Botulinum Neurotoxin B Inhibits Insulin-Stimulated Glucose Uptake into 3T3-L1 Adipocytes and Cleaves Cellubrevin Unlike Type A Toxin Which Failed To Proteolyze the SNAP-23 Present†

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ABSTRACT: Types A, B, and C1 botulinum neurotoxin (BoNT), a group of selective Zn^{2+}-dependent endoproteases, have been instrumental in demonstrating that their respective substrates [synaptosomal-associated protein with M_r = 25 kDa (SNAP-25), synaptobrevin (Sbr), and syntaxin] are essential for regulated exocytosis from nerve terminals and neuroendocrine cells. The colocalization of Sbr, or its homologue cellubrevin (Cbr), in the majority of the glucose transporter-isotype 4 (GLUT4)-containing vesicles from adipocytes implicates their involvement in insulin-stimulated glucose uptake, which results in part from enhanced fusion of these vesicles with the plasmalemma. In this study, exposure of cultured 3T3-L1 adipocytes to BoNT/B in a low-ionic strength medium was found to block insulin-evoked glucose uptake by up to 64%. BoNT/B was shown by immunoblotting to cause extensive proteolysis of Cbr and Sbr resulting in a significant blockade of the insulin-stimulated translocation of GLUT4 to the plasmalemma. This establishes that these two toxin substrates contribute to the insulin-regulated fusion of GLUT4-containing vesicles with the plasmalemma, at least in this differentiated 3T3-L1 clone. Although SNAP-25 was not detectable in the differentiated adipocytes, its functional homologue SNAP-23 is abundant and largely confined to the plasmalemma. SNAP-23 proved to be resistant to cleavage by BoNT/A. Consistent with these results, type A did not block insulin-induced glucose uptake, precluding a demonstration of its likely importance in this process.

Insulin stimulates glucose uptake into adipocytes by inducing the fusion of vesicles containing glucose transporters (GLUT)[1] with the plasmalemma (Zorzano *et al*., 1989). Many studies of this process have employed the 3T3-L1 fibroblast cell line which can be differentiated into an adipocyte-phenotype when treated with insulin, dexamethasone, and (isobutylmethyl)xanthine (IBMX) (Frost & Lane, 1985). The resultant cells respond to insulin with a rapid and significant increase in glucose transport [between ∼10-fold (Piper *et al*., 1991) and 15−20-fold (Frost & Lane, 1985; Calderhead *et al*., 1990), depending on the cell clone employed]. As well as containing a constitutive GLUT isotype 1, these differentiated cells also express GLUT4, the form specific to muscle and fat tissues where transport is dramatically stimulated by insulin (James *et al*., 1989). GLUT1 and GLUT4 have been shown by immunoprecipitation to reside largely on distinct vesicle populations in mature rat adipocytes (Zorzano *et al*., 1989) and 3T3-L1 adipocytes (Piper *et al*., 1991), though another study suggested that both isotypes can predominantly coexist in the same vesicular compartment of another clone of these cells (Calderhead *et al*., 1990). The demonstrated colocalization therein of synaptobrevin (Sbr; also known as vesicle-associated membrane protein, VAMP; Trimble *et al*., 1988; Elferink *et al*., 1989) and a closely related homologue cellubrevin (Cbr; McMahon *et al*., 1993) in the majority of GLUT4-containing vesicles raises the possibility of these being involved in mediating the insulin-regulated fusion mechanism (Cain *et al*., 1992; Volchuk *et al*., 1995) because Sbr is believed to be an essential component of neuroexocytosis. It interacts *in vitro* with two synaptic plasma membrane proteins (Söllner *et al*., 1993), syntaxin 1 A/B (Bennett *et al*. 1993) and synaptosomal-associated protein with M_r = 25 kDa (SNAP-25; Oyler *et al*., 1989); these are proposed to comprise the core of the docking/fusion complex which links synaptic small clear vesicles to the release sites at the active zones of nerve endings (Söllner *et al*., 1993; Hayashi *et al*., 1994), thereby providing the targetting specificity and/or the fusion apparatus necessary for transmitter release.

Support for that hypothesis has been gained through studies on the inhibition of exocytosis by tetanus toxin (TeTx) and serotypes of botulinum neurotoxin (BoNT/A−G). These are similar but immunologically distinguishable proteins produced by *Clostridium tetani* and *Clostridium botulinum*, respectively, and are composed of a disulfide-linked heavy chain (HC) and light chain (LC). HC is responsible for

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[1] Abbreviations: BoNT, botulinum neurotoxin; BSA, bovine serum albumin; Cbr, cellubrevin; CYT, cytosol; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GLUT, glucose transporter; HC, heavy chain; HDM, high-density microsomes; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethansulfonic acid; IBMX, (isobutylmethyl)xanthine; Ig, immunoglobulin; LC, light chain; LDM, low-density microsomes; LISM, low-ionic-strength medium; PBS, 10 mM potassium phosphate buffer, pH 7, containing 150 mM NaCl; PM, plasma membrane; Sbr, synaptobrevin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNAP-25, synaptosomal-associated protein of M_r = 25 kDa; TeTx, tetanus toxin.

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binding to high-affinity neuronal ecto-acceptors, subsequent internalization and translocation of the LC into the cytosol where LC blocks exocytosis [reviewed in Simpson (1986) and Dolly *et al*. (1992, 1994)]. The LCs of BoNTs and TeTx are Zn^{2+}-dependent endoproteases [reviewed in Montecucco and Schiavo (1993) and Niemann *et al.* (1994)]. Sbr is proteolyzed by TeTx and BoNT/B, /D, /F, and /G (Schiavo *et al*., 1992, 1993a,b; Link *et al*., 1992; Yamasaki *et al*., 1994); Cbr is also cleaved by TeTx and BoNT/B (McMahon *et al*., 1993; Foran *et al*., 1995). BoNT/A and /E proteolyze SNAP-25 at separate sites (Blasi *et al*., 1993a; Schiavo *et al*., 1993c), while BoNT/C1 cleaves both syntaxin (Blasi *et al*., 1993b) and SNAP-25 (Foran *et al*., 1996).

Although most studies on these toxins have been performed on neuronal preparations, Ca^{2+}-evoked catecholamine release can be blocked when BoNT is applied intracellularly into adrenomedullary chromaffin cells (Bittner *et al*., 1989; Marxen *et al*., 1991; Foran *et al*., 1995). Accordingly, all of the toxins' targets have been found in chromaffin cells and good correlations demonstrated between the levels of toxin-mediated substrate cleavage and inhibition of secretion (Foran *et al*., 1995; Lawrence *et al*., 1996), highlighting the importance of these proteins in regulated exocytosis from large dense-core vesicles. Boyd *et al*. (1995) reported a requirement of Sbr/Cbr and SNAP-25 for Ca^{2+}-dependent insulin secretion from one of two insulinoma cell lines tested. Using permeabilized 3T3-L1 adipocytes, Volchuk *et al*. (1996) has demonstrated that antibodies selective for syntaxin 4 inhibit insulin-stimulated glucose uptake (albeit only partially), but the susceptibility of this process to BoNT serotypes was not assessed. On the other hand, BoNT/B was found to cleave the majority of Cbr and Sbr-2 in streptolysin-O-permeabilized 3T3-L1 cells, yet this resulted in only minimal (15%) inhibition of the insulin-dependent component of glucose uptake (Tamori *et al*., 1996). To date, there is no evidence of a role in this process for the target of BoNT/A; SNAP-25, through its mRNA, has been detected in the cells (Jagadish *et al*., 1996); thus, it was warranted to address this question in this study. To extend the evidence for the importance of the BoNT substrates, attempts were made to maximize the possibility for toxin inhibition of insulin-induced glucose uptake by introducing BoNT/A, /B, or /C1 into intact 3T3-L1 cells via a method proven to be successful with chromaffin cells (Lawrence *et al*., 1996; Foran *et al*., 1996). In this way, Cbr and Sbr were shown to be concerned with regulated membrane fusion. In contrast, enhancement of glucose uptake by insulin was unaltered by BoNT/A, consistent with the observed absence of SNAP-25 and the presence on the plasmalemma of its functional homologue SNAP-23 (Ravichandran *et al*., 1996), a novel protein not cleaved by this toxin.

