Chloroquine inhibits glucose-transporter recruitment induced by insulin in rat adipocytes independently of its action on endomembrane pH

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In adipocytes, stimulation of glucose transport by insulin is mediated largely by translocation of the GLUT4 isoform of glucose transporters from an intracellular store to the plasma membrane. Most endomembrane compartments are endowed with H⁺-pumping ATPases, and the resulting luminal acidification is thought to play a role in vesicular traffic. Chloroquine (Clq), a permeant weak base, was used to test whether endomembrane pH is an important factor in GLUT4 translocation. Under conditions chosen to optimize Clq uptake, the weak base precluded insulin-induced GLUT4 translocation and the associated stimulation of glucose transport. Clq also effectively dissipated the ΔpH of acidic endomembrane compartments, assessed fluorimetrically. To define whether the intracellular GLUT4 storage compartment is acidic, immunoadsorption and immunoblotting experiments were performed to

determine whether glucose transporters and vacuolar-type H⁺ pumps coexist in the same membranes. Unexpectedly, H⁺ pumps were not detectable in vesicles bearing GLUT4. Moreover, dissipation of endomembrane ΔpH by monensin failed to inhibit insulin-stimulated GLUT4 translocation and hexose transport. Finally, the inhibitory effect of Clq persisted in the presence of monensin. We conclude that GLUT4 resides in an intracellular compartment devoid of H⁺ pumps. The insertion of this compartment into the plasmalemma is not regulated by transmembrane pH gradients. Clq impairs the stimulation of glucose transport by blocking translocation of GLUT4 by a pH-independent mechanism. Clq may provide a useful tool to elucidate the signalling or fusion steps involved in insulin-induced GLUT4 translocation.

INTRODUCTION

Following biosynthesis, glucose transporters in fat-cells are segregated into two functionally distinct subcellular compartments. A fraction of the transporters is located at the plasma membrane, where they mediate uptake of the hexose into the cell. A second component is found in an endomembrane compartment, which is recovered with low-density microsomes after cellular homogenization and fractionation (Karnieli et al., 1981). The precise nature of the intracellular organelle(s) containing the hexose transporters remains to be defined, but appears to consist of tubulovesicular elements, endosomes and coated vesicles (Robinson et al., 1992; Slot et al., 1991).

Two predominant isoforms of glucose transporters have been detected in mammalian adipocytes. In rodents the GLUT4 isoform constitutes over 90% of the glucose transporters, and the remainder is GLUT1 (Zorzano et al., 1989). The latter is located predominantly, though not entirely, at the plasma membrane, whereas GLUT4 can be detected in both the plasmalemmal and endomembrane compartments (Hudson et al., 1992; Shibasaki et al., 1992). It is now well established that redistribution of glucose transporters between intracellular and plasma-membrane locations mediates, at least in part, the physiological regulation of the rate of glucose uptake (Simpson and Cushman, 1986). Within minutes of stimulation with insulin, a sizable fraction of the endomembrane GLUT4 transporters are recruited to the adipocyte surface membrane (James and Pilch, 1988; James et al., 1989; Birnbaum, 1989; Holman et al., 1990). Translocation of GLUT4 glucose transporters between compartments has been confirmed by a variety of approaches,

including subcellular fractionation (James and Pilch, 1988; James et al., 1989; Birnbaum, 1989), electron microscopy (Slot et al., 1991; Smith et al., 1991; Robinson et al., 1992) and photolabelling of intact cells (Holman et al., 1990; Calderhead et al., 1990; Clark et al., 1991). Such incorporation of transporters into the plasmalemma is widely believed to occur by exocytic insertion of the endomembrane vesicles (Karnieli et al., 1981; Slot et al., 1991; Smith et al., 1991; Robinson et al., 1992). To date, there is no further knowledge of the mechanism of glucose-transporter translocation, and there are no tools to inhibit this mechanism selectively.

