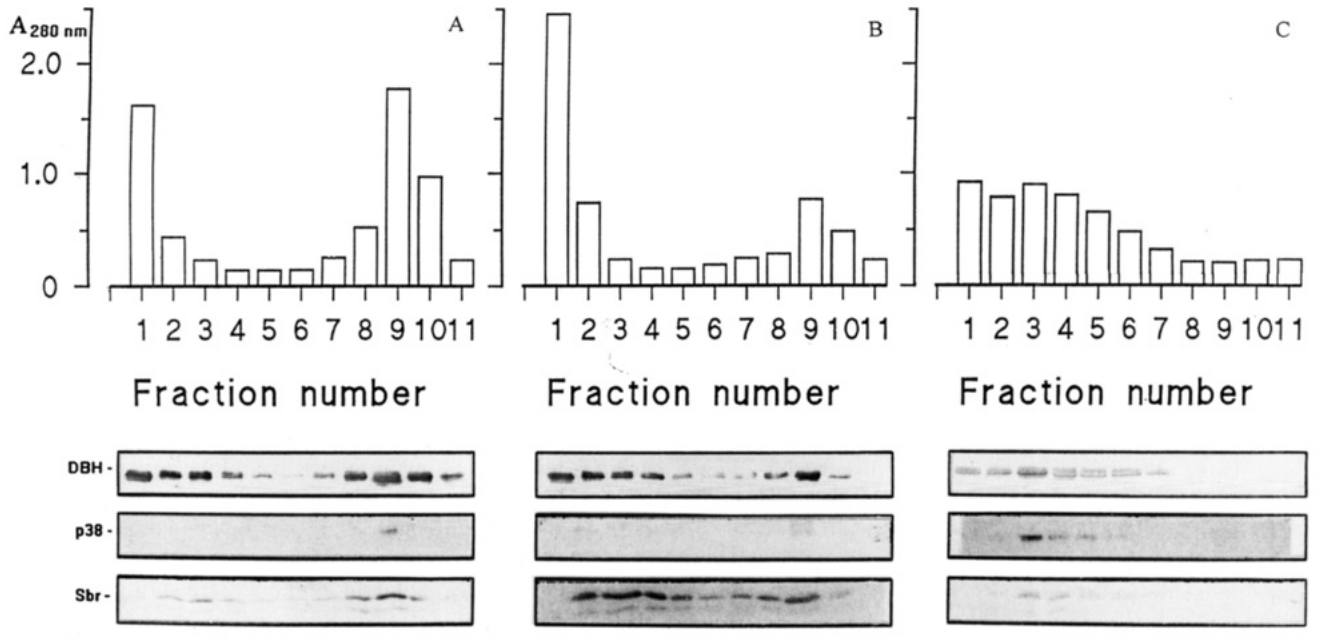

FIGURE 4: Density gradient centrifugation of subcellular fraction from adrenal medulla. CGs (A' 0.6 mg), partially lysed CGs (B; 0.6 mg; see methods), or S2P membranes (C; 0.9 mg) were subjected to centrifugation through a continuous sucrose gradient (0.4−2.0 M, including a 2.5 M sucrose pad). Fractions were collected from the top of the gradients, and A_{280} was monitored. The fractions were concentrated and subjected to Western blotting as in Figure 2. These results are typical of two or three independent experiments for each sample.

occupying a gradient position reminiscent of the lysed membranes of CGs but with the expected absence of the denser intact CGs. Athough it is likely that most of the Sbr immunoreactivity of this broad peak was contributed by CG ghosts membranes because of their notable content of DβH, the much higher p38 to Sbr ratio compared to CGs (see Figure 4A,B and Table 1) suggests that SLMVs coisolate with CG ghosts.