EXPERIMENTAL PROCEDURES

Materials. A 3T3-L1 fibroblast clone (obtained from ATCC; no CCL 92.1) was supplied by the European Collection of Animal Cell Cultures (Salisbury, U.K.). Cell culture reagents were from GIBCO BRL (Paisley, Scotland) and immobilon-P membrane was from the Millipore Corporation (Bedford, MA). High-purity digitonin was purchased from Novabiochem (U.K.). 2-Deoxy-D-[2,6-^{3}H]-glucose and ECL-antibody detection system were from Amersham International (Buckinghamshire, U.K.). Affinity-isolated anti-species-specific immunoglobin (Ig) conjugated with alkaline phosphatase or horseradish peroxidase, bovine serum albumin (BSA) fraction V, insulin, unlabeled 2-deoxy-D-glucose, dexamethasone, IBMX, plus all other reagents were obtained from Sigma Chemical Co. (Dorset, U.K.).

A monoclonal antibody against GLUT4 (clone 1F8; James *et al*., 1989) was from Genzyme (Kent, U.K.). Rabbit antiserum generated against a C-terminal peptide corresponding to amino acids 498−509 of rat GLUT4 (Piper *et al.*, 1991) was purchased from Biogenesis Ltd. (Dorset, U.K.). Affinity-purified Ig against the last 12 C-terminal residues of GLUT1 was kindly provided by Dr. Stephen Baldwin, University of Leeds. Antibodies were raised in rabbits against a soluble recombinant His^6-tagged mouse syntaxin 1A lacking the last 27 C-terminal residues (kindly provided by D. Liu, of this laboratory) and affinity-purified on immobilized antigen (Foran *et al*., 1996). Alternatively, identification of syntaxins 1A and B was carried out using a monoclonal antibody (HPC-1) purchased from Sigma. Affinity-purified Ig raised against residues 33−94 of human Sbr-2 (a region of amino acid sequence shared with Sbr-1 and Cbr; McMahon *et al*., 1993) was prepared, as detailed elsewhere (Foran *et al*., 1995). Likewise, a rabbit antiserum to the C-terminus of SNAP-25 (residues 195−206) was produced and affinity-purified, as described by Lawrence *et al*. (1996). Antisera were generated in rabbits against peptides corresponding to either the first 20 N-terminal residues of Cbr (MSTGVPSGSSAATGSNRRLC) or the last 11 C-terminal residues of SNAP-23 (CANARAKKLIDS), after being covalently conjugated through the additional cysteines incorporated onto amino groups present on BSA using the bifunctional cross-linking agent 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester. Specific Ig was affinity-isolated from immune sera using the immunogenic peptide coupled to iodoacetyl-Sepharose 4B [prepared as detailed in Lawrence *et al.* (1996)]. Each affinity-purified Ig was tested to ensure that it labeled the requisite band of appropriate M_r and bound to the respective antigen on blots in a manner that was preventable by inclusion of the immunogen (data not shown). Prior to use, all synthetic peptides were highly purified using reverse-phase high-performance liquid chromatography and their identities shown to be correct by mass spectrometry (expertly performed by Roy McDowell and Dr. Richard Easton, with thanks). BoNT/A and /B were purified by modifications of the process described for BoNT/F (Wadsworth *et al*., 1990), as specified in Shone *et al*. (1993). BoNT/B was fully nicked as described by Evans *et al*. (1986). BoNT/Cl 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 sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) with Coomassie staining of protein; also, they all exhibited the high levels of toxicity (assessed by mouse bioassay) previously reported.

Cell Culture and Adipocyte Differentiation. 3T3-L1 fibroblasts (passages 3−10 of the cells received) were seeded at 2×10^3 cells/cm^2 in 6- or 24-well tissue culture plates and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Two days after reaching confluence, differentiation of the cells was induced

for 48 h in DMEM (supplemented as above) containing 0.5 mM IBMX, 0.25 mM dexamethasone, and 1 μg/mL insulin, followed by a further 48 h period in the above medium lacking IBMX and dexamethasone. The cells were then maintained in DMEM (supplemented) without additions for 4 days with the medium being replaced every 2 days (Frost & Lane, 1985).

Exposure of the Cells to BoNT/A, /B, and /C1. Cultures of confluent 3T3-L1 fibroblasts or differentiated adipocytes were rinsed rapidly with a low-ionic-strength medium [LISM; consisting (in millimoles per liter) of 5 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5 $NaHCO_3$, 0.5 $CaCl_2$, 20 Hepes·NaOH, pH 7.4, 10 glucose, 220 sucrose, and 0.5% (w/v) BSA] before incubation at 37 °C for 24−48 h in the same buffer in the absence or presence of BoNT/A, /B, or /C1, at concentrations specified in the figure legends. After rinsing once with DMEM (supplemented as described above), the cells were re-equilibrated with fresh medium for 24 h at 37 °C before experiments were performed. In some initial experiments, attempts were made to poison cells by exposure to BoNT in DMEM.

Quantification of Glucose Uptake into Adipocytes. The uptake of 2-deoxy-D-[2,6-^{3}H]glucose was determined using methods outlined in Merrall *et al*. (1993) with minor modifications. After rinsing the cultures twice with serum-free DMEM, the cells (on 24-well plates) were equilibrated for 2−3 h at 37 °C with the same medium and washed three times briefly with 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS). The cells were then covered with an aliquot of buffer A [(in millimoles per liter): 136 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 10 Hepes·NaOH, pH 7.4, and 0.5% (w/v) BSA]. Following a 15 min preequilibration at 37 °C in buffer A, a further 20 min incubation was performed in the absence or presence of insulin (final concentration of 100 nM, determined to induce a near-maximal glucose uptake response in 3T3-L1 adipocytes). Uptake was measured over 5 min following addition of 2-deoxy-D-[2,6-^{3}H]glucose (100 μM; 1.5 μCi/mL), after which time the wells were washed four times briefly with ice-cold PBS. Cells were solubilized by the addition of 1 mL of 0.1% (w/v) SDS; after removal of an aliquot for protein determination (Markwell *et al.*, 1978), the radioactivity associated with each well was determined by scintillation counting. Results are expressed as picomoles of deoxyglucose translocated per milligram of protein per minute. In some experiments, the values measured for non-insulin-treated (basal) cells were subtracted from the requisite totals obtained in the presence of insulin to yield figures for the insulin-dependent deoxyglucose uptake; inhibition of the latter by toxin was calculated as a percentage of the requisite control level. Standard deviations for the insulin-dependent component were derived from the equation $[(SD + insulin)^2 + (SD - insulin)^2]^{1/2}$. Statistical analyses were performed throughout this study using Student's *t* test for paired data. The level of nonspecific uptake was measured following pretreatment of cells with a potent inhibitor of the glucose transporter, cytochalasin B [final concentration = 25 μM; Bloch (1973)]; this represented ~11% of the basal uptake and was subtracted from all the values presented.

