Proteolytic cleavage of cellubrevin and vesicle-associated membrane protein (VAMP) by tetanus toxin does not impair insulin-stimulated glucose transport or GLUT4 translocation in rat adipocytes

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Acute insulin stimulation of glucose transport in fat and skeletal muscle occurs principally as a result of the hormonal induced translocation of the GLUT4 glucose transporter from intracellular vesicular stores to the plasma membrane. The precise mechanisms governing the fusion of GLUT4 vesicles with the plasma membrane are very poorly understood at present but may share some similarities with synaptic vesicle fusion, as vesicle-associated membrane protein (VAMP) and cellubrevin, two proteins implicated in the process of membrane fusion, are resident in GLUT4-containing vesicles isolated from rat and murine 3T3-L1 adipocytes respectively. In this study we show that proteolysis of both cellubrevin and VAMP, induced by electroporation of isolated rat adipocytes with tetanus toxin, does not impair insulin-stimulated glucose transport or GLUT4 translocation. The hormone was found to stimulate glucose uptake by approx. 16-fold in freshly isolated rat adipocytes. After a single electroporating pulse, the ability of insulin to

activate glucose uptake was lowered, but the observed stimulation was nevertheless nearly 5-fold higher than the basal rate of glucose uptake. Electroporation of adipocytes with 600 nM tetanus toxin resulted in a complete loss of both cellubrevin and VAMP expression within 60 min. However, toxin-mediated proteolysis of both these proteins had no effect on the ability of insulin to stimulate glucose transport which was elevated approx. 5-fold, an activation of comparable magnitude to that observed in cells electroporated without tetanus toxin. The lack of any significant change in insulin-stimulated glucose transport was consistent with the finding that toxin-mediated proteolysis of both cellubrevin and VAMP had no detectable effect on insulin-induced translocation of GLUT4 in adipocytes. Our findings indicate that, although cellubrevin and VAMP are resident proteins in adipocyte GLUT4-containing vesicles, they are not required for the acute insulin-induced delivery of GLUT4 to the plasma membrane.

INTRODUCTION

One characteristic feature of insulin-sensitive tissues such as fat and skeletal muscle is that they express the GLUT4 glucose transporter which endows them with the ability to acutely up-regulate the transport of glucose across their surface membranes in response to insulin binding. Under circumstances when circulating insulin concentrations are low, the bulk of the cellular GLUT4 is sequestered within specialized tubulovesicular intracellular membrane structures [1–4]. On exposure of adipocytes or muscle to insulin, a proportion of the GLUT4 that is resident in these membranous structures is mobilized for insertion into the plasma membrane. The increased delivery and insertion of new GLUT4 molecules into the plasma membrane results in an increase in glucose-transport activity and forms the principal basis by which insulin stimulates glucose transport in both fat and skeletal muscle (for reviews see [5–7]).

Although the translocation hypothesis, based on the pioneering work of Cushman and Wardzala [8] and Suzuki and Kono [9], is now widely established, we still, however, know very little about the cellular mechanisms involved in the trafficking of GLUT4 from its intracellular storage pool to the cell surface. One current view is that the insulin-induced translocation of GLUT4 resembles regulated secretion, a process in which GLUT4 molecules, normally retained within a unique membrane organelle,

undergo regulated fusion with the cell surface on insulin stimulation [6]. This process shares some similarities with synaptic neurotransmitter release, which relies upon a number of proteins that comprise the fusogenic machinery. Synaptic vesicle fusion is initiated through interaction of proteins resident on neurotransmitter-containing vesicles, called v-SNAREs, and on the target membrane, called t-SNAREs [10,11]. The fusion of two different membranes is thought to be brought about by the attachment to this SNARE scaffold of a soluble *N*-ethylmaleimide-sensitive fusion attachment protein (SNAP). The SNAP proteins themselves allow the attachment of an *N*-ethylmaleimide-sensitive fusion protein (NSF). NSF is considered to be functionally very important since it not only contains an intrinsic ATPase activity that is thought to be important for ‘powering’ the membrane fusion process, but also possesses the binding site for SNAP proteins which are thought to help mediate the binding of NSF to membranes. The formation of the v/t-SNARE–SNAP–NSF complex may then trigger the hydrolysis of NSF-bound ATP which is speculated to initiate vesicular fusion [12,13].

