Botulinum E Toxin Light Chain Does Not Cleave SNAP-23 and Only Partially Impairs Insulin Stimulation of GLUT4 Translocation in 3T3-L1 Cells

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The stimulation of glucose uptake into fat and muscle by insulin results predominantly from the translocation of the glucose transporter, GLUT4, from an intracellular vesicle pool to the cell surface. Homologues of several key proteins known to be involved in the process of synaptic vesicle fusion have been identified on GLUT4 vesicles, including VAMP2 and cellubrevin. Syntaxin 4, SNAP-23 and/or SNAP-25 are also implicated in this process. Bacterial toxins that specifically cleave these proteins have been utilised to assess their involvement in cell function. We aimed to distinguish which of the SNAP isoforms are specifically involved in GLUT4 translocation. Here we show that both human (h) and mouse (m) SNAP-23, unlike SNAP-25, are not substrates for Botulinum E toxin light chain (BoNT/E). Furthermore, we demonstrate that microinjection of differentiated 3T3-L1 cells with BoNT/E inhibited insulin stimulation of GLUT4 translocation only slightly, 27%, whereas tetanus toxin light chain, that cleaves VAMP2, inhibited insulin stimulation of GLUT4 translocation by 80%. These studies therefore do not support a major role for SNAP-25 in insulin stimulation of GLUT4 translocation and place SNAP-23 as a prime candidate for a role in this process.   © 1997 Academic Press

Insulin stimulation of glucose transport in insulin-sensitive tissues results predominantly from the translocation of GLUT4 transporters, from a pool within tubovesicular membrane structures in the cell, to the cell surface (1,2), through a process that appears to share some similarities with regulated exocytosis (see 3, 4 for review). In particular, proteins that appear to be involved in docking and fusion of exocytic vesicles with the cell surface are present in insulin-sensitive cells and have been implicated in insulin-dependent GLUT4 trafficking. These proteins include VAMP 2 (5-15), syntaxin 4

(16,17) and lower levels of SNAP-25 (18), whose counterparts in neural cells comprise the fusogenic machinery for regulated trafficking of neurotransmitter to nerve terminals. These, or related proteins, have been implicated in regulated trafficking in several cell systems and appear remarkably conserved from yeast to man (3, 19, 20). A homologue of SNAP-25, that shares 72% homology with SNAP-25B (21-23), and which may exist in two forms (24), has also been recently described in insulin responsive tissues. This homologue, termed SNAP-23, has a more general tissue distribution than SNAP-25, is present in higher abundance than SNAP-25 in fat cells and has been localised to the plasma membrane (23). By analogy with the neural system it is a prime candidate as the third member of the trimeric complex involved in GLUT4 trafficking.

Evidence for a functional role for VAMP 2 and syntaxin 4 in GLUT4 translocation has been provided by studies that have included the introduction into cells of antibodies, peptides based on interaction domains, or fusion protein domains produced using recombinant techniques, each of which has been demonstrated to inhibit GLUT4 translocation (25-28). Bacterial toxins that cleave VAMP 2 have also been shown to inhibit GLUT4 translocation in all (12,25,27,29) but one study(15). This latter study found no effect of tetanus toxin (TeTx) cleavage of VAMP 2 on GLUT4 translocation when the toxin was electroporated into fat cells (15). The reason for the conflict between this and the other studies (12, 25, 27,29) is unclear. The involvement of SNAP-25 or 23 is equivocal. In a previous study (18), we reported only a very low level of SNAP-25 in 3T3L1 cells, inconsistent with a major role in GLUT4 translocation. Furthermore, we showed that insulin stimulation of GLUT4 translocation was insensitive to the effects of botulinum A toxin light chain (BoNT/A) (that cleaves SNAP-25)(25). In the same study, we showed that

botulinum D toxin light chain (BoNT/D) or TeTx (that cleave VAMP 2 and cellubrevin) inhibited insulin stimulation of GLUT4 translocation, implicating either or both of these isoforms in the process. Similar findings have been reported by Chen et al (29). Since BoNT/A cleaves only a 9 residue peptide from the C terminus of SNAP-25, it is possible that this may not be sufficient to block the interaction of other SNARE complex components (30,31).