Subcellular Fractionation of Cultured 3T3-L1 Cells, Chromaffin Cells, and Cerebral Cortex. 3T3-L1 fibroblasts, or those differentiated into adipocytes, were grown in 6-well plates (~$1-2 \times 10^6$ cells/well) and rinsed twice with ice-cold PBS before being homogenized in the presence of protease inhibitors and fractionated by centrifugation at 4 °C, as detailed for chromaffin cells (Lawrence *et al*., 1996). The homogenate was adjusted to 0.32 M sucrose and then centrifuged at 1000*g* max for 10 min to yield the P1 membrane pellet. The resultant supernatant (S1) was re-centrifuged at 15000*g* max for 20 min to yield a crude plasma membrane fraction (P2); the supernatant was centrifuged in a Beckman SW60Ti rotor at 260000*g* max for 1 h to sediment all remaining membranes (P3), which included high- and low-density microsomes plus the majority of GLUT4-containing vesicles. Where specified, total membranes were prepared by centrifuging the S1 supernatant in a Beckman SW60Ti rotor at 390000*g* max for 40 min. All membrane pellets were resuspended in 100 mM 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 10000*g* max for 10 min, and protein concentrations of the supernatants were determined by the colourimetic method of Markwell *et al*. (1978) and from absorbancies at 280 nm. Synaptosomal plasma membranes (termed synaptic membranes) were prepared from rat cerebral cortex using the method of Jones and Matus (1974).

Alternatively, high-density microsomes (HDM), low-density microsomes (LDM), and plasmalemma (PM)-enriched fractions were isolated, as described by Piper *et al*. (1991), from control and BoNT/B-treated adipocytes for the purpose of monitoring the translocation of GLUT4 from LDM to PM and HDM (in response to insulin) by immunoblotting (see below) and to study the subcellular localization of antigens. The high-speed supernatants containing the cytosolic proteins following the pelleting of LDM were precipitated using a chlorform−methanol procedure (Wessel & Flugge, 1984) prior to preparation for immunoblotting.

Permeabilization of Cells with Digitonin in Order To Observe the Proteolytic Activities of BoNT/A, /Cl, or /B. Bovine chromaffin cells were prepared from adrenal glands and maintained in culture, as described previously by Lawrence *et al*. (1994). These cells and differentiated 3T3-L1 adipocytes were washed three times with buffer A, prior to exposure in the absence or presence of prereduced 100 nM BoNT/A, /Cl, or /B during permeabilization at 37 °C for 30 min with 20 mM digitonin in KGEP buffer [139 mM potassium glutamate, 5 mM EGTA, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [PIPES], pH 7.0], which included 2 mM $MgCl_2$ and 2 mM ATP. BoNTs were reduced with 20 mM dithiothreitol in 25 mM Hepes·NaOH, pH 7.4, containing 0.15 M NaCl for 30 min at 37 °C before dilution in digitonin−KGEP and application to cells; neurotoxin-free control cells were exposed to the same final dithiothreitol concentration which never exceeded 1 mM. After incubation, the latter medium was aspirated and replaced with 50 mM $NaHCO_3$, pH 8.5, containing numerous protease inhibitors [detailed in Lawrence *et al*. (1996)], before homogenizing and sedimenting the total membrane fractions at 390000*g* max for 40 min. The resultant pellets were solubilized in SDS-containing buffers and processed for immunoblotting as described in the next section.

Immunoblotting and Quantitation of Antigens. Samples were subjected to SDS−PAGE (either 10 or 12.5% acrylamide), electrophoretically transferred to immobilon-P mem-

brane, and treated with blocking solution [20 mM Tris·HCl, pH 7.4, containing 150 mM NaCl, 4% (w/v) skimmed milk powder, 1% (w/v) BSA]. Western blotting was carried out as detailed by Foran *et al*. (1996), with minor modifications. Primary antibodies (at dilutions indicated in the figure legends) were incubated with the membranes overnight, followed by washing (three times briefly, followed by four 10 min washes) with the above-noted buffer containing 0.1% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20). Antibodies bound to the membranes were detected using either anti-species-specific Ig conjugated to alkaline phosphatase (visualized using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium) or horseradish-peroxidase-coupled anti-species-specific Ig (visualized by chemiluminesence using the ECL-detection system). For both methods, the secondary antibodies were incubated (at a 1:1000 dilution) for 2 h at 25 °C in blocking solution, followed by washing (as specified earlier) and subsequent visualization. Immunoblots were quantified by densitometric scanning, using image analysis software (NIH image V 1.57), and the resultant values were expressed as a percentage of that for the requisite toxin-free control.

RESULTS

BoNT/B and /C1, but Not /A, Inhibit Insulin-Stimulated Glucose Uptake by 3T3-L1 Adipocytes. Fibroblasts exhibited a low level of glucose uptake and insulin failed to significantly increase this value (Figure 1A). Differentiation of confluent cultures of 3T3-L1 fibroblasts into adipocytes (see Experimental Procedures) produced 2.4- and 6.4-fold increases in basal- and insulin-stimulated total glucose uptake compared to the values for undifferentiated fibroblasts (Figure 1A), in accordance with published data (see introductory section). Note that insulin gave a 3.6-fold increase in glucose uptake into differentiated adipocytes above the basal level (Figure 1A); the insulin-induced increment is somewhat low, probably due to the resting value being higher than that reported elsewhere. With the goal of investigating the effect of BoNT on insulin-stimulated glucose uptake, methodologies for introducing the toxins inside these cells were evaluated. As prolonged incubation of intact chromaffin cells with BoNT/A in a low-ionic-strength medium (LISM) achieved complete blockade of Ca^{2+}-evoked secretion, even though the high-affinity cholinergic ecto-acceptors are absent (Foran *et al*., 1995, 1996; Lawrence *et al*., 1996), this procedure was applied to adipocytes. Moreover, this intoxication is preferable to cell permeabilization protocols where loss occurs of cytosolic components essential for exocytosis, thereby reducing the levels of insulin-stimulated GLUT4-translocation and labeled glucose uptake by at least 50% (compared to intact cells) followed by entire loss of function if permeabilization exceeds 30 min (Clarke *et al*., 1994). In preliminary experiments, incubation of intact adipocytes with 200 nM BoNT/B in DMEM resulted in a reduction [30.7 ± 8.4% (±SD; $n = 4$; $p < 0.005$)] of the insulin-stimulated increment of glucose uptake relative to that for toxin-free control cells (Figure 1B). Notably, when treatment with BoNT/B was performed in LISM, an approximate doubling of the extent of inhibition [56.4 ± 5.8% (±SD; $n = 4$; $p < 0.001$)] of this same component of glucose uptake was observed (Figure 1B). Some variability was noted in the extents of inhibition (±SD; $n = 3$ or 4; all p values < 0.001), 43.0 ± 7.4, 43.1 ± 4.5, 45.7 ± 10.3, 53.3 ± 6.4, 57.6 ± 5.8, 64.7 ± 8.4 (average inhibition = 51.2% with 400 nM BoNT/B in LISM), of the insulin-stimulated increment for an additional six different batches of differentiated adipocytes. Recent studies by Tamori *et al*. (1996) which introduced BoNT/B using streptolysin-O-permeabilization achieved only minimal inhibition of this component (see introductory section). While the method of poisoning used herein was more effective than reported for streptolysin-O-permeabilized cells (Tamori *et al*., 1996), it did cause a reduction of 31.5 ± 9.1% (±SD; $n = 4$; $p < 0.005$; Figure 1B) in the control level of response to insulin. However, this loss is far less than that observed after permeabilization (Clarke *et al*., 1994; Tamori *et al*., 1996). Preincubation of cells with increasing concentrations of BoNT/B in LISM yielded a dose-dependent blockade of insulin-induced glucose uptake, with a smaller but significant decrease in the basal