In many cell types, vesicular components of exocytic pathways have been found to possess an acidic interior (for review, see Mellman et al., 1986). Luminal acidification of exocytic and other endomembrane compartments is generally thought to be the result of active proton pumping via vacuolar (V)-type ATPases (Forgac, 1989). The functional significance of the low internal pH of endomembranes has been extensively studied. Although the underlying mechanism is not well understood, it is apparent that disruption of organellar acidification often impairs endomembrane traffic (Mellman et al., 1986; Mollenhauer et al., 1990). Thus both exocytic and endocytic pathways are affected by treatment of cells with agents such as the alkali-cation/proton exchanger monensin (Mollenhauer et al., 1990, and references therein), or with acidotropic weak bases such as chloroquine (Clq) (Wibo and Poole, 1974).

The purpose of the present study was two-fold: (a) to define whether the intracellular compartment containing GLUT4 in adipocytes is acidified by V-type H⁺ pumps and (b) to determine

whether acidotropic agents such as Clq impair mobilization of GLUT4 by insulin.

MATERIALS AND METHODS

Materials

Collagenase and BSA used for adipocyte preparation and D-glucose uptake were purchased from Boehringer Mannheim. BSA, Clq, monensin and insulin were from Sigma. Acridine Orange was from BDH Chemicals. Protein A-Sepharose was obtained from Pharmacia. Immobilon-P was purchased from Millipore. The Enhanced Chemiluminescence (ECL) detection system, Hyperfilm, donkey anti-rabbit and sheep anti-mouse antibodies linked to horseradish peroxidase were all from Amersham. D-[¹⁴C]Glucose was purchased from New England Nuclear. All other reagents were of the highest purity available. The anti-GLUT4 antibody was prepared by immunizing rabbits with a synthetic peptide corresponding to the 12 C-terminal amino acids, conjugated to keyhole-limpet haemocyanin as described by James et al. (1989). The monoclonal 1F-8 antibody was obtained from East Acres Laboratories (Southbridge, MA, U.S.A.). The monoclonal anti-H⁺ATPase antibody (E11) was prepared as described previously (Hemken et al., 1992).

Preparation of isolated rat adipocytes

Isolation of adipocytes was performed by the method of Rodbell (1964), with the modifications of Weber et al. (1988). Epididymal fat-pads from Sprague-Dawley rats weighing 175–200 g were used. Isolated adipocytes were maintained in a Krebs-Ringer bicarbonate Hepes buffer (KRBH: 120 mM NaCl, 4 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM NaHCO₃, 30 mM Hepes, pH 7.4) containing 1% (w/v) fraction V BSA, 2.5 mM D-glucose and 200 nM adenosine.

The isolated cells were then subjected to one of the three following experimental protocols, as detailed in the Results and discussion section.

Protocol 1: cells were washed five times in KRBH containing 5% BSA and 200 nM adenosine, without glucose. The adipocytes were then incubated in the absence or presence of 100 nM insulin for 30 min or pretreated with 200 μ M Clq for 30 min before addition of 100 nM insulin and further incubation for 30 min.

Protocol 2: adipocytes were washed five times in KRBH containing 200 nM adenosine (Honnor et al., 1985) without BSA or glucose. The cells were then incubated in the absence or presence of 100 nM insulin for 30 min or pretreated with 200 μ M Clq for 30 min before addition of 100 nM insulin and further incubation for 30 min.

Protocol 3: adipocytes were washed five times in KRBH containing 200 nM adenosine, without BSA or glucose. Next, the cells were incubated in the absence or presence of 200 μ M Clq or 25 μ M monensin for 30 min, or with 25 μ M monensin for 5 min followed by addition of 200 μ M Clq and further incubation for 30 min. After the above preincubations, BSA was added to a final concentration of 5% (w/v) and the suspension allowed to incubate for 15 min before undergoing further treatment with or without 100 nM insulin for 30 min. As seen from the results, this protocol allowed for Clq action during the BSA-free incubation, and preserved its action during the BSA-containing incubation needed to allow for insulin action.