Functional support for the involvement of SNAP-23 or -25 in GLUT4 translocation would be provided if GLUT4 translocation was sensitive to toxin cleavage of the SNAP homologues. However, the toxin sensitivity of SNAP-23 has not previously been assessed in full. Chen et al (29)reported that SNAP-23 in 3T3-L1 cells was not cleaved by BoNT/A. This finding is not surprising since sequence alignment shows that SNAP-23 does not have the cleavage site for BoNT/A. Human SNAP-23 (hSNAP-23) does share the Arg-Ile cleavage site for BoNT/E cleavage. However the BoNT/E cleavage site in mouse SNAP-23 (mSNAP-23) is Lys-Ile. It is unclear whether this homology is sufficient for cleavage. Also, it is not clear whether the binding motifs for neurotoxins are sufficiently conserved in SNAP-23 to allow BoNT/E to bind SNAP-23 to enable cleavage to take place (32,33) and whether cleavage of either SNAP-25 or 23 by BoNT/E is sufficient to inhibit GLUT4 translocation. Here we show that BoNT/E cleaves SNAP-25, but does not cleave SNAP-23. We also show that BoNT/E has only a small effect on insulin stimulation of GLUT4 translocation, further indicating that SNAP-25 does not have a significant involvement in this process, and placing SNAP-23 as a prime candidate for this role.

MATERIALS AND METHODS

Cell culture. 3T3-L1 Fibroblasts obtained from the American Type Culture Collection (Rockville, MD) were maintained and passaged as pre-confluent cultures in DMEM (Sigma) with 10% newborn calf serum (CSL Ltd., Australia). Cells for differentiation were maintained at confluence for 48 h, then induced to differentiate by the addition of DMEM containing 5% foetal calf serum (FCS) (CSL Ltd., Australia), 4 μg/ml insulin, 0.25 mM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. After 72 h, induction medium was replaced with fresh FCS/DMEM containing 4 μg/ml insulin. Cells were used 7-14 days after differentiation, by which time >90% of the fibroblasts differentiated into mature adipocytes.

Microinjection. Cells grown to confluence and differentiated on coverslips were transferred to Krebs-Ringer bicarbonate Hepes buffer, pH 7.4, containing 2 mM pyruvate, 0.5% BSA and 2.5 mM glucose for 45 min. They were microinjected over a 30 min period using a Zeiss automated injection system (Carl Zeiss, Germany) coupled to an Eppendorf (Germany) microinjector. Over 200 cells were microinjected for each condition within any single experiment. Micropipettes were prepared using a Sutter (USA) P-97 micropipette puller. Reagents were dissolved in a buffer containing 5 mM sodium phosphate (pH7.2), 100 mM KCl for microinjection. Cells were transferred into fresh medium and allowed to recover for 60-90 min following injection of BoNT/A, E or TeTx at 0.6mg/ml, prior to stimulation

with insulin (100 nM) and analysis of GLUT4 translocation using the plasma membrane lawn assay (34,35). GLUT4 translocation in microinjected cells was compared to that in non-injected cells in the immediate vicinity on the same coverslip.

GLUT4 plasma membrane lawn assay. GLUT4 translocation was determined using the plasma membrane lawn assay as described by Robinson and James (34) with modifications described by Marsh et al (35). Briefly, after cell treatment, 3T3L1 cells grown on cover slips were washed in poly-L-lysine and hypotonically shocked with three washes in 1/3 membrane buffer (70 mM KCl, 5 mM MgCl$_2$, 3 mM EGTA, 1 mM DTT, 30 mM Hepes, pH 7.2). The cells were then sonicated in membrane buffer, using a probe sonicator (Microson, USA) at setting 0, to generate a lawn of plasma membrane fragments that remained attached to the cover slip. The fragments were then immunolabelled with polyclonal rabbit anti-GLUT4 antibody (R1159, 1/100) (36) followed by CY3-labelled goat anti-rabbit (Amersham, UK). Coverslips were visualised and imaged using a BioRad (USA) Lasersharp MRC-500 confocal laser scanning immunofluorescence microscope. Data were analysed using BioRad COMOS confocal imaging software.