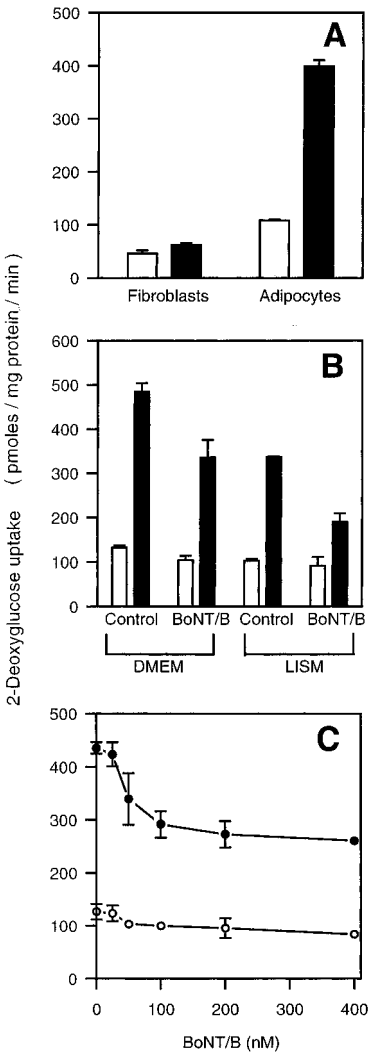

FIGURE 1: Inhibition by BoNT/B of glucose uptake into cultured fibroblasts and adipocytes. 3T3-L1 fibroblasts were grown to confluence in supplemented DMEM, and some wells were differentiated into adipocytes; basal (open symbols) and insulin-stimulated (closed symbols) uptake of [^{3}H]-2-deoxyglucose were measured as detailed in Experimental Procedures. In panel A, non-toxin-treated fibroblasts and adipocytes were used but, otherwise, exposed to LISM in the manner described for panels B and C. Adipocytes were preincubated for 24 h at 37 °C in the absence (control) or presence of fixed (panel B, 400 nM in DMEM or LISM) or varied (panel C) BoNT/B concentrations in LISM and equilibrated in DMEM for 1 day before measuring uptake of [^{3}H]-2-deoxyglucose. Values plotted are the mean (±SD; $n = 4$); error bars are omitted when they are smaller than the symbols.

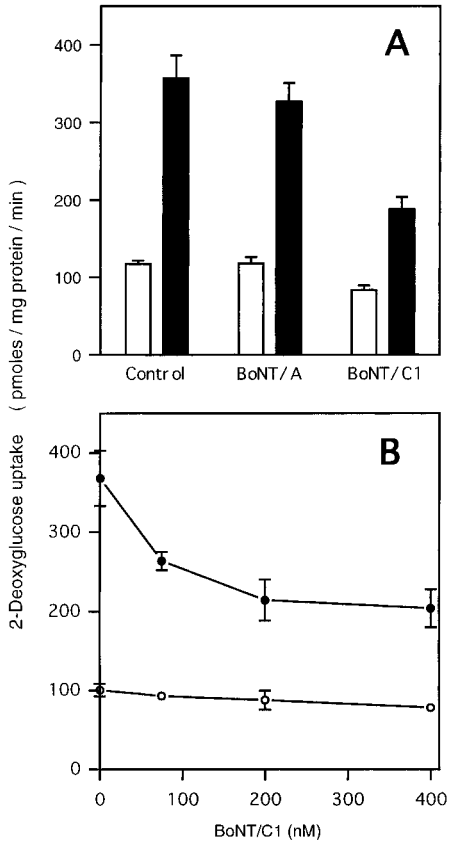

FIGURE 2: Treatment of adipocytes with BoNT/C1 but not /A reduces basal- and insulin-induced uptake of glucose. Adipocytes were incubated for 24 h in LISM at 37 °C in the absence (control) and presence of 400 nM BoNT/A, 400 nM /C1 (A) or various concentrations of the latter (B) before re-equilibration and measurement of basal- (open symbols) or insulin-induced (closed symbols) uptake of [^{3}H]-2-deoxyglucose, as detailed in Figure 1. Data are the mean (±SD; $n = 4$).

values [for the 400 nM BoNT/B concentration the basal value was lowered by 29.8 ± 5.1% (±SD; $n = 4$; $p < 0.005$); Figure 1C], consistent with the observations of Tamori *et al*. (1996). Attempts to achieve a more complete blockade of the insulin-stimulated glucose uptake by prolonging the exposure to toxin (up to 48 h) or performing multiple additions of toxin over that period proved unsuccessful (not shown).

Having demonstrated that insulin-dependent glucose uptake could be attenuated by BoNT/B and defined the optimal incubation conditions, the inhibitory effects of other BoNT types that have different substrates (see introductory section) were evaluated. Interestingly, a 24 h exposure of adipocytes to 400 nM BoNT/C1 in LISM lowered basal- and insulin-stimulated glucose uptake by 28.3 ± 3.9% (±SD; $n = 4$; $p < 0.025$) and 55.6 ± 4.4% (±SD; $n = 4$; $p < 0.005$), respectively (Figure 2A). Incubation of the cells with a range of BoNT/C1 concentrations in LISM resulted in a dose-dependent decrease of insulin-induced glucose uptake up to a maximum of 51.3 ± 9.2% (±SD; $n = 4$; $p < 0.005$); again, a lower reduction of basal uptake was seen (Figure 2B). Overall, the maximal inhibition of the insulin-stimulated increment of glucose uptake obtained in seven additional sets of experiments were (±SD; $n = 3$ or 4) 33 ± 5 ($p < 0.001$), 34 ± 9 ($p < 0.025$), 36 ± 13 ($p < 0.01$), 37 ± 7 ($p < 0.001$), 48 ± 15 ($p < 0.005$), 51 ± 14 ($p < 0.01$), and 64 ± 8 ($p < 0.005$) (average inhibition = 43%). In contrast, pre-

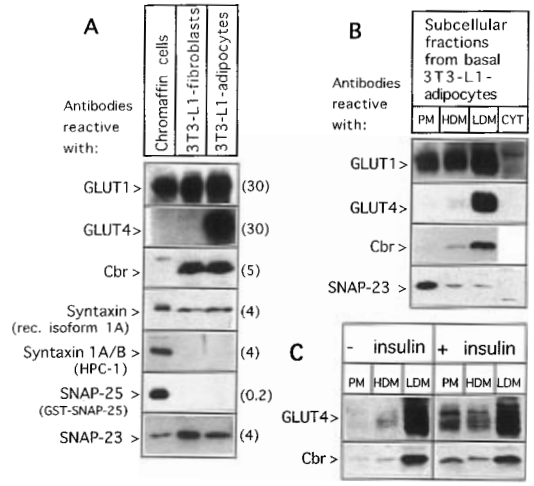

FIGURE 3: Distribution of GLUT-isoforms and BoNT substrates in membranes from 3T3-L1 fibroblasts, adipocytes, and chromaffin cells: SNAP-23 is plasmalemma-associated in 3T3-L1 adipocytes. Total membrane fractions from the specified cells or, alternatively, subcellular membrane fractions PM, HDM, and LDM as well as cytosol (CYT) were isolated from basal- or insulin-stimulated 3T3-L1 adipocytes (see Experimental Procedures). The amounts of membrane or cytosol proteins (indicated below) were subjected to SDS−PAGE, transfered to Immobilon-P membranes, and incubated overnight using the appropriate antibodies specified: anti-GLUT1 (C-terminal peptide); anti-GLUT4 (C-terminal peptide); anti-Cbr (N-terminal peptide); anti-SNAP-23 (C-terminal peptide); also, see figure. Binding of primary antibodies was detected using horseradish-peroxidase-conjugated species-specific secondary Ig, visualized using enhanced chemilumenescence (film exposure periods varied for different antibodies). In panel A, 30 μg of membrane protein blotted was loaded for fibroblast and adipocyte samples; however, the amounts of chromaffin cell sample blotted varied and are specified in brackets as micrograms of protein opposite the appropriate track. In panel B, subcellular fractions isolated by the method of Piper *et al.* (1991) were solubilized in equal volumes of SDS−buffer before immunoblotting using different amounts of protein (PM = 9, HDM = 12, LDM = 25, and CYT = 73 μg). In panel C, the quantities of membrane protein blotted were (PM = 20, HDM = 20, and LDM = 50 μg) for fractions isolated from basal- or insulin-stimulated adipocytes. These results are representative of immunoblots from two or three separate experiments performed using different cell samples.

exposure of three batches of differentiated adipocytes (exhibiting equivalent insulin-evoked glucose uptake responses as before) to 400 nM BoNT/A (for 24 or 48 h) in LISM failed consistently to give a significant reduction in the extent of glucose uptake in response to insulin (Figure 2A).