Although significant contamination by p38-containing SMLVs was excluded, even the most purified CGs contained a minute level of p38 [Table 1 and Lowe *et al.* (1988), Obendorf *et al.* (1988)]. When the Sbr and p38 contents in CGs and SCVs were determined by the [^{125}I]protein A labeling method, their concentrations in CGs relative to those in Sephacryl S-1000 HR-purified SCVs from bovine cerebral cortex were lower [8.9 ± 1.5% and 2.4 ± 1.1% (SEM; n =

4), respectively (Table 1)]. Western blots detected using alkaline phosphatase-conjugated secondary antibodies show these relative enrichments qualitatively (Figure 2D). However, when only the membrane proteins of CGs are considered in comparison to SCV (the latter do not contain lumenal storage proteins), the relative concentrations of Sbr and p38 compared to SCVs were ~35% and 9%, respectively (Table 1). The latter value is in very good agreement with that (<10%) determined for p38 by Obendorf *et al.* (1988).

BoNT/B-Mediated Proteolysis of Sbr and Cbr within Intact Chromaffin Cells Correlates with Its Blockade of Evoked Catecholamine Release. The above demonstration that Sbr and Cbr reside on the membrane of CGs warranted an examination of whether cleavage of both Sbr and Cbr by BoNT/B correlates with blockade of neurosecretion (Figure 5). Intact cultured chromaffin cells can be intoxicated by prolonged incubation with BoNT/B in a low-ionic strength buffer which greatly enhances its uptake (Marxen *et al.*, 1989). This method is preferable to permeabilization of cells where loss of secretory components might occur; also, the cells were returned to normal medium for 24 h before exocytosis was measured, thereby precluding any deleterious effect of exposure to the low-ionic strength buffer. The toxin presumably interacts with gangliosides on the extracellular surface and internalizes via a nonproteinaceous acceptor route. This method revealed a dose-dependent blockade by BoNT/B of neurosecretion to a maximium of >95% of that evoked from intact cells by 55 mM K^+ or from digitonin-permeabilized cells by 20 μM Ca^{2+} or 1 mM Ba^{2+} (Figure 5A). Inhibition was studied in conjunction with quantitative immunoblotting using [^{125}I]protein A detection to measure the amounts of Sbr/Cbr remaining uncleaved after various degrees of intoxication (Figure 5B). The radioactive contents of the excised [^{125}I]protein A-labeled sections of the blots from Figure 5B were expressed relative to those for toxin-free controls. A maximum toxin-mediated diminution of ~83% of Sbr and Cbr was recorded which corresponded to an almost complete block of exocytosis (Figure 5A,C). Two separate sets of experiments gave very similar results (Figure 5C) and highlight the strikingly close correlation between loss of Sbr/Cbr and the levels of exocytosis remaining. Additionally, examination of three batches of cells treated similarly with toxin consistently gave a ~70% reduction of Sbr and Cbr after exposure to a single concentration of BoNT/B (60 nM) and a corresponding ~80% blockade of exocytosis. The 36-kDa protein immunologically-related to Sbr remained uncleaved in BoNT/B-intoxicated cells (Figure 5B); its insensitivity to toxin treatment is reaffirmed in Figure 1D. Such BoNT/B-intoxicated cells showed no significant change in their protein staining patterns on SDS−PAGE (Figure 5D), which is in agreement with the toxin's selective proteolytic action. The presence of contaminating proteases in our purified toxin batches was monitored, using several metalloprotease substrates, and found to be negligible (Foran *et al.*, 1994). Furthermore, similar intoxication of cells with 60 nM BoNT/A produced no detectable alteration in the Sbr and Cbr levels (Figure 5E) yet resulted in significant blockade of exocytosis (~95%) of that evoked by Ba^{2+}.

