Functional studies in 3T3L1 cells support a role for SNARE proteins in insulin stimulation of GLUT4 translocation

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Insulin stimulation of glucose transport in the major insulin-responsive tissues results predominantly from the translocation to the cell surface of a particular glucose transporter isoform, GLUT4, residing normally under basal conditions in intracellular vesicular structures. Recent studies have identified the presence of vesicle-associated membrane protein (VAMP) 2, a protein involved in vesicular trafficking in secretory cell types, in the vesicles of insulin-sensitive cells that contain GLUT4. The plasma membranes of insulin-responsive cells have also been shown to contain syntaxin 4 and the 25 kDa synaptosome-associated protein (SNAP-25), two proteins that form a complex with VAMP 2. The potential functional involvement of VAMP 2, SNAP-25 and syntaxin 4 in the trafficking of GLUT4 was assessed in the present study by determining the effect on GLUT4 translocation of microinjection of toxins that specifically cleave VAMPs or SNAP-25, or microinjection of specific peptides from VAMP 2 and syntaxin 4. Microinjection of tetanus toxin light chain or botulinum D toxin light chain resulted in an 80 and 61 % inhibition respectively of insulin stimulation of GLUT4 translocation in 3T3L1 cells assessed using the plasma-membrane lawn assay. Botulinum A toxin light chain, which cleaves SNAP-25, was without effect. Microinjection of an N-terminal VAMP 2 peptide (residues 1–26) inhibited insulin stimulation of GLUT4 translocation by 54 %. A syntaxin 4 peptide (residues 106–122) inhibited insulin stimulation of GLUT4 translocation by 40 % whereas a syntaxin 1c peptide (residues 226–260) was without effect. These data taken together strongly suggest a role for VAMP 2 in GLUT4 trafficking and also for syntaxin 4. They further indicate that the isoforms of SNAP-25 isolated to date that are sensitive to cleavage by botulinum A toxin light chain do not appear to be involved in GLUT4 translocation.

INTRODUCTION

Insulin stimulation of glucose transport in the major insulin-responsive cell types, muscle and fat, occurs by the recruitment of glucose transporters, in particular GLUT4, from an intracellular low-density microsomal compartment to the cell surface. This process involves the formation of vesicles that fuse to the plasma membrane, through a process resembling regulated exocytosis (see [1] for a review). The mechanism of this stimulation remains unclear, although recent studies have demonstrated GTP stimulation of GLUT4 translocation [2] and the requirement of phosphatidylinositol-3-kinase [3–5]. Major advances have been made in understanding the process of regulated exocytosis, to a large extent in neutral cells, over recent years and this has been used as a model for similar processes in other cell types. In particular, several proteins have been identified that direct the trafficking of proteins to the correct cellular membrane location and this has led to the formulation of the SNARE hypothesis (where SNARE is soluble *N*-ethylmaleimide-sensitive factor attachment receptor) (reviewed in [6]). This hypothesis proposes the existence of unique forms of vesicle membrane (v-SNARE) and target membrane (t-SNARE) receptors that ensure the docking and fusion of vesicles with the appropriate target membrane. Syntaxins, 25 kDa synaptosome-associated protein (SNAP-25) and vesicle-associated membrane protein (VAMP) are three proteins identified in neural cells that form a complex around which the process of vesicle docking and fusion occurs, directing neurotransmitter release [6–8]. This mechanism appears to have wider applicability with the identification of these proteins in other cell types including the insulin-responsive tissues, muscle and fat [9–20]. The process has also been closely linked to that of insulin secretion since the demonstration not only of the presence of these proteins in pancreatic islets but also that pretreatment of islets with botulinum A toxin light chain (BoNT/A), which cleaves SNAP-25, results in the inhibition of Ca^{2+}-stimulated insulin release [21].

Several recent studies [9–18], including our own [19,20], have demonstrated the presence of vesicle fusion proteins in fat and muscle tissue. Members of the VAMP family (VAMP 2 and cellubrevin) and secretory carrier membrane proteins have been identified as components of GLUT4 vesicles [9,11–14,16–18]. Syntaxin 4 has been demonstrated as a component of the plasma membrane in muscle [15], and our studies have identified SNAP-25 as a component of fat cell plasma membranes, albeit at low levels [19]. Thus it is clear that most of the vesicle machinery identified in neural cells is present also in the major insulin-responsive cells.

The major task therefore is to establish the functional involvement of each of these proteins in the translocation of GLUT4 to and from the cell surface. Previous studies in this

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment receptors; t-SNARE, target-SNARE; v-SNARE, vesicle-SNARE; VAMP, vesicle-associated membrane protein; BoNT/D, botulinum D toxin light chain; TeTx, tetanus toxin light chain; $DiIC_{18}(5)$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; GTP[5], guanosine 5′-[γ-thio]triphosphate.