The basic concepts of the SNARE hypothesis have been the focus of considerable recent interest with regard to the trafficking of GLUT4 in both skeletal muscle and fat given that two v-SNAREs, cellubrevin and vesicle-associated membrane protein-2 (VAMP-2), have been shown to be expressed in these tissues

Abbreviations used: VAMP, vesicle-associated membrane protein; Te-Tx, tetanus toxin; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF-attachment protein.

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[14–16]. Moreover, the suggestion that SNAREs may be involved in the trafficking of GLUT4 has been strengthened by the observation that VAMP is colocalized with GLUT4 vesicles in rat adipocytes [14] and that both cellubrevin and VAMP-2 are components of intracellular GLUT4 vesicles in murine 3T3-L1 adipocytes [17]. However, although the presence of these SNARE proteins on GLUT4 vesicles may indicate their involvement in the fusion of such vesicles with the plasma membrane, it alone is not proof. This note of caution stems from the observations made by Link et al. [18], who reported that the proteolytic cleavage of cellubrevin, a protein that is expressed in the endocytic pathway of nearly all tissues, is not required for the NSF-dependent fusion of early endosomes *in vitro*. Therefore, in an attempt to assess whether VAMP and cellubrevin participate in the acute hormonal stimulation of glucose transport and GLUT4 translocation, we have taken advantage of the selective proteolytic activity that certain clostridial toxins show towards cellubrevin and VAMP [19,20]. We present novel evidence showing that the introduction of tetanus toxin (Te-Tx) into isolated rat adipocytes by electroporation results in the near-total loss of cellubrevin and VAMP expression and that under such circumstances the acute activation of glucose transport and GLUT4 translocation by insulin are not impaired.

MATERIALS AND METHODS

Isolation of rat adipocytes

Male Sprague–Dawley rats (180–200 g, Bantin & Kingman, Hull, U.K.) were used throughout. Animals were killed by cervical dislocation and epididymal fat-pads removed. Adipocytes were isolated by digestion in Krebs–Ringer–Bicarbonate (KRB) buffer pH 7.4, supplemented with 2 % BSA (fraction V; Sigma, Poole, Dorset, U.K.) containing 0.1 % collagenase (Sigma) by the method of Rodbell [21]. Collagenase digestion was performed at 37 °C for 45 min, after which period digested tissue was filtered through a nylon mesh (180 µm pore size). The filtrate containing isolated adipocytes was washed three times in KRB buffer before finally being resuspended in an appropriate volume of this buffer.

Adipocyte fractionation

Plasma membranes and low-density microsomes from adipocytes, which had been preincubated with either human insulin (500 nM, Sigma) or vehicle (0.9 % NaCl) alone, were prepared as described previously [22]. Briefly, isolated adipocytes were homogenized in ice-cold TES buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM PMSF) using ten strokes of a Dounce type-A glass homogenizer. The resulting homogenate was centrifuged at 16000 g for 15 min. The supernatant (S1) was retained on ice and the pellet from this spin resuspended in TES buffer and spun again at 16000 g for a further 15 min. The resulting pellet was gently homogenized and layered on to a 1.2 M sucrose cushion and centrifuged at 100000 g for 70 min. Plasma membranes from this spin were recovered from the top of the sucrose cushion and diluted 10-fold in TES buffer and re-pelleted by centrifugation at 45000 g for 45 min. Plasma membranes were resuspended in 0.1 ml of TES buffer. The supernatant S1 was centrifuged at 48000 g to pellet high-density microsomes and the resulting supernatant from this spin isolated and centrifuged for a further 70 min at 200000 g to pellet low-density microsomes. The total protein content of each subcellular membrane fraction was determined using the Bradford method [23].