Toxin preparation. Plasmids containing the neurotoxin light (L) chains of Tetanus toxin (TeTx), Botulinum A (BoNT/A) and -/E (BoNT/E) toxins were obtained from Professor H. Niemann, Hanover, Germany. The L chain genes containing a C-terminal His$_6$-tag were expressed in *E. coli* M15[pREP4] and were purified by binding to Ni-NTA resin (Qiagen).

Preparation of bacterially expressed SNAP-25 and SNAP-23. Human SNAP-25A was expressed as a GST-fusion protein using the expression vector pGEX2T in *E. coli* strain BL21(DE3). Human SNAP-25B (gift from Thomas Sudhof, HHMI, Dallas, TX)(37), hSNAP-23 (gift from Paul Roche, NIH, Bethesda, MD)(21) and mSNAP-23 (syndet, gift from Guilla Baldini, Columbia University, New York, NY (22)) were expressed as GST-fusion proteins using the expression vector pGEX4T-1 in *E. coli* strain DH5α. Recombinant GST-fusion proteins were purified from *E. coli* lysates by glutathione-sepharose chromatography using standard procedures.

Statistical analyses. Statistical analyses were performed using Student's paired *t* test. Results are expressed as the mean $\pm$ S.E.M. where appropriate. Statistical significance was determined at the 0.05 level.

RESULTS AND DISCUSSION

BoNT/E Does Not Cleave SNAP-23

The ability of recombinant BoNT/E light chain to cleave mSNAP-23, hSNAP-25A and B expressed as GST-fusion proteins was determined (Figure 1). The data presented in Figure 1a show that incubation of SNAP-25A or B with BoNT/E resulted in a slight decrease in molecular weight (approx. 3-4kD). This decrease in molecular weight was reproducible over several analyses and is consistent with the cleavage of a 26 residue peptide from the C-terminus of the protein, as reported previously (38,39). By contrast, mSNAP-23 was not cleaved by BoNT/E. Figure 1b shows the results of a separate study demonstrating that hSNAP-23 was also not cleaved by BoNT/E, whereas SNAP-25B was, consistent with the data in Figure 1a. Interestingly, hSNAP-23 ran on SDS-PAGE at a slightly higher molecular weight than mSNAP-23. The reason for this is unclear, but may relate to charge differences in the two molecules, particularly at the C-terminus.
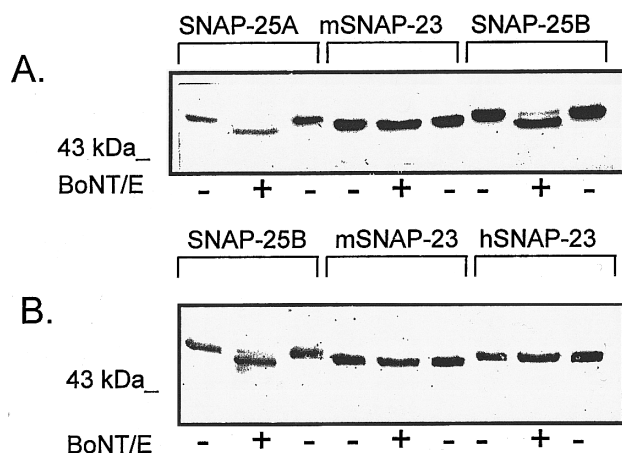
A.

SNAP-25A mSNAP-23 SNAP-25B

43 kDa_

BoNT/E   -   +   -   -   +   -   -   +   -

B.

SNAP-25B mSNAP-23 hSNAP-23

43 kDa_

BoNT/E   -   +   -   -   +   -   -   +   -

FIG. 1. Cleavage of SNAP-23, -25A and 25B by BoNT/E. The neurotoxin L chains at approximately 200 nM concentration in 20 mM HEPES buffer pH 7.5, 100 mM NaCl, were incubated with the GST-fusion protein (1μg) at 37°C for 2 h. SDS loading buffer was then added and an aliquot was electrophoresed on 15% SDS-PAGE. Standard proteins markers from Pharmacia were run, and the molecular weight marker, carbonic anhydrase, is indicated.