Unless otherwise specified, all incubations were at 37 °C. After treatment by one of the above protocols, the cells either were used for estimation of the rate of glucose uptake, or underwent homogenization and subcellular fractionation.

Purification of plasma membranes (PM) and low-density microsomes (LDM)

The adipocytes were washed twice in 20 mM Tris / HCl (pH 7.4) / 1 mM EDTA / 255 mM sucrose at 4 °C and homogenized in the same buffer. Subcellular fractions were prepared as described by Simpson et al. (1983), and stored at –70 °C unless used for immunoadsorption of GLUT4-containing vesicles, in which case they were used immediately after purification.

Immunoadsorption of GLUT4-containing vesicles

Immunoprecipitation of vesicles containing GLUT4 was performed with freshly prepared LDM, by using a protocol described previously by Piper et al. (1991) for 3T3-L1 adipocytes. The microsomes were resuspended in buffer containing 20 mM Hepes, 1 mM EDTA, 255 mM sucrose, 100 mM NaCl, pH 7.4 (HESN buffer) and protein concentration was determined as described by Peterson (1977). LDM samples (200 μ g of protein) were mixed with 40 μ l of either anti-GLUT4 antiserum or non-immune serum and the volume was adjusted to 1 ml. The suspension was allowed to incubate overnight at 4 °C with gentle mixing. The samples were then layered on top of a 0.4 M sucrose cushion (8 ml) that was underlaid with 1.5 M sucrose in HESN buffer (2 ml) and centrifuged at 100 000 g for 4 h. The membranes were recovered from the 0.4 M/1.5 M sucrose interface and mixed with 100 μ l of a 20% (w/v) Protein A-Sepharose suspension which had been preincubated with BSA (3 mg/ml in HESN buffer) under constant mixing for 2 h at room temperature. Beads and membranes were next coincubated overnight at 4 °C with gentle mixing. The membrane-Sepharose suspension was then centrifuged at 10 000 g for 10 s. The supernatant, containing unbound membranes, was removed and centrifuged at 210 000 g for 2 h and the resulting membrane pellet was resuspended in Laemmli sample buffer. The pellet containing the Sepharose-bound membranes was resuspended in HESN buffer and layered on a 0.7 M sucrose cushion (0.5 ml) and centrifuged at 10 000 g for 3 min. The cushion was aspirated and 30 μ l of Laemmli sample buffer added to the pellet to release the bound membranes.

SDS/PAGE and immunoblotting

The protein concentration of all fractions was determined by the method of Peterson (1977). SDS/PAGE (10% acrylamide in the separating gel) was performed as described by Laemmli (1970). Unless otherwise specified, 25 μ g of PM or LDM was loaded per lane. In immunoadsorption experiments, the entire precipitate was loaded on the gel. After electrophoresis, the samples were transferred to Immobilon-P as described by Burnette (1981). If blots were to be probed for both GLUT4 and the 31 kDa subunit of the V-type H⁺ATPase they were cut in half, horizontally, between the 32.5 kDa and 49.5 kDa molecular-mass markers. The upper half was probed for GLUT4 and the lower for V-type ATPase. GLUT4 blots were blocked overnight at 4 °C in PBS containing 1% (w/v) BSA and 0.2% (w/v) Triton X-100, pH 7.4. All subsequent incubations were in the same buffer. The blots were incubated for 60 min at room temperature with polyclonal anti-GLUT4 antiserum diluted 1/4000 in buffer, washed three times, incubated in a 1/30 000 dilution of donkey anti-rabbit antibody linked to horseradish peroxidase for 60 min at room temperature, and finally washed three times in the above buffer and once in PBS. Where indicated, monoclonal anti-GLUT4 (1F8) was used as the primary antibody (1/1000 dilution). In this case, the secondary antibody was horseradish-

peroxidase-coupled sheep anti-mouse IgG (1/10000 dilution). The blots were then transferred to ECL detection reagent for 1 min, removed, and the excess solution was shaken off. The blots were covered by plastic wrap and exposed to Hyperfilm for 30–60 s.