Immunoisolation of GLUT4 vesicles

Low-density microsomal membrane protein (200 µg) was incubated with 5 µl of non-relevant rabbit serum or 5 µl of anti-GLUT4 antibody (East Acres Biologicals, Southbridge, MA, U.S.A.) for 2 h at 4 °C. GLUT4-containing vesicles were complexed with Protein A–Sepharose beads (100 µl of a 50 % packed suspension) by end-over-end mixing at 4 °C for 2 h. The immune complexes were isolated by centrifugation at 12000 g for 30 s at room temperature. The resulting immune pellet was washed twice in PBS before a wash with 1 M NaCl/1 mM EDTA/50 mM Hepes, pH 7.4, followed by two final washes in PBS before resuspension in Laemmli buffer. The immune supernatant was centrifuged at 190000 g for 30 min to pellet unprecipitated membranes, which were subsequently resuspended in Laemmli buffer.

Te-Tx activation and adipocyte electroporation

Before its introduction into rat adipocytes whole Te-Tx (obtained from Dr. Ulrich Weller, University of Mainz, Mainz, Germany) was first reduced using 10 mM Hepes, pH 7.2, containing 10 mM dithiothreitol and 50 mM NaCl at 37 °C for 30 min and then activated by addition of 250 µM ZnCl₂ [24]. Rat adipocytes were electroporated using a Bio-Rad gene pulser based on a procedure previously described [25]. Briefly, 0.2 ml of adipocyte suspension in KRB buffer was placed in 0.4 cm gap electroporation cuvettes (Bio-Rad) and mixed with 10 µl of activated Te-Tx (final concentration in cuvette 300 or 600 nM) or 10 µl of activation buffer (for control cells). Permeabilization was achieved by using a capacitance of 960 µF and an electric field of 200 V/cm (one shock). Electroporated adipocytes were transferred to Eppendorf tubes and incubated for 1 h at 37 °C before cell lysis or for use in uptake studies.

2-Deoxyglucose transport in isolated adipocytes

2-Deoxyglucose transport was measured in isolated adipocytes by a previously reported procedure [26]. Briefly, 400 µl of adipocyte cell suspension was preincubated with or without insulin (500 nM) for 30 min at 37 °C. Incubations were started by addition of 50 µM 2-deoxy-D-[³H]glucose (Dupont; specific radioactivity 26.2 Ci/mmol) for 1 min for control cells and 30 s for cells that had been pre-exposed to insulin. Uptake assays were terminated by the rapid centrifugation of 300 µl of the uptake suspension through a 100 µl dinonyl phthalate oil cushion (Fluka) for 20 s. Non-specific cell-associated radioactivity was corrected for by the inclusion in the uptake medium of [¹⁴C]mannitol (Dupont; specific radioactivity 54.5 mCi/mmol).

Western-blot analysis

Adipocytes were homogenized in ice-cold lysis buffer containing 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 % Triton X-100, 10 mM 2-mercapthoethanol and 1 mM PMSF. Homogenates were centrifuged at low speed to remove cell debris. Whole cell lysates or subcellular membranes prepared by adipocyte fractionation were subjected to SDS/PAGE on 10 % resolving gels as described by Laemmli [27] for GLUT4 or on 10 % polyacrylamide Schagger gels [28] for cellubrevin. Proteins were transferred on to nitrocellulose membranes (Amersham), blocked with BSA and probed overnight at 4 °C with polyclonal antibodies against GLUT4 (1:500 dilution), cellubrevin (1:500

dilution of either MC16, kindly provided by Dr. P. De Camilli, Yale University, New Haven, CT, U.S.A. or D204, a gift from Dr. H. McMahon, MRC Laboratory of Molecular Biology, Cambridge, U.K.) and a monoclonal antibody against VAMP (1:200 dilution; Chemicon Ltd). Primary antibody detection was carried out using a horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham). Autoradiographs were quantified using a Bio-Rad 670 Imaging Densitometer.

Statistical analyses

Where appropriate, statistical analysis was carried out using a two-tailed Student's *t* test, and data were considered significant at *P* values < 0.05.