The inability of BoNT/A to cleave SNAP-23 could be predicted from sequence alignment that showed that the homologous cleavage site in SNAP-25, Gln-Arg, was Ala-Arg in hSNAP-23 (21)and Thr-Arg in mSNAP-23 (22). However, the cleavage site for BoNT/E, Arg-Ile, twenty six residues in from the C terminal tail of SNAP-25, was maintained in hSNAP-23 and homologous (Lys-Ile) in mSNAP-23. It might have been expected therefore that SNAP-23 would have been a substrate for BoNT/E. Recently Weimbs *et al* (40) identified a conserved domain of approximately 60 amino acids in the t-SNARE superfamily that was repeated twice in SNAP-25-like proteins. This domain is predicted to form a coiled coil. Based on this data, we compared the predicted cleavage site topology of SNAP-23 and -25. This analysis showed significant differences in the nature of the amino acids immediately flanking the cleavage site. Montecucco and coworkers (32, 33) identified a common neurotoxin recognition sequence, in addition to the cleavage sites specific for each neurotoxin cleavage site that they termed the SNARE motif. This motif is characterised by the presence of three negatively charged residues and three hydrophobic residues spaced in such a way that an Edmundson wheel plot shows a negatively charged surface contiguous to a hydrophobic face. SNAP-25 contains four such motifs (Figure 2) spanning residues 21-30, 35-44, 49-58 and 145-154 upstream of the BoNT/E and -/A cleavage sites. These are conserved to a large extent in SNAP-23 but each motif is disrupted to some extent (Figure 2). The results of the present study suggest that this disruption and/or differences around the cleavage site are significant enough to prevent the binding and/or cleavage by BoNT/E.

BoNT/E Only Partially Impairs Insulin Stimulation of GLUT4 Translocation

Since neither BoNT/A or -/E cleaved SNAP-23, it is not possible to use the toxins to assess whether SNAP-23 is involved in the process of insulin stimulation of GLUT4 translocation. However, toxin sensitivity can be used to assess the role of SNAP-25. Previous studies from this laboratory demonstrated that BoNT/A does not affect insulin stimulation of GLUT4 translocation (25). However, BoNT/A has previously been shown to be less effective than BoNT/E in inhibiting either insulin secretion from pancreatic islets (41), or Ca^{++}-activated fusion of large dense core vesicles with the plasma membrane in PC12 cells (30) despite its demonstrable cleavage of SNAP-25. Indeed partly because of this, Banerjee et al (30) proposed that the SNAP-25 residues in the BoNT/E cleaved region between 181-197 might be important in a postdocking step.

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mSNAP-23   S1     VTDESLESTR
hSNAP-23   S1     ITDESLESTR
hSNAP-25A  S1     LADESLESTR
hSNAP-25B  S1     LTDESLESTR
                  21         30

mSNAP-23   S2     LAIESQDAGI
hSNAP-23   S2     LAIESQDAGI
hSNAP-25A  S2     LVEESKDAGI
hSNAP-25B  S2     LVEESKDAGI
                  35         44

mSNAP-23   S3     MLDEQGEQLN
hSNAP-23   S3     MLDEQKEQLN
hSNAP-25A  S3     MLDEQGEQLE
hSNAP-25B  S3     MLDEQGEQLE
                  49         58

mSNAP-23   S4     EMEENLTQVG
hSNAP-23   S4     EMEENLTQVG
hSNAP-25A  S4     EMDENLEQVS
hSNAP-25B  S4     EMDENLEQVS
                145          154
                  xh--xh-xhp