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laboratory have demonstrated the interaction of a recombinant syntaxin 4 fusion protein with GLUT4 vesicles and the enhancement of this interaction by incubation with a recombinant SNAP-25 fusion protein, supporting the potential involvement of these proteins [19]. It has also been recently reported that preincubation of streptolysin O-permeabilized 3T3L1 cells with botulinum B toxin partially inhibited GLUT4 translocation and glucose transport, providing the first functional support for the involvement of these proteins in GLUT4 translocation [17]. The present study confirms and extends this important finding using alternative methodology. Microinjection techniques were used to deliver light chains of tetanus toxin (TeTx) and botulinum D toxin (BoNT/D), which cleave members of the VAMP family (VAMP 2 and cellubrevin), as well as BoNT/A, which specifically cleaves SNAP-25. The study shows that TeTx and BoNT/D inhibit GLUT4 translocation, whereas BoNT/A is without effect. Moreover we show that preincubation of TeTx or BoNT/D with GLUT4 vesicles prevents interaction of the vesicles with syntaxin 4 *in vitro*. We also demonstrate that microinjection of specific VAMP 2 and syntaxin 4 peptides inhibits GLUT4 translocation. These data indicate that VAMP 2 and/or cellubrevin and syntaxin 4 are involved in the mechanism of GLUT4 translocation whereas SNAP-25 isoforms that are sensitive to BoNT/A probably are not.

MATERIALS AND METHODS

Cell culture

3T3L1 fibroblasts obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were maintained and passaged as preconfluent cultures in Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 10 % newborn calf serum (CSL Ltd.). Cells for differentiation were maintained at confluence for 48 h, then induced to differentiate by the addition of DMEM containing 5 % fetal calf serum (CSL Ltd.), 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 fetal calf serum/DMEM containing 4 μg/ml insulin. Cells were used 7–14 days after differentiation, at which more than 90 % of 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 45 min period using a Zeiss automated injection system (Carl Zeiss) coupled to an Eppendorf microinjector. Micropipettes were prepared using a Sutter P-97 micropipette puller. Reagents were dissolved in a buffer containing 5 mM sodium phosphate, pH 7.2 and 100 mM KCl for microinjection. Cells were transferred into fresh medium and allowed to recover for 20 min after guanosine 5′-[γ-thio]-trisphosphate (GTP[γS]) microinjection or for 60–90 min after injection of peptides or toxins, at the concentrations indicated, before stimulation with insulin (100 nM) and analysis of GLUT4 translocation using the plasma-membrane lawn assay. GLUT4 translocation in microinjected cells was compared with 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 [22] with modifications described by Marsh et al. [23]. Briefly, after cell treatment, 3T3L1 cells grown on coverslips were washed in poly(L-lysine), hypotonically shocked with three washes in 1:3 membrane buffer (70 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, 30 mM Hepes, pH 7.2) and sonicated using a probe sonicator (Microson) at setting 0, in membrane buffer, to generate a lawn of plasma-membrane fragments that remained attached to the coverslip. The fragments were then immunolabelled with polyclonal rabbit anti-GLUT4 antibody (R1159; 1:100 dilution) [24] and either FITC-labelled goat anti-rabbit (Silenus, Australia), or CY3-labelled goat anti-rabbit (Amersham). Later experiments used the CY3 antibody because imaging was brighter. Coverslips were visualized and imaged using a Bio-Rad Lasersharp MRC-500 confocal laser scanning immunofluorescence microscope. Data were analysed using Bio-Rad COMOS confocal imaging software. Each study was conducted independently over approximately 1 month to minimize variability. Efforts were made to maintain confocal microscope gain settings over that period, although alterations in these as well as differences in 3T3L1 populations contribute particularly to between-study variability.

Interaction studies

Low-density microsomes were prepared from rat epididymal fatpads or from differentiated 3T3 L1 cells by homogenization and differential centrifugation as previously described [19,24–27]. The microsomes were incubated with purified glutathione S-transferase (GST)–syntaxin 4 and GST–SNAP-25 as previously published [19]. Protein interactions were monitored by the binding of phycoerythrin–streptavidin (DAKO, Carpinteria, CA, U.S.A.) to biotinylated proteins. GLUT4-containing microsomes were identified by staining with antibody 1F8 [28] and FITC-labelled goat anti-mouse immunoglobulin (Silenus), and microsomal membranes were identified by staining with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate $DilC_{18}(5)$ (Molecular Probes, Eugene, OR, U.S.A.). Toxin proteins (1 μg) were preincubated with microsomes (28 μg of protein) for 1 h at room temperature in HES buffer (20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4) before the addition of biotinylated syntaxin 4 (8 μg) or syntaxin 4 plus biotinylated SNAP-25 (6.4 μg) and the incubation continued at 4 °C overnight before the microsomes were washed by centrifugation (100000 ***g*** for 60 min) in a Beckman TL 100 ultracentrifuge before the addition of fluorescent labels.

Flow-cytometric analysis of the protein binding was carried out as previously described [19].

Toxin preparation

Plasmids containing the neurotoxin light chains of TeTx, BoNT/A and BoNT/D were obtained from Professor H. Niemann, Tubingen, Germany. The light-chain genes containing a C-terminal His_6 tag were expressed in *Escherichia coli* M15[pREP4] and were purified by binding to Ni^{2+}–nitrilotriacetate resin (Qiagen). Cleavage of the GST-fusion proteins were carried out as previously described [29].

Peptide synthesis

Peptides were synthesized using an Applied Biosystems 430A peptide synthesizer coupled with FastMoc strategy. Purity was checked by reverse-phase HPLC and their integrity confirmed by amino acid analysis and matrix-assisted laser-desorption ionization MS. Three peptides were examined: N-terminal VAMP 2, 1–26, SATAATAPPAAPAGEGGPPAPPPPNLC; syntaxin 4,

106–122, CPQKEADENYNSVNTRM (both of these included cysteine linkers); syntaxin 1c, 226–260, QPQGAFLKSCPEPQPNPEEGALWSSGAPGPAGRDD.