RESULTS AND DISCUSSION

Cellubrevin is associated with GLUT4 vesicles in rat adipocytes

One central postulate of the SNARE hypothesis is that the regulated fusion of two different membranes requires the pairwise matching of a v-SNARE on the donor membrane and an appropriate t-SNARE on the acceptor membrane [29]. The finding that cellubrevin and VAMP-2, two v-SNAREs, are associated with GLUT4 vesicles in 3T3-L1 adipocytes implies that some of the fundamental concepts of the SNARE hypothesis may be applicable to the insulin-regulated fusion of GLUT4 vesicles. Although the association of VAMP with GLUT4 vesicles in rat adipocytes has been previously reported, it remains unknown whether cellubrevin is also associated with GLUT4 in rat adipocytes. We therefore immunoprecipitated GLUT4 vesicles from a light microsomal fraction of rat adipocytes and screened the isolated immune pellet using anti-cellubrevin antibodies. Western-blot analysis of the GLUT4 vesicles pellet revealed that cellubrevin was co-precipitated (Figure 1). Under the experimental conditions employed the efficiency with which GLUT4 vesicles were precipitated from the low-density microsomal

Figure 1 Immunoprecipitation of GLUT4-containing vesicles from adipocyte low-density microsomes

Vesicles were isolated as described in the Materials and methods section using an anti-GLUT4 antibody. The immune pellet (IP) and immune supernatant (IS) were subjected to SDS/PAGE and immunoblotted with GLUT4 and cellubrevin antibodies. Bars represent quantitative analysis of GLUT4 (■) and cellubrevin (▨) abundance in the IP and IS (values are means $\pm$ S.E.M. from three separate experiments). A representative immunoblot of the precipitation is shown above the histogram. Cellubrevin is only co-precipitated with GLUT4 when a GLUT4 antibody (G4) is used and not when control (C) non-immune rabbit serum is used.

Figure 2 Effects of activated Te-Tx on the abundance of GLUT4, VAMP and cellubrevin in adipocyte lysates

(A) Isolated rat adipocytes were electroporated as indicated in the Materials and methods section in the presence of 300 or 600 nM activated Te-Tx and incubated at 37 °C for 1 h before cell lysis. Representative Western blots showing the effect of Te-Tx on the abundance of GLUT4, VAMP and cellubrevin in rat adipocyte lysates are shown. (B) Western blot showing cellubrevin abundance in lysates from: fresh non-electroporated adipocytes (lane 1); adipocytes electroporated in the presence of a recombinant C-fragment (residues 865–1315) of the Te-Tx heavy chain (lane 2); non-electroporated adipocytes incubated in the presence of activated Te-Tx (lane 3); adipocytes electroporated in the absence of Te-Tx (lane 4); adipocytes electroporated in the presence of 300 nM Te-Tx (lane 5).

fraction was typically in excess of 70%. In contrast non-immune rabbit serum did not precipitate GLUT4 (Figure 1). However, despite the relatively high degree of GLUT4 vesicle precipitation, the extent to which cellubrevin was co-precipitated was observed to be low ($21 \pm 5\%$; mean $\pm$ S.D. from three separate experiments). This finding indicates that, although cellubrevin is associated with GLUT4 (Figure 1), a substantially larger pool of cellubrevin may be resident in other undefined intracellular membrane compartments.

Proteolytic cleavage of cellubrevin and VAMP using Te-Tx

In an attempt to assess whether cellubrevin and VAMP play some crucial role in the trafficking of GLUT4 vesicles we developed a method to ablate the expression of these two v-SNAREs in the intact adipocyte. Both cellubrevin and VAMP have been shown to be good target substrates for neurotoxins derived from *Clostridium tetani* [19,20]. Te-Tx consists of two polypeptide chains (a heavy and a light chain) interconnected by a disulphide bridge which, when reduced, in the presence of zinc, activates the proteolytic endopeptidase activity of the light chain. Non-neuronal cells, such as adipocytes, do not possess binding sites for clostridial neurotoxins and therefore they are unable to penetrate or traverse the plasma membrane. However, electroporation of adipocytes transiently creates pores in the cell membrane, thereby allowing the toxin to enter the intracellular compartment. Western blotting of cell lysates, which had been prepared 1 h after electroporation of adipocytes in the presence of either 300 or 600 nM active Te-Tx, revealed that, at the lower toxin concentration, there was complete proteolysis of cellubrevin and more than 95% loss of VAMP immunoreactivity. At higher toxin concentrations (600 nM) VAMP expression was completely ablated indicating that the toxin had efficiently cleaved both v-SNAREs in the adipocyte within 1 h (Figure 2A). The VAMP antibody used in the present study does not discriminate between VAMP-1 and VAMP-2, but, given that only VAMP-2 has been