Detection of GLUT1, GLUT4, Cbr, and Sbr, but Not Syntaxin 1A/B or SNAP-25, in 3T3-L1 Adipocytes. Immunoblotting revealed similar contents of GLUT1 (the ubiquitously expressed isoform) in total membrane fractions isolated from adrenomedullary chromaffin cells, 3T3-L1 fibroblasts and differentiated adipocytes (Figure 3A) as expected (Piper *et al*., 1991). Note that all blotting was performed with a constant amount (30 μg) of protein from undifferentiated and differentiated 3T3-L1 cells whereas the signal intensities in many cases necessitated use of lower amounts of chromaffin cell membranes, noted in Figure 3. In contrast, the levels of GLUT4 and the BoNT substrates varied enormously in the isolated membranes from these cell types (Figure 3A). An Ig reactive with GLUT4 only labeled a protein band of approximately 50 kDa in the adipocyte samples (Figure 3A), consistent with the known tissue distribution of the latter antigen and indicative that dif-

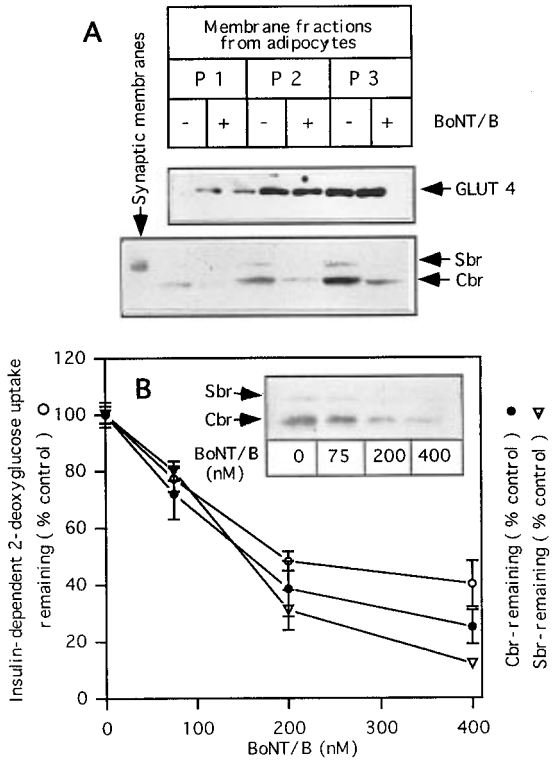

FIGURE 4: Comparison of the BoNT/B concentration dependencies for inhibition of insulin-stimulated glucose uptake by adipocytes and cleavage of Cbr and Sbr. Adipocytes were pre-exposed to 400 nM (A) or various BoNT/B concentrations (B) in LISM for 24 h at 37 °C. After washing, the cells were incubated in DMEM for 24 h at 37 °C before measurement of insulin-dependent glucose uptake (B) or preparation of membrane fractions which were subjected to Western blotting (A, B inset) for GLUT4 (monoclonal 1F8), Cbr, and Sbr [anti-(peptide residues 33−94 of Sbr-2) Ig] using the conditions given in Figure 3 legend. Note that in panel A, different amounts of protein were run for P1 (80 μg), P2 (40 μg), and P3 (20 μg) membrane pellets whereas in panel B inset an identical quantity of P3 membrane protein (40 μg) was used in all the gel tracks. The levels of immunoreactivity detected on the blots for each of the antigens were quantified by densitometric scanning and corrected for background. The values obtained for uptake of glucose (picomoles of [^{3}H]2-deoxyglucose per milligrams of protein per minute) under basal conditions were subtracted from the totals measured in the presence of insulin to yield the insulin-dependent component for control and each of the toxin-treated samples. The resultant insulin-dependent uptake and the content of Cbr and Sbr in the toxin-treated P3 membranes (data from panel B inset), each expressed relative to the requisite mean control value, are plotted in panel B (±SD; $n = 3$); the values were presented in this way to facilitate a direct comparison of the toxin's effects on both parameters.

ferentiation into the correct phenotype from the original fibroblast lineage had occurred (Calderhead *et al.*, 1990; Piper *et al.*, 1991). After differentiation of the fibroblasts into adipocytes, equivalent levels of expression of Cbr were observed in both cell types (Figure 3A), as reported previously by Martin *et al.* (1996). A faint band of higher mobility (equivalent in size to Sbr) was detectable in chromaffin cells which probably represents Sbr because the last four amino acids of the 20-mer Cbr peptide used as immunogen are common in Sbr-1 and -2 (McMahon *et al.*, 1993); as shown later in Figure 4A, Sbr is a minor component relative to Cbr, which is much more abundant.

Further immunoblotting using an affinity-purified antibody raised against a recombinant His^6-tagged rat syntaxin 1A (termed rec. Syntaxin 1A in Figure 3A) revealed weak signals for total membranes isolated from both fibroblasts and adipocytes; the mobilities coincided with that of syntaxin identified in chromaffin cell membranes (Figure 3A), though the latter possessed a far higher content because 7.5-fold less protein was blotted (see legend to Figure 3). Although the syntaxin isoform was not identified, the lack of reactivity with high concentrations of a monoclonal antibody (HPC-1) selective for syntaxin 1A/B (Volchuk *et al.*, 1996) excludes these isoforms. Thus, this polyclonal Ig raised against rec. syntaxin 1A must be exhibiting additional reactivity toward other syntaxin isoforms (Bennett *et al.*, 1993).

SNAP-25 was not detectable on blots of the membrane fraction of either 3T3-L1 fibroblasts or adipocytes despite the use of a highly avid, affinity-purified Ig raised against a recombinant glutathione *S*-transferase (GST)−SNAP-25 fusion protein for detection, together with visualization by the uniquely sensitive ECL-system and prolonged exposure times (Figure 3A). As vastly smaller amounts of membrane protein from chromaffin cell (0.2 μg) relative to the 30 μg of 3T3-L1 cell membranes produced a very strong SNAP-25 signal after only a short exposure period, this establishes that SNAP-25 does not occur in 3T3-L1 cells at least at a level several hundred-fold lower than that in chromaffin cells. Consistently, blockade of insulin-stimulated glucose uptake was not observed with BoNT/A and only attained following pre-exposure of adipocytes to BoNT/B or /C1, as noted above.