mSNAP-23   C      QIQKITEK
hSNAP-23   C      QIKRITDK
hSNAP-25A  C      QIDRIMEK
hSNAP-25B  C      QIDRIMEK
                188         195
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FIG. 2. SNAP-25 and -23 toxin binding domains and BoNT/E cleavage site. The four toxin binding domains are indicated (adapted from refs. 31,32), termed S1-S4. The residues around the BoNT/E cleavage site RI are also shown, termed C.
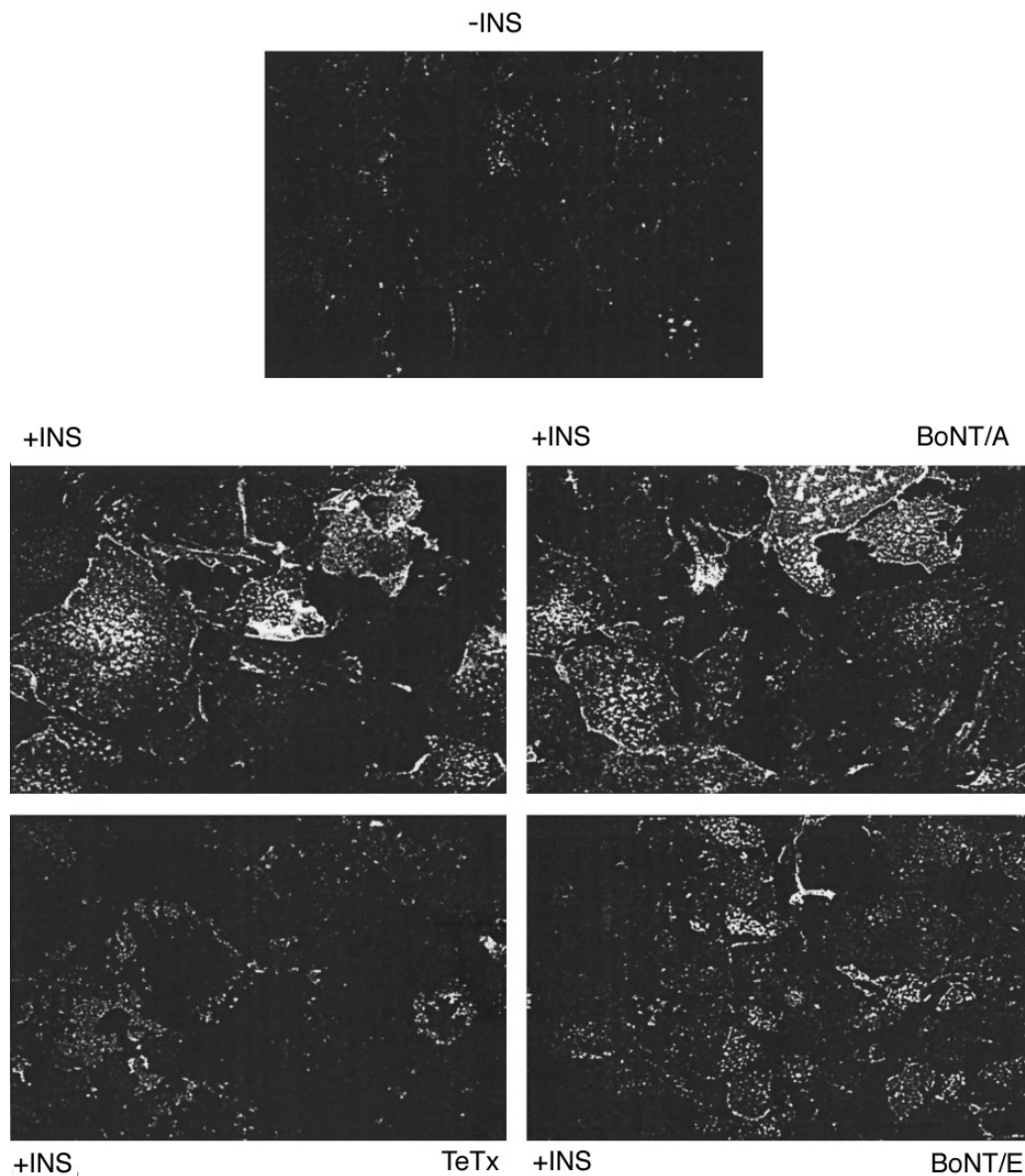
FIG. 3. Effects of microinjection of TeTx, BoNT/E or -/A on insulin stimulation of GLUT 4 translocation in 3T3L1 cells. Differentiated 3T3L1 cells were preincubated in Krebs Ringer bicarbonate, HEPES, 0.5% BSA, pH 7.4, for 60 min. Cells were then microinjected with the toxins at 0.6 mg/ml in 5 mM sodium phosphate, 100 mM KCl, pH 7.2 prior to incubation with insulin and determination of GLUT4 lawn fluorescence as described in Table 1. Representative lawns following the indicated treatments of cells are shown.