Blots to be probed with E11 antibody were blocked overnight at 4 °C in OTA (PBS containing 1% ovalbumin, 0.05% Tween-20 and 5 mM Na₂S₂O₃, pH 7.4). After blocking, the blots were incubated in E11 supernatant for 60 min at room temperature, washed five times with PBS containing 0.05% Tween-20, and re-blocked with OTA for 10 min at room temperature, whereupon sheep anti-mouse antibody linked to horseradish peroxidase was added to a final dilution of 1/4000 and the blots were allowed to incubate for 60 min at room temperature, then washed ten times with PBS containing 0.05% Tween-20. The blots were finally transferred to the ECL detection reagent for 1 min and the excess solution was shaken off. After wrapping in plastic, the blots were exposed to Hyperfilm for 30–60 s. Autoradiograms were quantified by laser densitometry using the PDI Quantity One scanning program.

D-Glucose uptake

Glucose uptake was assayed by the procedure of Kashiwagi et al. (1983) as modified by Marette and Bukowiecki (1989). Briefly, after being subjected to one of the protocols described above, the cells were incubated for 30 min at 37 °C at a concentration of $(4-8) \times 10^6$ cells/ml in KRBH containing 5% BSA, 200 nM adenosine and 400 nM D-[¹⁴C]glucose (57.5 nCi). At these low concentrations of glucose, transport is rate-limiting and uptake measurements reflect trans-membrane transport rather than subsequent glucose metabolism. The reaction was stopped by addition of cytochalasin B to a final concentration of 25 μ M. The adipocytes were then overlaid with 150 μ l of mineral oil, centrifuged, collected, and their radioactivity content was quantified by liquid scintillation. Non-specific uptake was measured in the continuous presence of 25 μ M cytochalasin B. Each determination was performed in quadruplicate.

Staining with Acridine Orange

Isolated adipocytes were washed five times in KRBH containing 200 nM adenosine without BSA and then incubated in the same medium at a concentration of $(0.5-1) \times 10^6$ cells/ml in the absence or presence of 200 μ M Clq (30 min) or 25 μ M monensin (5 min) at 37 °C. The cells were then stained with Acridine Orange at a final concentration of 1 μ M for 10 min at room temperature before being examined under a Nikon Diaphot inverted fluorescence microscope with a 485 nm excitation filter, 505 nm dichroic mirror and 530 nm long-pass emission filter.

RESULTS AND DISCUSSION

Table 1(a) shows results of measurements of D-glucose transport in suspended adipocytes. As expected, insulin induced a large (~ 5-fold) increase in the rate of hexose uptake when the cells were incubated in the presence of BSA. As reported by others (Simpson et al., 1983; Birnbaum, 1989; James et al., 1989), this stimulation is associated with migration of GLUT4 transporters from an endomembrane storage compartment (contained in the LDM) to the PM (see Table 1b). Such translocation is thought to account, at least partially, for the elevation of the rate of transport (Karnieli et al., 1981; Simpson and Cushman, 1986; James and Pilch, 1988; Zorzano et al., 1989; Calderhead et al., 1990). Pretreatment with Clq, an acidotropic weak base, had no effect

on the basal rate of transport (results not shown; see below), and exerted only a small, marginally significant, inhibition of the insulin-stimulated flux, consistent with earlier findings (Oka et al., 1987). A small, statistically insignificant, decrease in the translocation of GLUT4 to the PM was associated with the effects of Clq in BSA-containing solution (Table 1b).