Statistical analyses

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

RESULTS

The activities of recombinant BoNT/D and TeTx to cleave VAMP 2 and cellubrevin, and of BoNT/A to cleave SNAP-25 were confirmed by their ability to cleave GST–VAMP 2 and GST–SNAP-25 (Figure 1). The molecular mass of GST–VAMP 2 was markedly reduced after incubation of the protein with either TeTx or BoNT/D. When GST–SNAP-25 was incubated with BoNT/A there was only a slight decrease in molecular mass of the protein, although this was consistent over several cleavage analyses, two of which are shown in Figure 1. This was consistent with the reported cleavage of a nine-residue peptide from the C-terminus of the protein [30]. Thus, the TeTx, BoNT/D and BoNT/A preparations were active *in vitro* and therefore used for microinjection studies.

Glucose-transporter translocation was assessed using the plasma-membrane lawn assay [22,23]. Initially, the validity of the microinjection technique was determined by assessing the ability of GTP[γS] microinjected into differentiated 3T3L1 cells to mimic insulin stimulation of glucose-transporter translocation

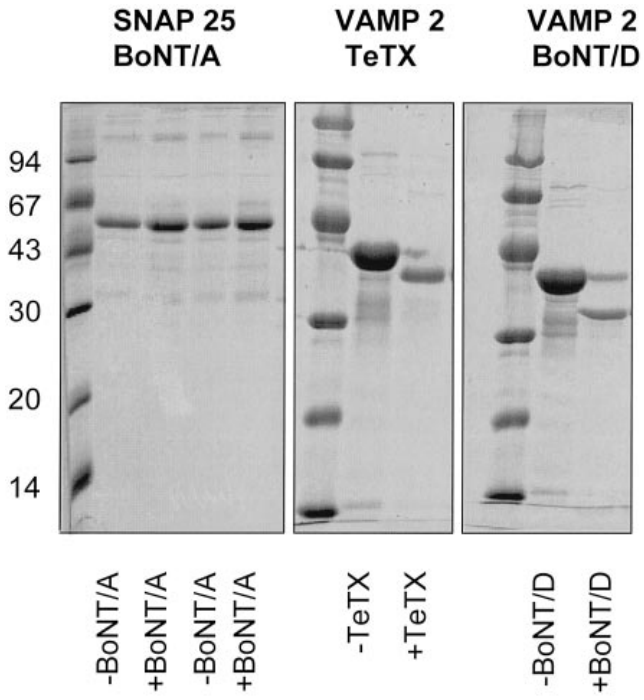

Figure 1 Cleavage of SNAP-25 by BoNT/A and VAMP 2 by TeTX and BoNT/D

The neurotoxin light chains at approx. 200 nM concentration in 20 mM Hepes buffer, pH 7.0, were incubated with the GST-fusion protein (30 μg) at 37° for 1–2 h. SDS loading buffer was then added and an aliquot electrophoresed on an SDS/15% polyacrylamide gel. Standard protein markers from Pharmacia were run in the left lanes and the molecular masses (kDa) are indicated.

Table 1 Effect of microinjection of GTP[γS] on GLUT4 translocation

Differentiated 3T3L1 cells were preincubated in Krebs–Ringer bicarbonate/Hepes buffer, pH 7.4, containing 0.5% BSA for 2 h. Cells were then microinjected with 2 mg/ml GTP[γS] in 5 mM sodium phosphate/100 mM KCl, pH 7.2, or buffer alone (basal). The bathing buffer was then changed and the cells allowed to recover for 20 min. Alternatively, cells were stimulated with 100 nM insulin for 15 min before assessment, in each case, of GLUT4 translocation by the lawn assay (see the Materials and methods section). Results are means ± S.E.M. from four separate experiments in which the relative fluorescence of three or more independent images of plasma-membrane lawns at a magnification of × 400 were determined as detailed in the Materials and methods section.

Cell incubation conditions	GLUT4 translocation (relative fluorescence)
Basal	2.86 ± 0.55
+ Insulin	14.81 ± 1.65
GTP[γS] microinjected	11.18 ± 1.65

(Table 1). Insulin elicited a 5.6-fold increase in plasma-membrane lawn immunofluorescence after immunoprobing lawns with a polyclonal GLUT4 antibody, R1159 [24], and FITC-labelled secondary antibody, consistent with translocation of GLUT4 to the plasma membrane after insulin stimulation. Microinjection of GTP[γS] caused a 4.5-fold increase in plasma-membrane GLUT4 fluorescence, which is not significantly different from that elicited by insulin. Microinjection buffer (100 mM KCl, 5 mM sodium phosphate, pH 7.4) alone, without GTP[γS], had no effect on insulin stimulation of plasma-membrane GLUT4 fluorescence and, by implication, its translocation to the plasma membrane.