The BoNT/E toxin sensitivity of insulin stimulation of GLUT4 translocation was assessed (Figure 3 and Table 1). BoNT/E or -/A was microinjected into differentiated 3T3-L1 cells as described in the Methods and the effect of insulin on GLUT4 translocation in the microinjected cells was assessed using the GLUT4 lawn assay. Figure 3 shows representative fluorescence of the plasma membrane lawns after toxin treatments. Table 1 shows quantification of this fluorescence over 3-7 separate experiments. Incubation of cells with insulin for 15 minutes caused a four fold stimulation of Iawn fluorescence. The level of insulin-induced GLUT4

fluorescence was unaffected by prior microinjection of cells with BoNT/A, as previously reported (25). The level of insulin-induced GLUT4 fluorescence in cells microinjected with BoNT/E was only 27% lower than cells that had not been injected with toxin whereas TeTx microinjection decreased insulin stimulation of GLUT4 lawn fluorescence by 80%, down to a level not significantly different from basal. This result is consistent with only a minor role at best for SNAP-25 in insulin stimulation of GLUT4 translocation. The TeTx sensitivity of insulin stimulation of GLUT4 translocation confirms our previous findings (25) and support a

TABLE 1

Effects of Microinjection of TeTx, BoNT/E or -/A on Insulin Stimulation of GLUT 4 Translocation in 3T3-L1 Cells

Treatment	Microinjection	n	GLUT4 lawn fluorescence (relative fluorescence)	p (cf insulin treated)
Basal	—	7	11.67 ± 1.57	<0.001
Insulin	—	7	43.26 ± 6.20	
Insulin	BoNT/A	7	46.54 ± 6.61	<0.2
Insulin	BoNT/E	6	34.60 ± 5.87	<0.01
Insulin	TeTx	3	17.98 ± 5.46	<0.05

Differentiated 3T3L1 cells were preincubated in Krebs Bicarbonate HEPES buffer, pH 7.4 containing 0.5% BSA and microinjected with 0.6 mg/ml TeTx, BoNT/A or -/E as indicated in 5 mM sodium phosphate, 100 mM KCl, pH 7.2, or buffer alone (basal), the bathing buffer was changed, and the cells were allowed to recover for 60–90 min prior to stimulation with 100 nM insulin for 15 min as indicated. The plasma membrane level of GLUT4 was then assessed by the GLUT4 lawn assay as described in the Methods. Plasma membrane GLUT4 lawn was quantitated using BioRad COMOS software. The pooled results of 3–7 experiments are shown in which four separate image determinations were quantitated for each condition within any signle experiment. P compared to insulin stimulated in the second row is shown.

role for VAMP2 in GLUT4 translocation. The minor effect of BoNT/E is consistent with the low level of SNAP-25 in these cells, as reported previously (18). The low BoNT/E sensitivity of insulin stimulation of GLUT4 translocation thus presents SNAP-23 that is not cleaved by BoNT/E as the prime candidate as a t-SNARE in this process. Analysis of the effects of micro-injection of antibodies to SNAP-23 or peptides that disrupt association might shed light on the role of this protein in GLUT4 translocation.

In conclusion, previous localisation studies (5-18) and functional studies in which proteins (including peptides or fusion proteins to interacting domains, antibodies and/or toxins) have been introduced to cells (12,15, 25-29,42), all clearly implicate syntaxin 4 and VAMP2 in the process of insulin stimulation of GLUT4 translocation. The present study provides the first attempt to assess the functional involvement of SNAP-23 and -25 in GLUT4 translocation. Previous studies demonstrating the tissue localisation of SNAP-23 (21-23), together with the present study demonstrating the BoNT/A and -/E toxin insensitivity of SNAP-23 and only small effects of BoNT/E on insulin stimulation of GLUT4 translocation, place SNAP-23 as the prime candidate for the third member of the fusion complex involved in insulin stimulation of GLUT4 translocation.

ACKNOWLEDGMENTS

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