reported to be expressed in 3T3-L1 adipocytes [17], it is our view that the observed immunoreactivity in our adipocyte lysates is likely to be attributable to VAMP-2. It is also interesting to note that, when we prepared cell lysates 6 h after electroporation with 600 nM Te-Tx, we were still unable to observe any immunoreactivity against cellubrevin and VAMP (results not shown). These findings signify that the effects of the active toxin persist long after its introduction into adipocytes and are consistent with previous reports showing that the inhibitory effects of the toxin on exocytosis in cultured chromaffin cells can be sustained for many days after its introduction into the cell [30].

In order to ensure that the loss of cellubrevin and VAMP was solely attributable to the effects of the toxin we (i) electroporated adipocytes in the presence of an inactive recombinant C-fragment (residues 865–1315) of the toxin heavy chain and (ii) incubated freshly isolated rat adipocytes that had not been subjected to electroporation with activated Te-Tx for 1 h. Under both circumstances the integrity of the immunoreactive cellubrevin band in adipocyte lysates was strongly preserved, suggesting that the proteolytic susceptibility of cellubrevin (and presumably that of VAMP as well) was dependent on the active toxin light chain gaining full access to the intracellular compartment (Figure 2B). The observation that activated Te-Tx when incubated with non-electroporated adipocytes did not proteolytically cleave cellubrevin is consistent with the view that non-neuronal cells do not possess cell surface binding sites that would allow the effects of the toxin to be mediated [31]. The observed loss of cellubrevin and VAMP in our experiments occurs over the same time frame as previously reported in semi-intact CHO cells [32] and that measured *in vitro* in rat skeletal muscle [16], cultured rat muscle cells [16] and murine 3T3-L1 adipocytes [17]. No significant changes were observed in the protein composition pattern between cells electroporated in the absence or presence of Te-Tx based on Coomassie Blue staining of SDS/polyacrylamide gels or of Ponceau-S-stained nitrocellulose membranes (results not shown). More importantly, expression of GLUT4 remained unaffected in those cells showing complete loss of cellubrevin and VAMP after treatment with 600 nM Te-Tx (Figure 2A), indicating that the effects of the toxin were selective for these v-SNAREs.

Effects of cellubrevin and VAMP cleavage on insulin-stimulated glucose transport and GLUT4 translocation

Having established a method that allowed us to selectively ablate the expression of cellubrevin and VAMP in fully intact fat-cells, we subsequently tested whether these proteins played a role in the activation of glucose transport in response to insulin. In order to ensure that adipocytes that had been electroporated with a single electric pulse had fully resealed before use in glucose-uptake studies, we assessed the ability of such cells to exclude [¹⁴C]mannitol, an extracellular reference marker of equivalent molecular size to glucose. Data from several experiments revealed that adipocytes that had been shocked either in the absence or presence of Te-Tx resealed completely and that the amount of ¹⁴C radioactivity associated with electroporated cells was not significantly different (< 5 %) from that associated with freshly isolated fat cells that had not been subjected to an electroporating shock (results not shown).

Electroporation of rat adipocytes *per se* had no significant effect on basal glucose uptake (3.6 ± 0.4 fmol/min per 100 cells, mean $\pm$ S.E.M., *n* = 7) when compared with control adipocytes not subjected to a pulse shock (2.8 ± 0.3 fmol/min per 100 cells, mean $\pm$ S.E.M., *n* = 4). Insulin induced a significant stimulation of glucose uptake in both non-shocked adipocytes (approx. 16-

Table 1 Effects of Te-Tx on insulin-stimulated glucose transport in rat adipocytes

Isolated rat adipocytes were subjected to a single electroporating pulse as described in the Materials and methods section in the absence or presence of either 300 or 600 nM activated Te-Tx. Adipocytes were then incubated at 37 °C for 1 h before exposure to 500 nM insulin or saline for a period of 30 min at 37 °C. After this time 2-deoxyglucose transport was assayed as described in the Materials and methods section. Values represent means $\pm$ S.E.M. with the number of individual experiments indicated in parentheses. * Statistically significant change compared with the respective basal value (*P* < 0.01 by Student's *t* test).