The effects of BoNT/A, BoNT/D and TeTx on insulin stimulation of GLUT4 translocation were assessed after microinjection into differentiated 3T3L1 cells (Figure 2, Table 2). Each toxin or microinjection buffer was injected into more than 200 cells over a 30 min period and the cells were maintained at 37 °C for 60 min thereafter. Insulin was then added to the cells for a further 15 min before the preparation of plasma-membrane lawns and assessment of GLUT4 associated with the membranes by immunoprobing with R1159 anti-GLUT4 antibody and CY3-labelled secondary antibody. Figure 2 shows representative fluorescence of the plasma-membrane lawns after toxin treatment, and Table 2 shows quantification of this fluorescence over several experiments. Insulin elicited a 3.3-fold increase in plasma-membrane lawn fluorescence. Microinjection buffer again had no effect on this stimulation, similar to the GTP[γS] study. Microinjection of BoNT/A also had no effect on the ability of insulin to stimulate plasma-membrane GLUT4 immunofluorescence and therefore the association of GLUT4 with the plasma membrane. By contrast, insulin stimulation of plasma-membrane GLUT4 immunofluorescence after BoNT/D treatment was only 1.9-fold, or approximately half that of untreated cells. These data indicate that BoNT/D inhibited GLUT4 translocation. However, in no experiment was total blockage of GLUT4 translocation achieved with this toxin under these experimental conditions. TeTx, like BoNT/D, inhibited insulin stimulation of plasma-membrane fluorescence and therefore GLUT4 translocation. After TeTx treatment, insulin stimulation of plasma-membrane lawn GLUT4 immunofluorescence was only 1.5-fold that of basal unstimulated cells, and in fact not significantly different from lawns of unstimulated cells. In two of seven experiments, there was no difference in GLUT4 immunofluorescence of TeTx lawns after insulin stimulation compared with basal, and therefore total inhibition of GLUT4 translocation could be inferred. The

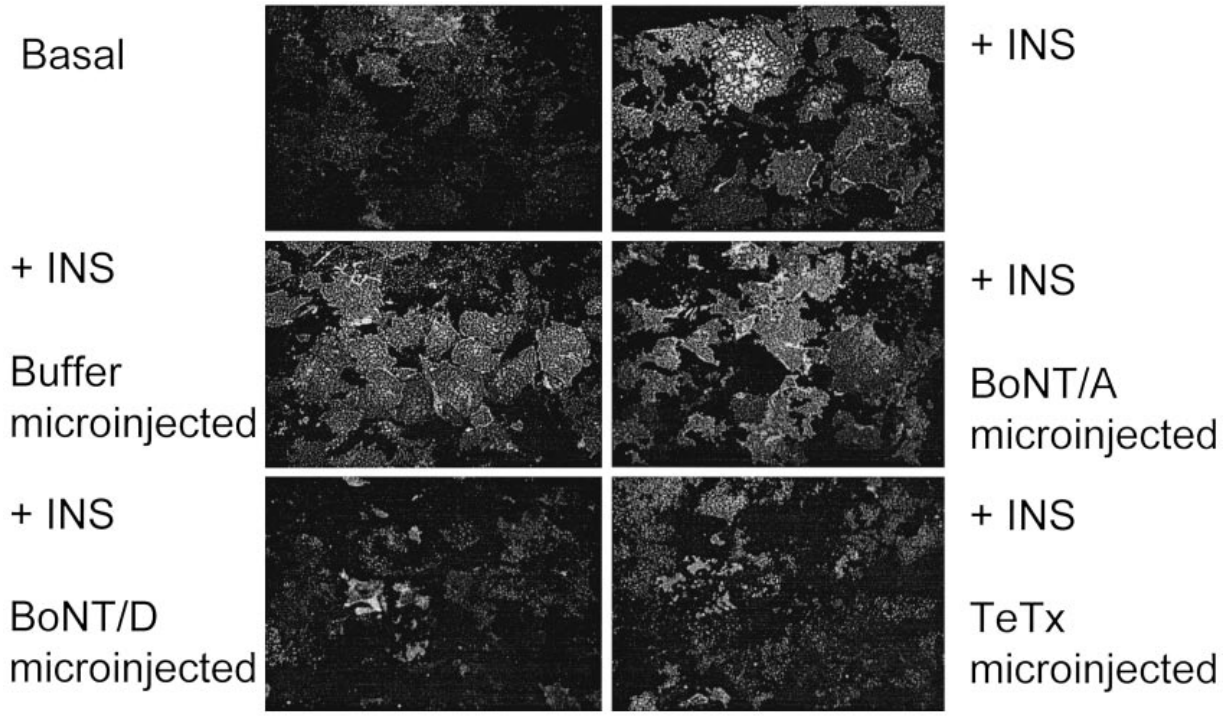

Figure 2 Effect of microinjection of BoNT/D, TeTx and BoNT/A on insulin stimulation of GLUT4 translocation

Differentiated 3T3L1 cells were preincubated in Krebs–Ringer bicarbonate/Hepes/0.5% BSA, pH 7.4, for 60 min as described in the legend to Table 1. Cells were then microinjected with the toxins at 1 mg/ml in 5 mM sodium phosphate/100 mM KCl, pH 7.2, and the incubation buffer was changed. The cells were allowed to recover for 60–90 min before stimulation or not with 100 nM insulin, as indicated (INS), for 15 min. GLUT4 translocation was then determined as described in the Materials and methods section by the plasma-membrane lawn assay with CY3-labelled secondary antibody. Representative lawns after the indicated treatments of cells are shown.

Table 2 Effect of microinjection of BoNT/D, TeTx and BoNT/A on insulin stimulation of GLUT4 translocation

Plasma-membrane lawns, prepared after toxin microinjection of cells as indicated in Figure 2, were quantified for lawn fluorescence using Bio-Rad COMOS software. The pooled results of *n* experiments are shown in which three separate image determinations were quantified within any single experiment. Results are means ± S.E.M. for the number of experiments indicated. *P* compared with insulin stimulation in the second row is shown (ns, not significant).