BSA was included in the assays discussed above in order to scavenge fatty acids that are released from the preparation and which are known to exert an inhibitory effect on the stimulation of hexose uptake by insulin (Hardy et al., 1991). It was conceivable that, in addition to binding fatty acids, BSA could also be scavenging Clq from the medium, precluding its action on the cells. To avoid this possibility, experiments like those described above were repeated in the absence of BSA. The results are summarized in the right-hand column of Table 1(a). Removal of the protein had little effect on the basal rate of D-glucose uptake, but largely eliminated the stimulatory action of insulin, probably due to the presence of fatty acids. Interestingly, though smaller than observed in the presence of BSA, substantial insulin-induced translocation of GLUT4 from LDM to PM was observed even in the absence of BSA (Table 1b). Thus, under these conditions sizable translocation occurred without concomitant stimulation of transport. In medium devoid of BSA, Clq inhibited hexose transport below basal levels, and virtually eliminated the translocation of GLUT4 (Tables 1a and 1b).

Although Clq seemingly impaired the insulin-induced translocation of GLUT4 in the absence of BSA, its effects on stimulated transport could not be determined, ostensibly due to

Table 1 BSA precludes the effects of chloroquine on glucose transport and GLUT4 translocation

(a) D-Glucose uptake determinations. Adipocytes were preincubated with or without 0.2 mM Clq in the presence (+ BSA; protocol 1 in the Materials and methods section) or absence (– BSA; protocol 2 in the Materials and methods section) of 5% BSA. After 30 min, the cells were treated without (Bas) or with 100 nM insulin (Ins), (Clq→Ins) as indicated, and the uptake of radiolabelled D-glucose was measured over a 30 min period as detailed in the Materials and methods section. Data are means $\pm$ S.E.M. of 5 experiments. (b) GLUT4 distribution determinations. Cells were pretreated with or without Clq in the presence (protocol 1) or absence (protocol 2) of BSA for 30 min as described for (a). After a 30 min incubation in the absence or presence of insulin, the reaction was terminated, and the cells were homogenized and fractionated. Samples of PM (30 μ g of protein/lane) or LDM (15 μ g of protein/lane) containing identical amounts of protein were analysed by electrophoresis and immunoblotted using polyclonal anti-GLUT4 antibody. The autoradiograms were scanned and the optical density was normalized to that of untreated (Bas) samples. Data are means $\pm$ S.E.M. of 4 experiments. Clq→Ins: an arrow is used to indicate the sequence of additions in all Tables.

(a)	D-Glucose uptake (pmol/30 min per 10 ⁶ cells)	
	Conditions	<div>+ BSA</div> <div>— BSA</div>
Bas	8.53 ± 0.54	8.92 ± 0.41
Ins	41.43 ± 3.13	10.88 ± 1.52
Clq→Ins	32.78 ± 2.25	4.92 ± 0.87

(b)	Glut4 distribution (relative density)			
	<div>+ BSA</div>		<div>— BSA</div>	
Conditions	PM	LDM	PM	LDM
Bas	1.00	1.00	1.00	1.00
Ins	7.75 ± 2.17	0.27 ± 0.03	3.65 ± 0.19	0.57 ± 0.05
Clq→Ins	6.21 ± 1.41	0.13 ± 0.03	1.30 ± 0.16	1.00 ± 0.03

Table 2 Effect of chloroquine on D-glucose uptake and GLUT4 distribution following preincubation without BSA

For D-glucose uptake determinations, adipocytes were preincubated with or without 0.2 mM Clq in the absence of BSA. After 30 min, the cells were treated without (Bas) or with 100 nM insulin (Ins), (Clq→Ins) as indicated in the presence of 5% BSA (protocol 3 in the Materials and methods section) and the uptake of radiolabelled D-glucose was measured over a 30 min period as detailed in the Materials and methods section. Data are means $\pm$ S.E.M. of 3 experiments. For GLUT4-distribution determinations, cells were treated as above (protocol 3). Samples of PM (30 μ g of protein/lane) or LDM (15 μ g of protein/lane) were analysed by electrophoresis and immunoblotted using polyclonal anti-GLUT4 antibody. The autoradiograms were scanned and the optical density was normalized to that of untreated (Bas) samples. Data are means $\pm$ S.E.M. of 4 experiments.