SNAP-23 Is Expressed in 3T3-L1 Adipocytes, Fibroblasts, and Chromaffin Cells. A functional non-neuronal homologue of SNAP-25, termed SNAP-23, which exhibits the expected affinities for syntaxin isoforms 1−4 and Sbr-isoforms has recently been cloned and detected in a wide variety of tissues (Ravichandran *et al.*, 1996). While SNAP-23 is homologous to SNAP-25 (see Discussion), it could not be detected with two different antibodies reactive with SNAP-25 (Figure 3A and see later). Therefore, to assess whether SNAP-23 is expressed in 3T3-L1 adipocytes, immunoglobins were generated against the last 11 C-terminal residues of its deduced sequence (see Materials). For the first time, the affinity-purified Ig detected SNAP-23 in all of the membranes (Figure 3A). The specificity of this labeling was established by its eradication when blotting was performed in the presence of an excess of the immunogenic peptide; on the other hand, the C-terminal SNAP-25 peptide (500-fold molar excess) used to generate antibodies against that distinct protein (see Materials) failed to alter the level of signal (data not shown). The electrophoretic mobility is similar to that of SNAP-25; optimization of electrophoresis conditions to enable maximum resolution showed that SNAP-23 had a slightly lower M_r than SNAP-25, as expected from their deduced sequences which differ in length by only seven residues (data not shown). The differentiation of fibroblast to adipocyte appeared to reduce the abundance of SNAP-23 (Figure 3A; note that 7.5-fold less protein from the chromaffin cell sample was loaded onto the gel). Thus, SNAP-23 alone occurs in 3T3-L1 cells whereas it coexists with SNAP-25 in chromaffin cells.

SNAP-23 Resides Predominantly on the Plasmalemma of 3T3-L1 Adipocytes. To examine the subcellular location of SNAP-23, the well-defined HDM, LDM, and highly enriched PM-fractions were isolated (see Experimental Procedures) by Piper *et al.* (1991). SNAP-23 was found to be most abundant by far in PM-enriched fractions compared to all

the other subfractions from 3T3-L1 adipocytes (Figure 3B; please note that the least amount of PM-membrane protein was blotted). This predominant PM location closely parallels that found for syntaxin-2 using the same subcellular fractionation protocol (Volchuk *et al.*, 1996; Timmer *et* al., 1996). Immunoblotting with established LDM-markers GLUT4 and Cbr demonstrated that very little SNAP-23 coexisted in this defined LDM fraction (Figure 3B). GLUT1 was found in PM, HDM, and LDM fractions (Figure 3B); when allowance is made for the different quantities of protein used from each fraction (see Figure 3 legend), the relative contents of GLUT1 approximate to those found by Piper *et al*. (1991). Further validation of subcellular fractionation is provided by the known translocation of GLUT4 from LDM to PM-enriched fractions in response to insulin (Figure 3C). As expected (Piper *et al.*, 1991; Volchuk *et al.*, 1995), under the basal condition, very low levels of GLUT4 and Cbr were detected in the subsequently isolated PM and HDM fractions relative to LDM; following insulin stimulation, much larger amounts of GLUT4 and Cbr were found in PM-containing fraction and to a lesser extent in HDM (Figure 3A; note the different quantities of protein used for each fraction). In the case of the cytosol from adipocytes, no appreciable amount of SNAP-23 was detected (Figure 3B), consistent with membrane anchoring as established for SNAP-25 by thioester-linked palmitoylation (Oyler *et al*., 1989); the cysteines responsible for this are conserved in SNAP-23 (Ravichandran *et al*., 1996). In support of this proposal, dissociation of SNAP-23 immunoreactivity from 3T3-L1-membranes occurred only in the presence of detergent and not high ionic strength (0.6 M NaCl) or temporary exposure to pH 3 buffer (data not shown), consistent with lipid-anchored proteins (Oyler *et al.*, 1989).

BoNT/B-Mediated Blockade of Insulin-Stimulated Glucose Uptake in Adipocytes Is Associated with Proteolysis of Cbr and Sbr. In order to reliably conclude that the BoNT/B-induced inhibition of insulin-stimulated glucose uptake into adipocytes results from the direct and selective cleavage of Cbr and Sbr, these two parameters were measured in the same samples of cells. Further to the above-noted detection of Cbr (Figure 3A), the actual abundance of both bands was detected on Western blots with an antibody raised against a peptide, residues 33−94 of Sbr-2 (that usefully recognizes Sbr-1, Sbr-2, and Cbr; Foran *et al*., 1995). The less prevalent and slower migrating reactivity seen represents Sbr-isoforms, consisitent with the identical mobility to that present in rat brain synaptic membranes (Figure 4A). On the other hand, the more intense, lower M_r band is absent from synaptic membranes (Figure 4A) and comigrates on gels with Cbr. Adipocytes were poisoned by a 24 h exposure to 400 nM BoNT/B in LISM and allowed to equilibrate for 1 day in DMEM before measurement of insulin-evoked glucose uptake. Aliquots of the cells were harvested, homogenized, and fractionated by differential centrifugation (see Experimental Procedures) to enrich the Cbr, Sbr, and GLUT4 immunoreactivities in P2 and P3 pellets (Figure 4A,B). Relative to the P1 pellet, P2 and P3 membrane fractions exhibited respective increases of ∼3.3- and ∼8.9-fold in the combined Cbr and Sbr immuno-reactivities and elevations of ∼4.8- and ∼14-fold in GLUT4 blot intensities (Figure 4A; please note different amounts of protein were loaded for P1, P2, and P3.); these high values for the latter accord with the predominant location of these proteins in the LDM fraction. Cells that had been exposed to BoNT/B displayed a reduction in the combined signals for Cbr and Sbr of 67.2 ± 6.2% and 78.7 ± 7.7% (% errors; $n = 3$) from P2 and P3 pellets, respectively, compared to toxin-free controls (Figure 4A), as established by densitometric scanning of the blots. This same toxin treatment resulted in a blockade of the insulin-stimulated increment of glucose uptake of between 43 and 64% relative to non-toxin-treated cells (see earlier). Immunoblotting for the BoNT/B-insensitive marker GLUT4 on vesicles (as indicated by its undiminished signal in toxin-treated cells), which also contain Cbr/Sbr immunoreactivities (Cain *et al*., 1992; Volchuk *et al*., 1995), demonstrated equivalent enrichments of GLUT4 vesicles in P2 or P3 membrane pellets derived from cells preincubated in the absence or presence of toxin (Figure 4A). Examination of the concentration dependence for BoNT/B affecting both parameters showed a reasonably good correlation (Figure 4B). Due to the potential error inherent in densitometric analysis of immunoblots that was used to quantify the amounts of BoNT/B substrates remaining, absolute quantitation is precluded.

Subcellular Redistribution of GLUT4 and Cbr/Sbr in Response to Insulin Is Blocked by Pre-Exposure to BoNT/B. Experiments were performed to ascertain if BoNT/B-mediated blockade of insulin-stimulated glucose uptake arose from the inability of GLUT4-containing vesicles to fuse with the cells surface due to loss of Cbr/Sbr, rather than a nonspecific action of the toxin on another step in glucose transport. These involved measurement of insulin-induced translocation of GLUT4 and Cbr/Sbr from LDM to PM-enriched fractions, prepared by differential and density-gradient centrifugation (see Experimental Procedures) from BoNT/B-poisoned or toxin-free adipocytes. In nonstimulated adipocytes, cultured in the LISM in the absence of BoNT/B, the largest amount of GLUT4 was recovered in LDM (Figure 5A); please note that 2-fold less LDM protein than for PM-enriched fraction was immunoblotted. In response to insulin, the amounts of GLUT4 and Cbr/Sbr in the PM-enriched fraction increased, accompanied by a decrease in their content in LDM (Figure 5A). This response is consistent with a molecular mechanism by which insulin promotes glucose uptake due to the fusion of GLUT4-containing vesicles with the cell surface. Quantitation of immunoblots from three different fractionations using densitometric scanning gave an average increase of 100.8 ± 36.1% (±SD; $n = 3$; $p < 0.005$) in the GLUT4 reactivity of the PM-enriched fractions and a 50.2 ± 12.6% (±SD; $n = 3$; $p < 0.005$) decrease in that for LDM relative to insulin-free controls (Figure 5B). These values approximate to those measured in other studies performed using 3T3-L1 adipocytes (Volchuk *et al*., 1995), although a much higher increase in GLUT4 translocation to PM has been obtained using adipocytes prepared from rat epipidymal fat pads [e.g., Zorzano *et al.* (1989)]. The corresponding redistribution of GLUT4 and Cbr/Sbr observed following insulin-treatment of 3T3-L1 adipocytes accords with their colocalization on the major vesicle subpopulation (Volchuk *et al.*, 1995; Martin *et al*., 1996). The somewhat lower elevation by insulin of the GLUT4 content of PM-enriched fraction compared to the observed increase in 2-deoxy-D-glucose uptake (3-4-fold following LISM treatments; see above) is most likely due to a contamination of our PM preparation with small amounts of GLUT4-containing vesicles. This may have arisen