Treatment	Microinjection	*n*	GLUT4 lawn fluorescence (relative fluorescence)	*P*
Basal	—	7	10.87 ± 1.84	< 0.001
Insulin	—	7	36.00 ± 3.98	—
Insulin	Buffer	7	34.35 ± 3.75	ns
Insulin	BoNT/A	4	34.88 ± 4.91	ns
Insulin	TeTX	7	16.07 ± 1.70	< 0.001
Insulin	BoNT/D	4	20.64 ± 3.17	< 0.02

Table 3. Effect of microinjection of peptides derived from unique regions of VAMP 2, syntaxin 4 and syntaxin 1c on insulin stimulation of GLUT4 translocation

Peptides derived from the N-terminus of VAMP 2 [VAMP 2-(1–26)], syntaxin 4 [syntaxin 4-(106–122)] and syntaxin 1c [syntaxin 1c-(226–260)] were microinjected at 10 mg/ml and experiments performed and analysed as described for the toxins in Figure 2 and Table 2 except that in some experiments FITC-labelled secondary antibody was used whereas in others CY3-labelled secondary antibody was used. Thus data are expressed as percentage of insulin-stimulated GLUT4 lawn fluorescence instead of relative fluorescence. Results are means ± S.E.M. for the number of experiments indicated. *P* compared with insulin stimulation is shown (ns, not significant).

Treatment	Microinjection	*n*	GLUT4 lawn fluorescence (% of insulin-stimulated)	*P*
Basal	—	9	38 ± 5	< 0.001
Insulin	Buffer	6	105 ± 8	ns
Insulin	GST	3	117 ± 11	ns
Insulin	VAMP-2-(1–26)	3	47 ± 6	< 0.002
Insulin	Syntaxin 4-(106–122)	7	61 ± 5	< 0.001
Insulin	Syntaxin 1c-(226–260)	3	116 ± 15	ns

inhibition of GLUT4 translocation by BoNT/D and TeTx suggests the involvement of VAMP 2 and/or cellubrevin in this process, whereas the lack of effect of BoNT/A, which cleaves SNAP-25, implies that SNAP-25 is probably not involved. VAMP 1 is insensitive to cleavage by BoNT/D and TeTx and therefore the implication of the present studies is that VAMP 1 is not involved.

The potential involvement of VAMP 2 and syntaxin 4 in GLUT4 translocation was further assessment by examination of the effects of unique peptide sequences derived from the vesicle fusion proteins on insulin stimulation of GLUT4 translocation (Table 3). GST and microinjection buffer were injected as controls and had no effect on insulin stimulation of GLUT4 plasma-membrane immunofluorescence (GLUT4 translocation). An N-terminal VAMP 2 peptide (residues 1–26), which had been shown previously to inhibit neurotransmitter release from

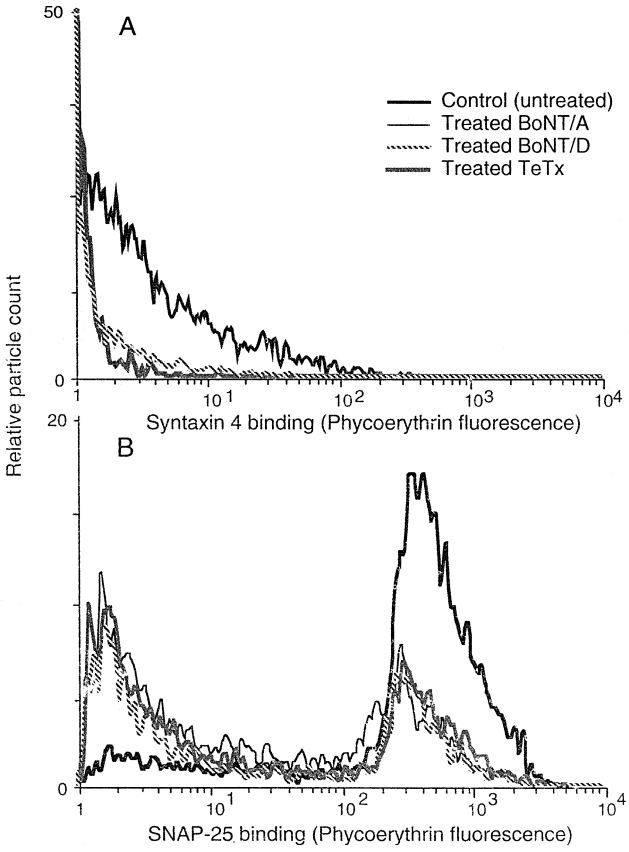

Figure 3 Flow-cytometric analysis of the binding of GST–syntaxin 4 (A) and GST–SNAP-25 (B) to low-density microsomes after toxin treatment

Microsomes were prepared and binding of proteins was carried out as described in the Materials and methods section. Protein binding was detected by phycoerythrin–streptavidin binding to biotinylated proteins. The microsomes were pretreated with toxins (BoNT/D, TeTx) (**A,B**), or GST–SNAP-25 was pretreated with BoNT/A (**B**) before protein binding was carried out. Specific binding of the proteins to microsomes was detected by correlation of phycoerythrin fluorescence with fluorescence of the membrane dye $DiIC_{18}(5)$.