DISCUSSION

Prior to this study, Sbr was believed not to be a component of CGs, a deduction based largely on the failure to detect it on LDCVs in neurons, by immunogold electron microscopy (Baumert *et al.*,1989). However, subcellular fractionation revealed that the majority of Sbr and Cbr coisolated with CGs. Furthermore, size-exclusion chromatography of CGs was performed to eliminate any contamination by SLMVs, a possibility raised by immunodetection of their marker, p38, in chromaffin cells (Navone *et al.*, 1986). The bulk of the Sbr reactivity remained within the void volume of the gel filtration column, demonstrating its occurrence on large diameter granules and excluding SLMVs which would have been included in the gel. The ability of this technique to remove vesicles of similar diameter to SLMVs was established by the clear separation of SCVs from the position where CGs eluted (Figure 3). Further evidence for Sbr and Cbr being on CGs was obtained by centrifugation on continuous sucrose gradients (rather than the discontinuous system used preparatively), based on the known differences in the densities of CGs, p38-enriched SLMVs (Obendorf *et al.*, 1988), and other membrane fractions, *i.e.*, coated vesicles (Maycox *et al.*, 1992) or plasma membranes (Meyer & Burger, 1979). Whereas Sbr/Cbr comigrated with CGs and their ghost membranes, the highest p38 content occurred in the S2P fraction. Thus, the Sbr/Cbr in the CG fraction cannot be ascribed to contamination by SLMVs; also, the S2P fraction gave a much larger ratio of p38 to Sbr compared to CGs, typical of SLMVs (De Camilli & Jahn, 1990). If the Sbr/Cbr of CGs arose by adherence or fusion of SLMVs, this would result in them inheriting the same p38/Sbr ratios, but this was not observed.

After having demonstrated minimal, if any, contamination of the purified CGs by SMLVs, a comparision was made of the relative amounts of Sbr and p38 in SCVs compared to CGs for functional reasons and, also, because a minimal level of p38 was recorded in the latter (Figures 3A and 4A), consistent with a previous report (Lowe *et al.*, 1988; Obendorf *et al.*, 1988). Use of a radiolabeled protein A detection method allowed these to be measured directly. Intact CGs were found to contain far less Sbr/Cbr and p38 than SCVs (~9% and ~2%, respectively); however, when only the membranes of CGs were considered, these values increased about 4-fold. This demonstrated presence of Sbr and Cbr in CGs accords with the recent detection of Sbr in amylase secretory vesicles from exocrine cells in the pancreas (Braun *et al.*, 1994). It has also been reported to be present on glucose transporter-containing vesicles from rat adipocytes (Cain *et al.*, 1992). Thus, these collective studies have established that this important protein occurs in neuronal, endocrine, exocrine, and other cell types that perform regulated exocytosis.

The observed location of the vast majority of Sbr and Cbr in CGs, and that their selective breakdown by BoNT/B closely correlates with its blockade of catecholamine release, offers strong evidence that either or both are essential components for catecholamine exocytosis. Following introduction of BoNT/B into intact chromaffin cells, almost complete blockade (~95%) of exocytosis ensued; thus, it is uncertain why ~17% of Sbr/Cbr reactivity remains without equivalent levels of secretion, an observation that could be interpreted to suggest other targets. One possibility is that a limiting level of Sbr exists below which exocytosis does not occur. Alternatively, this fraction of Sbr/Cbr might not have been cleaved because of being inaccessible in a membrane compartment or a newly-synthesized pool, neither of which are involved in the exocytotic pathway. As the