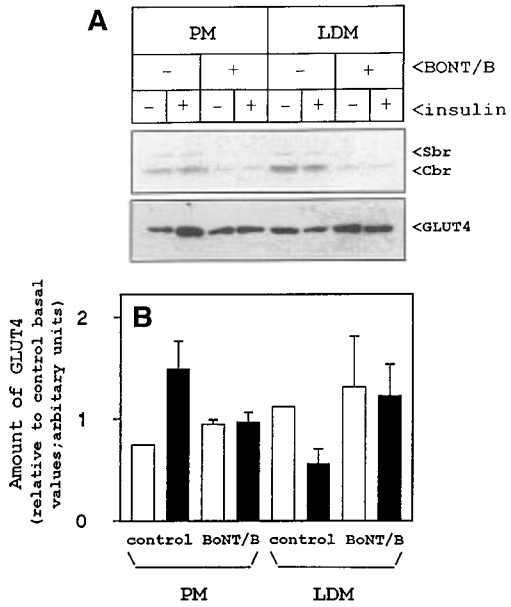

FIGURE 5: Pre-exposure of adipocytes to BoNT/B blocks the subcellular redistribution of GLUT4 that is induced by insulin. Adipocyte cultures were exposed to LISM in the absence or presence of 400 nM BoNT/B for 24 h at 37 °C, followed by incubation for another 24 h in toxin-free DMEM supplemented as detailed in Experimental Procedures before manipulations. The cells were equilibrated in serum-free DMEM for 2−3 h at 37 °C and washed twice with PBS followed by buffer A, before being incubated for 20 min in the absence or presence of 100 nM insulin. After immediate harvesting, the cells were homogenized in buffer containing protease inhibitors and LDM or PM-enriched membrane fractions isolated, as detailed in Experimental Procedures. In panel A, the membrane fractions were subjected to immunoblotting for Cbr/Sbr [anti-(peptide residues 33−94 of Sbr-2) Ig] and GLUT4 (monoclonal 1F8), using the conditions specified in Figure 3 legend. Note that different amounts of protein were run for PM (40 μg) and LDM (20 μg) samples. Panel B shows the average levels (±SD) of GLUT4 immunoreactivies on Western blots of the membrane fractions from basal (open bars) and insulin-stimulated (closed bars) cells from three independently performed sets of experiments (as in part A), quantified using densitometric scanning and expressed relative to normalized control basal values.

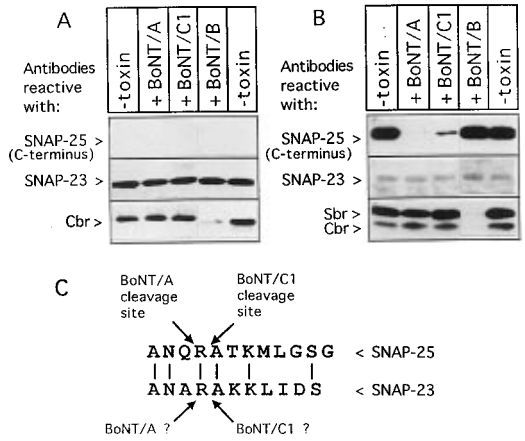

FIGURE 6: Cleavage of SNAP-25 by BoNT/A or /C1 and Sbr/Cbr by type B upon application to permeabilized cells: resistance of SNAP-23 to proteolysis by BoNTs**.** 3T3-L1 adipocytes (A) or chromaffin cells (B) were exposed in the absence or presence of 100 nM prereduced BoNTs during permeabilization at 37 °C for 30 min with digitonin−KGEP buffer (as detailed in Experimental Procedures). Equal amounts of SDS-solubilized total membrane fractions isolated subsequent to toxin exposures (50 μg in panel A and 20 μg in panel B) were subjected to immunoblotting (as detailed in the legend to Figure 3) using the appropriate antibodies: anti-(SNAP-25 C-terminal peptide); anti-(SNAP-23 C-terminal peptide); anti-(Cbr/Sbr; residues 33−94 of Sbr-2); anti-(Cbr N-terminal peptide). Binding of primary antibodies was detected using horseradish-peroxidase-conjugated secondary antibodies and visualized using enhanced-chemiluminescence. In panel C, the C-terminal sequences of SNAP-25 and SNAP-23 are aligned; the vertical lines indicate amino acid identity.

because the fractionation methods used proved less efficient as only small quantities of intoxicated cells were available.

Following BoNT/B-poisoning of intact adipocytes by exposure in LISM, insulin-stimulated GLUT4 translocation from LDM to PM fractions was greatly reduced, compared to toxin-free controls; also, an extensive diminution of Cbr/Sbr was noted in the LDM and PM-enriched fractions (Figure 5A). Quantitation of Western blots from three different BoNT/B-intoxications revealed average insignificant values of only 2.5 ± 7.3% (±SD; $n = 3$; $p > 0.5$) increase in GLUT4 content of the PM-enriched fractions in response to insulin and a corresponding minimal decrease of 6.7 ± 31.2% (±SD; $n = 3$; $p > 0.5$) in LDM (Figure 5B). Curiously, the amount of GLUT4 in PM-enriched fractions appeared to increase somewhat after BoNT/B treatment (Figure 5B) while reducing the basal level of glucose uptake (Figure 1C); the basis of this anomaly is unclear. Thus, it has been demonstrated directly that the insulin-stimulated fusion of GLUT4-containing vesicles with the cell surface is extensively blocked following BoNT/B-mediated proteolyses of Cbr/Sbr in intact adipocytes.

SNAP-23 Is Resistant to Proteolytic Scission by BoNT/A or /C1, the Neurotoxins That Cleave SNAP-25. Observation of SNAP-23 on PM in 3T3-L1 adipocytes is consistent with its possible involvement in insulin-regulated fusion of GLUT4-containing vesicles. Therefore, this warranted an examination of whether it could be proteolyzed by either BoNT/A or /C1 (see introductory section). The highly efficient method for achieving proteolysis of SNAP-25 by applying the toxins to digitonin-permeabilized chromaffin cell monolayers (e.g., 20 nM BoNT/A or /C1 cleaves the majority of SNAP-25 in 30 min; Lawrence *et al.*, 1997, and data not shown), was performed on cultured 3T3-L1 adipocytes, and the extent of proteolysis was monitored by immunoblotting using a total membrane fraction. For this purpose, Ig was generated against a peptide encompassing the last 11 C-terminal residues of SNAP-23 (see Materials) with the expectation that cleavage of the latter by either BoNT/A or /C1 (Figure 6C) would result in disappearance of the immunoreactive signal on Western blots. To ensure that this was indeed the case, several important control experiments were performed with this Ig. Firstly, it was demonstrated that the synthetic peptide CANAR (at a 500-fold molar excess) which comprises the N-terminus of the peptide immunogen (see Materials), corresponding to the C-terminus of SNAP-23 (Figure 6C) failed to perturb the binding of Ig on blots of total adipocyte membranes. Moreover, the anti-SNAP-23 Ig did not bind to CANAR peptide immobilized on resin (not shown). Therefore, the reactivity of this antibody is totally dependent on the residues positioned on the C-terminal side of the putative cleavage sites for BoNT/A and /C1 (Figure 6C) and, thus, toxin cleavage would diminish the immunoreactive signal. Treatment of permeabilized adipocytes for 30 min with high concentrations (up to 100 nM) of BoNT/A or /C1 failed to produce a significant reduction in SNAP-23 immunoreactivity in five separate experiments (Figure 6A). In contrast,