Aplasia ganglia [31], inhibited insulin stimulation of plasma-membrane GLUT4 immunofluorescence by 54% compared with cells that were not microinjected. A syntaxin 4 peptide (residues 106–122), based on a unique region of syntaxin 4, likewise inhibited GLUT4 translocation (GLUT4 immunofluorescence inhibited by about 40%). On the other hand, a syntaxin 1c peptide (residues 226–260), based on a unique region of syntaxin, was without effect. The syntaxin 1c data, together with those obtained by microinjection of GST, served as useful controls for the effects of syntaxin 4 and VAMP 2. These data provide functional support for a role for syntaxin 4 as a t-SNARE for VAMP 2 in trafficking of GLUT 4 vesicles. This possibility was further investigated by flow-cytometric analysis.

Previous studies from this laboratory [19] demonstrated the use of flow cytometry as a tool to measure protein interactions with GLUT4-containing low-density microsomes of particle size consistent with them being GLUT4 vesicles. Specifically, the interaction of syntaxin 4 with GLUT4-containing microsomes was demonstrated, as well as the enhancement of SNAP-25 binding by syntaxin 4. The effects of BoNT/A, BoNT/D and TeTx on the interaction of GST–syntaxin 4 and SNAP-25 with GLUT4-containing microsomes is shown in Figure 3. Figure 3(A) shows flow-cytometric analysis of the binding of biotinylated GST–syntaxin 4 to GLUT4-containing microsomes (1F8, anti-GLUT4 antibody-positive microsomes) after toxin treatment. Both BoNT/D and TeTx inhibited syntaxin 4 association with GLUT4-positive microsomes, as shown by a shift to the left of syntaxin 4 fluorescence (detected with phycoerythrin) associated with them. GLUT4-positive microsomes incubated in the absence of syntaxin 4 showed no phycoerythrin fluorescence (results not shown). Figure 3(B) shows the binding of labelled SNAP-25, in the presence of syntaxin 4, to GLUT4-positive microsomes after toxin treatment. In this case, cleavage of SNAP-25 with BoNT/A inhibited SNAP-25 associated with GLUT4-positive microsomes as shown by decreased SNAP-25 fluorescence (right peaks, Figure 3B) and an increase in negative fluorescence (left peaks). For clarity, data from GLUT4 vesicles incubated in the absence of biotinylated SNAP-25 is not shown, but was similar to that of the negative toxin peaks (left peaks). BoNT/D and TeTx, which cleave VAMP, also decreased SNAP-25 fluorescence, consistent with the association of SNAP-25 with GLUT4-positive microsomes occurring via binding to syntaxin 4. When BoNT/D was added to the vesicles at the same time as syntaxin 4 and SNAP-25, the binding of the proteins was still reduced (results not shown). Interestingly, the syntaxin 4-based peptide that inhibited GLUT4 translocation in the microinjection studies (Table 3) was without effect on the association of syntaxin 4 with GLUT4-positive microsomes (results not shown).

Double labelling of microsomes with syntaxin-4 and GLUT-4 antibody (Figure 4) indicated that the majority, 81.4%, of the microsomes detected were GLUT-4-positive and bound syntaxin-4 (quadrant 2 in Figure 4A), although a proportion, 16.7%, did not bind syntaxin-4 (quadrant 4 in Figure 4A). Treatment with BoNT/D reduced the proportion of microsomes that bound syntaxin 4 (Figure 4B) from 81.4 to 24.5%, although the proportion of GLUT-4-positive microsomes was not reduced (98.1 and 99.6% respectively; the sum of quadrants 2 and 4). TeTx treatment resulted in essentially the same pattern as BoNT/D treatment (results not shown).

DISCUSSION

Insulin stimulation of glucose transport in fat and muscle involves the translocation of vesicles containing the insulin-responsive glucose transporter, GLUT4, to the cell surface. Much work over recent years has been directed towards identifying those molecules in addition to GLUT4 that reside in this vesicle compartment as a means to begin to understand the mechanism by which the vesicle might be translocated in response to insulin (see [1] for a review). Recent studies have identified the presence in GLUT4 vesicles of an aminopeptidase of unknown function, vp-165 [32–34], a GTPase, Rab 4 [35,36], phosphatidylinositol 3-kinase [37,38] and two families of proteins, VAMPs and secretory carrier membrane proteins [9,11–14,16–19], which appear to be involved in trafficking events between distinct membrane compartments in response to stimuli. Indeed, studies from this laboratory [19,20] and those of others [9–18] indicate that much of the machinery needed to form active fusion complexes for trafficking between different cellular compartments are present in insulin-responsive cells and/or tissues, including muscle and fat.

Recently, two studies have pointed to the functional involvement of VAMP 2 in glucose-transporter translocation. These studies showed that incubation of streptolysin O-permeabilized 3T3L1 cells with BoNT/D or BoNT/B, which cleave VAMP isoforms, inhibited GLUT4 translocation [17,39]. BoNT/C, which cleaves syntaxins 1, 2 and 3, was without effect [39]. In the present study, the involvement of VAMP isoform(s)