Condition	Relative 2-deoxyglucose transport
Non-electroporated adipocytes	
Basal	1.0
Insulin	$15.75 \pm 1.40^*$ (4)
Electroporated adipocytes	
Basal	1.0
Insulin	$4.63 \pm 0.26^*$ (6)
Insulin + 300 nM Te-Tx	$5.79 \pm 1.05^*$ (3)
Insulin + 600 nM Te-Tx	$4.66 \pm 0.23^*$ (4)

Figure 3 Representative Western blot showing the effects of insulin and Te-Tx on the subcellular distribution of GLUT4 in electroporated rat adipocytes

Adipocytes were electroporated in the absence or presence of 600 nM activated Te-Tx and then incubated at 37 °C for 1 h. After this period adipocytes were incubated in the absence or presence of 500 nM insulin at 37 °C for a further 30 min before cell fractionation. Then 7.5 µg of the plasma-membrane (PM) or low-density-microsomal (LDM) protein was applied to gels and analysed by SDS/PAGE and Western blotting as described in the Materials and methods section.

fold, Table 1) and adipocytes that had been subjected to a single electroporating pulse (approx. 5-fold, Table 1). The decrease in the magnitude of the stimulation appeared to be solely due to the electroporation event and is in line with previous findings, showing that this procedure lowers the overall stimulation of glucose uptake by insulin in adipocytes [33]. No significant changes in basal glucose uptake were observed in adipocytes in which cellubrevin and VAMP expression had been ablated by electroporating cells with 600 nM activated Te-Tx (basal uptake in the presence of Te-Tx was 3.1 ± 0.3 fmol/min per 100 cells; mean $\pm$ S.E.M., *n* = 4). Surprisingly, however, we also observed that the loss of cellubrevin and VAMP in Te-Tx-treated adipocytes had no impact on the acute activation of glucose uptake by insulin. The magnitude of the stimulation (approx. 5–6-fold) was comparable with that observed in fat-cells that had been electroporated in the absence of Te-Tx (Table 1). Since the stimulation of glucose uptake in response to insulin was very similar irrespective of whether adipocytes had been electroporated in the absence or presence of Te-Tx, we tested whether the translocation of GLUT4 in adipocytes remained functional

after electroporation. Figure 3 shows that the translocation of GLUT4 from low-density microsomes to the plasma membrane remains intact in adipocytes electroporated in the absence and presence of 600 nM Te-Tx and that the magnitude of the translocation is not affected by the treatment of cells with the toxin.

Recently it was shown that VAMP-2 is required for the cAMP-dependent exocytic release of amylase from the rat parotid acinar cell and that toxin-mediated proteolysis of VAMP-2 correlates in a linear manner with the inhibition of amylase release from acinar cell [34]. The fact that we observed complete loss of cellubrevin and VAMP and that this was found to have no effect on the magnitude of the hormonal stimulation of glucose transport or GLUT4 translocation suggests that these proteins are unlikely to be involved in the acute insulin-induced trafficking of GLUT4 vesicles to the plasma membrane. However, whilst this manuscript was in preparation, Tamori et al. [35] reported that cellubrevin and VAMP-2 may have a role to play in the translocation of GLUT4 in 3T3-L1 adipocytes. This proposition was based on the finding that, when botulinum neurotoxin serotype B was introduced into semi-intact 3T3-L1 cells, using streptolysin-O, both VAMP-2 and cellubrevin were degraded by the toxin. Under these circumstances the authors reported that the magnitude of the insulin stimulation of glucose transport fell from 1.8-fold (observed in cells permeabilized in the absence of the toxin) to 1.5-fold (when toxin was present) and that these changes were paralleled by a small but detectable reduction in GLUT4 translocation [35]. These findings are inconsistent with our data and may be attributable to differences in cell type. However, it is difficult to reconcile the very low stimulation of glucose uptake reported by these investigators in their permeabilized 3T3-L1 cells (which under normal circumstances usually respond to insulin with up to a 15-fold increase in glucose uptake) considering the magnitude of GLUT4 translocation observed in control and toxin-treated cells [35]. The low hormonal stimulation of glucose uptake in the study by Tamori et al. may be the result of using semi-intact cells. If this is true, the interpretation, based on the relatively small changes (< 17%) in insulin-stimulated glucose transport, that cleavage of cellubrevin and VAMP-2 results in inhibition of GLUT4 translocation needs to be further substantiated.