Cbr in thesesame cells was cleaved by BoNT/B, establishing that this experimental protocol afforded exposure of this susceptible substrate to toxin (Figure 6A). Unlike adipocytes that were devoid of reactivity with an antibody against a C-terminal peptide of SNAP-25 (Lawrence *et al.*, 1996) (Figure 6A) the relatively high signal in chromaffin cells was virtually abolished by the same exposure to BoNT/A or /Cl; likewise, Cbr and Sbr immunoreactivities were obliterated by BoNT/B (Figure 6B). However, the detectable SNAP-23 signal in the permeabilized chromaffin cells remained unaltered after the 30 min incubation with any of the three toxins (Figure 6B), confirming the observation with adipocytes. The demonstrated absence of SNAP-25 and the resistance of SNAP-23 to BoNT-mediated proteolyses are consistent with the inability of BoNT/A to inhibit insulin-elicited enhancement of glucose uptake into 3T3-L1 adipocytes.

DISCUSSION

The purpose of this study was to ascertain whether the three intracellular targets for BoNT serotypes participate in the enhancement by insulin of glucose uptake into 3T3-L1 adipocytes. Thus, methodology was optimized for the introduction of these toxins into the cultured intact cells that lack the high-affinity ecto-acceptors, known to be unique to the susceptible terminals of peripheral cholinergic neurones [reviewed by Dolly *et al.* (1994)]. While permeabilization of other cell types with digitonin or streptolysin-O provides access to the toxins (see introductory section), loss of essential cytosolic components can occur which results in the decline of exocytosis (Terbush & Holz, 1986; Lawrence *et al.*, 1994; Clarke *et al.*, 1994). Furthermore, such permeabilization methods offer very limited periods for manipulation before total decline of cellular functions. For these reasons, prolonged incubation of adipocytes with BoNTs in LISM was adopted because this protocol (Marxen *et al.*, 1991) has proved so successful for the abolition of exocytosis from adrenochromaffin cells by BoNT/A, /B, or /C1 (Marxen *et al.*, 1991; Foran *et al.*, 1995, 1996); Marxen *et al*. (1989) have proposed that BoNT/A interacts with gangliosides on the cell surface in a manner that leads to internalization. It is noteworthy that the LISM protocol did not significantly reduce the number of 3T3-L1 cells attached to the culture plates, as determined by microscopy and determination of nucleic acid content. Despite a noted drop in the level of insulin-elicited glucose uptake by the cells after the LISM treatment, the activity preserved exceeds that found following permeabilization (Tamori *et al.* 1996; Clarke *et al* 1994). Furthermore, exposure to the BoNT in LISM did not alter the number of cells adhering to the plates as judged by the content of toxin-resistant LDM marker protein GLUT4.

When applied in this way, BoNT/B yielded significant blockade of insulin-induced uptake of glucose into 3T3-L1 adipocytes and a corresponding cleavage of Cbr and Sbr, unlike the discrepancy reported by Tamori *et al*. (1996). BoNT/Cl also partially inhibited glucose uptake and reduced the signal seen with the polyclonal anti-syntaxin/A Ig. The observed inhibitory effect of BoNT/Cl is attributable to the syntaxin cleavage rather than its alternative substrate SNAP-25 (Foran *et al*. 1996) because of this being shown herein to be absent from 3T3-L1 cells and the homologue SNAP-23 is BoNT-resistant. The unavailability of Ig selectively-reactive toward the syntaxins 2, 3, and 4 precluded identification of the isoforms cleaved, though 1A/B are excluded by their absence. Additionally, as GLUT4 translocation to the cell surface in response to insulin was shown to be extensively blocked following pre-exposure to BoNT/B, it can reasonably be deduced that its targets Cbr/Sbr are concerned with insulin-regulated fusion of GLUT4-containing vesicles with the plasmalemma. The apparent susceptibility of that response to BoNT/C1 could entail their interaction with a type C1-sensitive syntaxin in a manner similar to that proposed for neuro-exocytosis (Söllner *et al.*, 1993). Although syntaxins 2, 3, and 4 have been demonstrated in 3T3-L1 cells (Volchuk *et al.*, 1996; Timmers *et al.*, 1996), only isoform 4 has been implicated in insulin-stimulated glucose uptake because its specific Ig antagonized this process (Volchuk *et al.*, 1996). However, this isoform is not cleaved by BoNT/C1 (Schiavo *et al.*, 1995) while syntaxins 2 and 3 are reported unable to associate with Sbr and Cbr (Calakos et al., 1994; Pevsner *et al.*, 1994; Timmers *et al.*, 1996). Clearly, much effort from many laboratories is required to resolve this complex but fundamental mechanism.

The aforementioned difficulty in accurately quantifying toxin-induced inhibition of glucose uptake in batches of cells and cleavage of the targets precluded a truly quantitative correlation being sought with the apparent higher level of blockade of GLUT4 trafficking produced by BoNT/B. Consequently, our data do not exclude the existence of a subpopulation of GLUT4-containing vesicles in 3T3-L1 adipocytes lacking Sbr and/or Cbr as detected by electron microscopy (Martin *et al.*, 1996) which may mediate a component of insulin-stimulated glucose uptake.

In the case of SNAP-25, use of two different highly avid antibodies together with the uniquely sensitive ECL-method failed to detect this protein in 3T3-L1 adipocytes. Accordingly, BoNT/A proved unable to alter glucose uptake. However, an important novel outcome of this investigation was the detection of SNAP-23 in 3T3-L1 adipocytes almost entirely in the PM consistent with its proposed function as a docking/fusion protein (Ravichandran *et al.*, 1996) and the observed lack of susceptibility to cleavage by BoNT/A or /C1, even under conditions for intact and permeabilized cells in which the toxins readily proteolyzed their substrates. Indeed, analogous to SNAP-25, this homologue can associate with syntaxin- and Sbr-isoforms *in vitro*. Importantly, SNAP-23 while being 59% identical in amino acid sequence to SNAP-25 exhibits extensive diversity at its C-terminus (Ravichandran *et al.*, 1996; see also Figure 6C), the region which encompasses the cleavage sites of BoNT/A and /C1 in the latter protein, including an amino acid substitution in the aligned sequences converting the peptide bond (Q^{197}−R^{198}) cleaved by type A (Schiavo *et al.*, 1993c) to A−R. Notably, a similar substitution in the BoNT/B and TeTx cleavable Q−F bond present in rat Sbr-2 to V−F creates a protein profoundly resistant to scission (Schiavo *et al.*, 1992). Similarly, the significant amino acid sequence differences noted between SNAP-23 and SNAP-25 could account for the observed inability of BoNT/A to cleave SNAP-23. Likewise, BoNT/C1 was unable to proteolyze the latter, despite conservation of the R−A bond shared with the SNAP-25 sequence (see Figure 6C), which is the demonstrated site of C1 cleavage in the latter protein (Niemann, 1996).

In conclusion, the collective findings presented herein establish that while some proteins are likely to be shared by the fusion pathways for synaptic vesicles and GLUT4-containing-vesicles, sufficient differences exist in the relative abundancies and the precise homologues involved to underlie the characteristic features of these mechanisms.

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