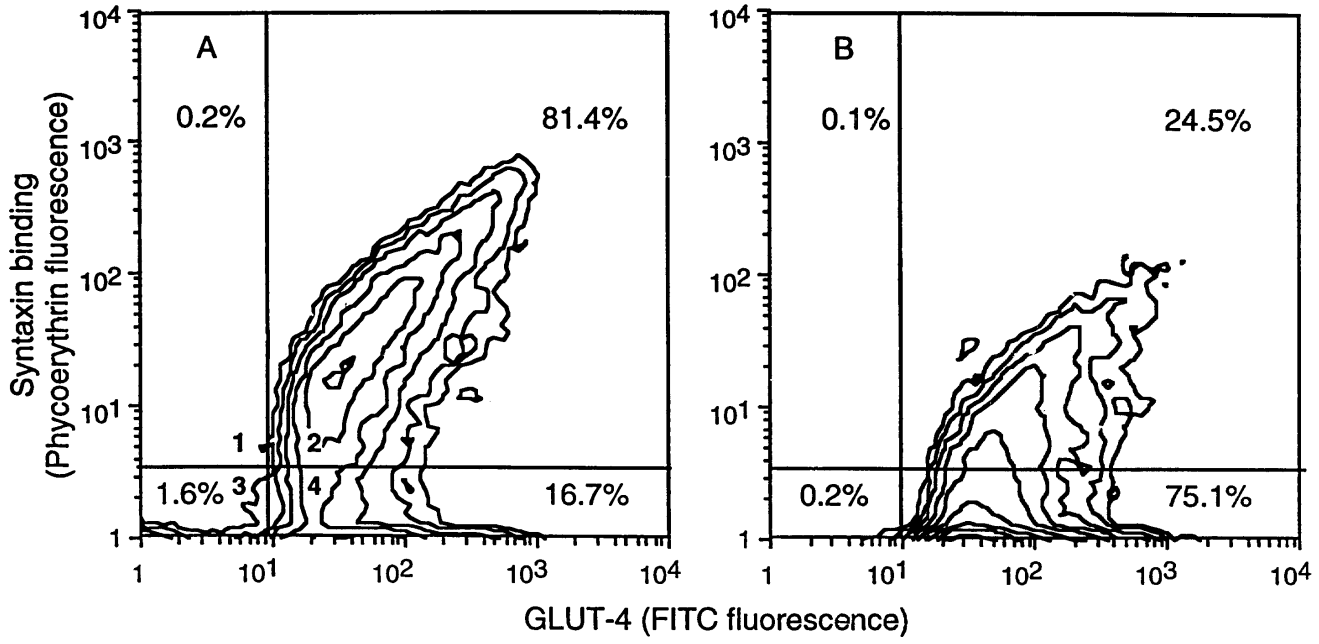

Figure 4 Two-colour flow cytometric analysis of the effect of BoNT/D on GLUT4 carrying low-density microsomes

Low-density microsomes were fluorescently labelled with monoclonal antibody 1F8 (specific for GLUT4 [28]) as described in the Materials and methods section. Detection of syntaxin-4-binding and BoNT/D treatment were carried out as described in the Materials and methods section. Contours indicate the counts of microsomes possessing relative levels of FITC and phycoerythrin fluorescence. The percentages of microsomes in each quadrant, identified by numbers in the corner of each plot, are shown. The quadrants indicate: 1, GLUT4-negative, syntaxin-4-positive; 2, GLUT4-positive, syntaxin-4-positive; 3, GLUT4-negative, syntaxin-4 negative; 4, GLUT4-positive, syntaxin-4 negative. (**A**) Untreated microsomes; (**B**) BoNT/D-treated microsomes.

in GLUT4 translocation is demonstrated using alternative methodology. First we showed that BoNT/D and TeTx microinjected into 3T3L1 cells inhibit GLUT4 translocation. Secondly we demonstrated that an N-terminal VAMP 2 peptide, shown previously to inhibit neurotransmitter release [31], also inhibited GLUT4 translocation. Our results to date do not support a role for SNAP-25 in GLUT4 translocation, since BoNT/A, which cleaves SNAP-25, was without effect on insulin stimulation of GLUT4 translocation, despite the protein being detected in these cells previously [19].

These results demonstrating the effects of BoNT/D and TeTx in GLUT4 translocation and the inhibition of GLUT4 translocation with an N-terminal VAMP 2 peptide, and two previous studies [17,39] using BoNT/B and BoNT/D firmly establish the involvement of VAMP isoform(s) in GLUT4 translocation. The specific VAMP(s) involved cannot be proved conclusively from these studies. However, TeTx inhibited GLUT4 translocation by more than 80%. Since this neurotoxin does not cleave VAMP 1 [40], it is difficult to ascribe a major role for this isoform in GLUT4 translocation. Consistent with this, Volchuk et al. [14] were unable to detect VAMP 1 at the protein level with specific antibodies in skeletal muscle or in differentiated L6 muscle cells, although a previous study of ours [19] and Ralston et al. [13] demonstrated the presence of VAMP 1 mRNA in human fat and skeletal muscle. This isoform was not detected in mRNA transcripts from rat skeletal muscle [14]. VAMP 2 and cellubrevin, on the other hand, have been isolated as components of GLUT4 vesicles in several studies [9,16,17]. Their levels are increased on differentiation of L6 muscle cells to myotubes [14] and of 3T3L1 cells into the fatty state [16], and they have been shown to translocate to the plasma membrane in response to insulin, although not to the same extent as GLUT4 [16,18]. Volchuk et al. [16] immunopurified cellubrevin containing vesicles and found them to contain GLUT4 but no detectable VAMP 2 and hypothesized the existence of at least two populations of GLUT4 vesicles. Whether these will distinguish exocytic from endocytic pathways remains to be determined. Certainly in neural cells VAMP 2 has been shown to participate in regulated exocytosis [41], whereas cellubrevin is ubiquitously expressed and involved in endosomal recycling [42]. Our data on VAMP 2 peptide likewise do not distinguish between these possibilities, since potential cellubrevin interaction may be compromised by the VAMP 2 peptide, although the cellubrevin N-terminus does not contain the proline-rich motif identified as a component of the inhibitory activity of regulated exocytosis in *Aplasia* [31].