Since we are unable to alter either basal or insulin-stimulated glucose transport or GLUT4 translocation in rat adipocytes after proteolysis of cellubrevin and VAMP, the question remains: what is the functional role of these v-SNARE molecules which are associated with GLUT4 vesicles in adipocytes? One possibility is that cellubrevin and VAMP may be required for GLUT4 sorting between intracellular compartments rather than for the hormone-induced exocytosis of GLUT4 vesicles to the cell surface. The recent finding that 3T3-L1 adipocytes possess at least two distinct intracellular GLUT4 compartments, an endosomal recycling pool and an insulin-responsive storage organelle [36], raises the possibility that GLUT4 may be sorted and delivered from the recycling compartment into the storage organelle and that such a process may be dependent on cellubrevin and/or VAMP. Alternatively, it is plausible that cellubrevin and VAMP may participate in some cellular function unrelated to GLUT4 trafficking. Bi et al. [37] have recently shown that in sea-urchin eggs the rapid resealing of the plasma membrane in response to injury-induced damage is a synaptobrevin-dependent process, given that the resealing of the plasma membrane was significantly impaired by prior treatment of sea-urchin eggs with Te-Tx. In this respect it is noteworthy that Steinhardt et al. [38] have suggested that the membrane repair process may make use of membrane vesicles not specifically

tailored for the repair process. However, the suggestion that GLUT4 vesicles containing cellubrevin and VAMP could be utilized for membrane resealing, although attractive, remains very speculative at present.

If the fundamental concepts of the SNARE hypothesis are applicable to GLUT4 translocation, then it is conceivable that other as yet unidentified SNARE proteins may be involved in this process. The sensitivity of v-SNARE isoforms to bacterial toxins is known to be variable [39] and consequently GLUT4 vesicles may harbour SNARE proteins that are insensitive to Te-Tx but which mediate membrane-fusion events in an NSF- and α -SNAP-dependent manner. An alternative possibility, which cannot be excluded, is that fusion of GLUT4 vesicles with the plasma membrane may operate independently of NSF and α -SNAP. In MDCK cells, for example, two distinct membrane-fusion mechanisms operate within the same cell; the trafficking of vesicles from the endoplasmic reticulum to the Golgi to the basolateral membrane appears to be dependent upon NSF and α -SNAP, whereas vesicle transport to the apical membrane is independent of these factors [40]. Examples of NSF-independent mechanisms have also been documented in yeast in which the homotypic fusion of endoplasmic reticulum tubules is independent of NSF and α -SNAP [41]. Whether the proteins operating in the apical pathway of MDCK cells or in the homotypic fusion process in yeast represent a new class of fusogenic proteins, which may also operate in the trafficking of proteins such as GLUT4, remains to be investigated.

In summary, we have shown that cellular proteolysis of cellubrevin and VAMP does not impair the acute activation of glucose transport or GLUT4 translocation by insulin in isolated rat adipocytes. Precisely what function cellubrevin and VAMP perform with regard to intracellular GLUT4 trafficking remain currently unknown. Addressing this issue and the question of whether the insulin-induced targeting of GLUT4 vesicles to the cell surface relies on another class of fusogenic proteins should prove invaluable in furthering our conceptual understanding of how the transport of these vesicles is regulated during normal physiological and insulin-resistant circumstances.

We thank Dr. Isabelle Hainault (INSERM U177, Paris, France) for useful discussions, Dr. Pietro De Camilli (New Haven, CT, U.S.A.) and Dr. Harvey McMahon (MRC Laboratory of Molecular Biology, U.K.) for generously providing us with cellubrevin antisera. E.H. is supported by a postdoctoral fellowship from the British Diabetic Association. J.C.A. is an MRC postdoctoral fellow and H.S.H. is the recipient of a Wellcome Trust University Award. This work was supported by research grants from the British Diabetic Association, the Medical Research Council and The Wellcome Trust.

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