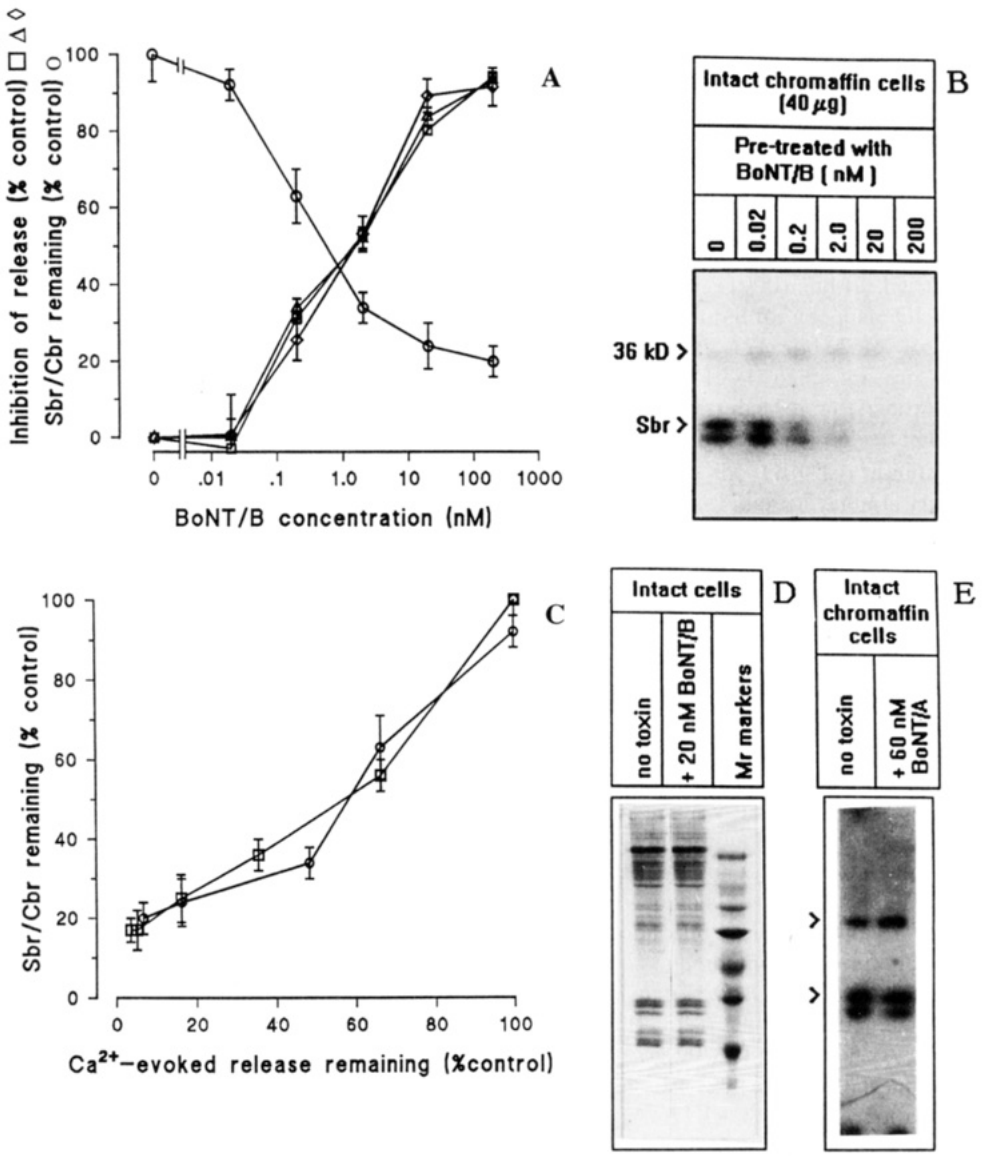

FIGURE 5: BoNT/B-mediated proteolysis of Sbr/Cbr in chromaffin cells and its dose-dependent blockade of Ca^{2+}-evoked secretion. Cells were incubated at 37 °C for 24 h in low-ionic strength buffer in the absence or presence of BoNT/B (A−D) or BoNT/A (E) and allowed to recover in culture medium at 37 °C for 24 h. Catecholamine release was evoked by 55 mM K^+ from intact cells (◇), or by permeabilization in a buffer containing 20 μM digitonin and either 20 μM Ca^{2+} (□), or 1 mM Ba^{2+} (△) (outlined in Methods) and measured after 15 min in aliquots of the bathing fluid (from three wells). The catecholamine remaining within cells in some wells was quantified in an aliquot after extraction with 1% (v/v) Triton-X-100. A representative value of total catecholamine content was calculated from the sum of the amounts remaining and that released. Secretion was expressed as a percentage of this value. BoNT/B inhibition of secretion was expressed (A) relative to that recorded for toxin-free control cells (±SD; $n = 4$). The amounts of Sbr/Cbr immunoreactivities in toxin-treated cells relative to toxin-free controls shown were quantified by [^{125}I]protein A detection of bound anti-Sbr antibody. The combined samples from six wells treated with each toxin concentration were solubilized in SDS containing buffer and concentrated by chloroform/methanol precipitation, prior to subjecting equal amounts of proteins to SDS−PAGE. After transfer to PVDF membranes, the samples were blotted with anti-Sbr IgG and amounts of the latter bound were quantified by labeling with iodinated protein A followed by autoradiography (B). The labeled bands of Sbr/Cbr were excised from the PVDF membranes and counted in a γ-spectrophotometer. Their radioactive contents were expressed (±SEM; $n = 4$) relative to those for toxin-free control cells and displayed (A) together with the values calculated for toxin-mediated inhibition of catecholamine release. Equivalent results from two independent BoNT/B dose dependency studies (○, □), are plotted in panel C. (E) Western blot of the amounts of Sbr/Cbr in BoNT/A (60 nM)-intoxicated cells (exhibiting ~95% inhibition of Ba^{2+}-evoked catecholamine release) compared to toxin-free control cells, visualized through autoradiography using the same methods as employed for BoNT/B (B); the arrows indicate the positions of the antibody-reactive 36-kDa and Sbr bands. (D) Equal amounts of solubilized protein from control and 20 nM BoNT/B-intoxicated cells used in panel B were subjected to SDS−PAGE and silver stained for total protein; protein markers used had the following M_r (kDa): 14.2, 20.1, 24, 29, 36, 45, and 66.