The above studies implicate VAMP 2 as a v-SNARE in GLUT4 translocation. Only syntaxins 1 and 4 have been shown to interact with VAMP 2 [43,44]. Syntaxins 1a and 1b have not been detected in insulin-responsive cells, however, at the protein level at least, and only low levels of syntaxins 2 and 3 have been observed [10]. This is consistent with the lack of effect of BoNT/C on GLUT4 translocation reported by Cheatham et al. [39]. Recently, syntaxin 4 was demonstrated to be expressed in insulin-responsive cell lines, as well as muscle and fat [15,19]. Its expression was increased after differentiation of L6 muscle cells to form myotubes, consistent with increased insulin responsiveness after differentiation [15]. It thus represents a potential candidate for the so-called t-SNARE. In support of this contention, it was recently found that anti-(syntaxin 4) antibodies inhibit GLUT4 translocation [45,46]. The present results extend these studies by the demonstration that a unique syntaxin 4 peptide (residues 106–122) inhibits GLUT4 translocation. This was somewhat surprising since the sequence lies outside known interaction domains [44,47] (see [48] for a review). Consistent

with this, we found no effect of this peptide on the association of syntaxin 4 with GLUT4 vesicles by flow cytometry. It provides the interesting possibility, however, that this region of syntaxin 4, which was selected for its low sequence identity with other syntaxins, represents an as yet undescribed interaction site with another protein. One must be careful with this interpretation since it is possible that the peptide sequence may exert a non-specific effect. In any case, the microinjection and flow-cytometry data support the potential involvement of syntaxin 4 in GLUT4 translocation.

We had previously shown [19] that recombinant expressed syntaxin 4 fusion proteins were capable of interacting specifically with GLUT4 vesicles, as analysed by flow cytometry. The present study, again using flow-cytometric analysis, demonstrates that this interaction of syntaxin 4 with GLUT4 vesicles can be markedly reduced by preincubation of low-density microsomal fractions (which contain GLUT4 vesicles) with TeTx or BoNT/D. These results therefore indicate that the BoNT/D and TeTx sensitivity seen in the *in vivo* microinjection studies resides within the GLUT4 vesicle where VAMP 2 and/or cellubrevin are known to reside. They further show that the syntaxin 4 interaction is insensitive to cleavage by BoNT/A, as would be expected from the previously demonstrated specificity of BoNT/A for SNAP-25 [30]. Previously we showed that recombinant SNAP-25 interacted with GLUT4 vesicles in the presence of syntaxin 4 and enhanced syntaxin 4 binding to GLUT4 vesicles [19]. This enhanced binding was reduced by both preincubation of the recombinant SNAP-25 with BoNT/A and preincubation of the microsomes with BoNT/D and TeTx. BoNT/D was still effective when added to microsomes at the same time as the proteins, possibly suggesting that VAMP on the microsomes is not protected from cleavage in the presence of syntaxin and SNAP. We were unable to detect any inhibition of GLUT4 translocation by BoNT/A *in vivo*, however, despite detecting SNAP-25 previously. Thus, although the machinery to form the analogous core complex to neural cells exists in insulin-responsive cells, namely VAMP, syntaxin and SNAP-25, GLUT4 translocation in response to insulin appears to function independently of SNAP-25 unless the low levels of SNAP-25 in the cells were protected from cleavage by BoNT/A. Alternatively, the recently described SNAP-23, which appears to have a fairly ubiquitous tissue distribution, may function in this role [47]. Its sensitivity to BoNT/A was not reported. The ability of syntaxin 4 to interact directly with GLUT4 vesicles seen in our flow-cytometry study indicates that fusion of the vesicles with the plasma membrane can occur without the involvement of SNAP-25 or -23. Timmers et al. [18] have provided evidence to support the involvement of *N*-ethylmaleimide-sensitive factor and α-SNAP in this interaction. Whether these or as yet unidentified molecules enhance the efficiency of SNARE complex-formation and the association of GLUT4 vesicles with the plasma membrane remains to be determined. Certainly, other non-neural forms of accessory proteins involved in vesicle trafficking have been identified, including synaptotagmin [49], Munc18 [50], Rabs [35,36,51,52] and Rab GDP dissociation inhibitor [53]. The extent to which these may be involved in GLUT4 trafficking remains to be determined.

In conclusion, this study provides functional support for the involvement of VAMP 2 and/or cellubrevin in insulin-stimulated GLUT4 translocation as the v-SNARE on GLUT4 vesicles. It further supports the possible role of syntaxin 4 in this process as a potential t-SNARE on the plasma membrane. The fact that BoNT/A had no effect on insulin stimulation of GLUT4 translocation appears to rule out a role for the currently described isoforms of SNAP-25 in this process.

We thank Professor H. Niemann (BFAV, Tubingen, Germany) for the gift of the vectors containing the light-chain genes for the toxins used in these experiments, Mr. Alan Kirkpatrick for the preparation of the peptides, Dr. J. Proietto (University of Melbourne, Australia) for the polyclonal anti-GLUT4 antibody (R1159), Professor David James (University of Queensland, Australia), Dr. L. Cosgrove and Dr. M. Frenkel for many helpful discussions and Ms Annette Alafaci for technical assistance.

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Received 9 September 1996/28 November 1996; accepted 16 January 1997