intoxication was performed over a long time scale and because the formation of hetero-oligomeric complexes between Sbr and other proteins is reversible, there would have been ample opportunity for the substrate's complete proteolysis. The absolute maximal inhibition seen does not, however, detract from the strikingly close correlation seen between the losses of Sbr/Cbr and the levels of exocytosis remaining in BoNT/B-poisoned cells. A requirement for relatively high BoNT/B concentration and long preincubation for maximal blockade compared to its *in vitro* potency on the mouse hemidiaphragm (de Piava *et al.*, 1993) probably reflects the lower efficiency of toxin internalization, due to the absence of high-affinity toxin acceptors on chromaffin cells. The inhibition of exocytosis by BoNT/B is consistent with its selective cleavage of Sbr/Cbr because intoxicated cells showed no detectable change in the overall protein

staining pattern on SDS−PAGE, thereby excluding a non-specific toxin-mediated or contaminating protease-mediated proteolytic mechanism. Detailed enzymological studies to date suggest that BoNT/B and TeTx are highly selective proteases requiring very large minimal peptide substrates of 35 and 62 residues, respectively, for notable hydrolysis (Shone *et al.*, 1993; Foran *et al.*, 1994).

Although there are considerable differences between the regulated neurosecretion pathways for SCVs and that occurring from CGs in chromaffin cells, many of these specializations may be contributed by other distinct components that interact with the core proteins (Sbr, syntaxin 1A, and SNAP-25). Although the latter has been characterized in neurons [reviewed by Dolly *et al.* (1994)], in a very recent report, such a putative docking/fusion complex has been shown between their counterparts from chromaffin cells (Roth & Burgoyne, 1994). This lends further support to the role of Sbr in catecholamine release, demonstrated herein, contrary to the deductions from indirect experiments cited in the literature (*cf.* introduction). Future studies examining proteolysis of syntaxin 1A and SNAP-25 by BoNT/A and -/C1 may offer evidence for their involvement in Ca^{2+}-evoked catecholamine exocytosis, as established herein for Sbr/Cbr.

ACKNOWLEDGMENT

The authors are grateful to Dr. C. Shone for his provision of BoNT/A and -B in addition to the Sbr peptides. Prof. D. Apps is thanked for providing antiserum to DβH. Critical reading of the manuscript by Drs. K. Foster, M. J. Duggan, A. Ashton, and A. de Paiva is much appreciated.

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BI9425866