Conditions	D-Glucose uptake (pmol/30 min per 10 ⁶ cells)	GLUT4 distribution (relative density)	
		PM	LDM
Bas	7.81 $\pm$ 0.94	1.00	1.00
Ins	44.85 $\pm$ 3.46	3.60 $\pm$ 0.19	0.55 $\pm$ 0.05
Clq→Ins	11.56 $\pm$ 0.82	1.27 $\pm$ 0.14	1.00 $\pm$ 0.03

the presence of inhibitory factors that are normally scavenged by the serum protein. To overcome this difficulty, an experimental protocol was designed whereby the cells would be initially equilibrated with or without the acidotropic agent in the absence of scavenging protein, followed by a second stage wherein the stimulatory effect of insulin on transport was assayed in the presence of BSA (protocol 3). Clq was present also during the latter stage. The results of these experiments are summarized in Table 2. Under these conditions, insulin still effectively stimulated D-glucose uptake in the absence of Clq [from 7.8 ± 0.9 to 44.8 ± 3.5 pmol/30 min per 10⁶ cells (means $\pm$ S.E.M. of 3 experiments)]. As before, such stimulation was accompanied by translocation of GLUT4 from the LDM to the PM (Table 2). The basal (unstimulated) rate of transport was unaffected by Clq (7.8 ± 0.9 versus 7.1 ± 1.2 pmol/30 min per 10⁶ cells). In contrast with the results in Table 1, however, preincubation with Clq obliterated the stimulatory effect of the hormone on hexose transport (Table 2). Such inhibition was paralleled by a marked decrease in the extent of translocation of GLUT4 to the PM (Table 2). Thus, under conditions where Clq was able to interact with the cells in the absence of scavengers, a profound inhibition of the effects of insulin on glucose-transporter mobilization and function was observed.

Acidotropic agents are thought to alter membrane traffic by disruption of pH gradients in acidic vesicular compartments (Wibo and Poole, 1974; Mollenhauer et al., 1990). In endosomes, Golgi, clathrin-coated vesicles and other endomembrane structures, V-type H⁺ pumps are responsible for the maintenance of the internal acidic milieu (Mellman et al., 1986; Forgac, 1989). The precise identity of the GLUT4-containing organelle remains to be defined, and could potentially include acidic organelles. Interference with intravesicular H⁺ buildup could conceivably account for the inhibitory effects of Clq on GLUT4 translocation. Therefore, we wished to investigate the possibility that the GLUT4-containing vesicles might be endowed with V-type H⁺ pumps. To test this possibility, the fraction of LDM containing GLUT4 was precipitated by immunoadsorption with polyclonal antibodies raised against the C-terminus of GLUT4 (see the Materials and methods section for details) and tested for the presence of V-type H⁺ pumps. The results of these experiments are shown in Figure 1. The effectiveness and specificity of the

Figure 1 Immunoadsorption of vesicles bearing GLUT4

LDM were immunoprecipitated (IMMPT) as described in the Materials and methods section using either anti-GLUT4 antibodies (GT-4) or non-immune serum (NIS). The resulting pellet (P) was washed through a sucrose cushion. The vesicles remaining in the supernatant (S) were sedimented. Both samples were then solubilized and used for electrophoresis and immunoblotting (IMMPT) using anti-GLUT4 antibodies (a) or anti-H⁺ATPase antibodies (b). The result shown is representative of three similar experiments.

immunoprecipitation protocol are documented in Figure 1(a), where pellets and supernatants of vesicular suspensions precipitated with anti-GLUT4 antibody (GT-4) or with non-immune serum (NIS) were analysed by electrophoresis and immunoblotting using anti-GLUT4 antibodies. The monoclonal anti-GLUT4 antibody IF-8 was used for blotting, to obviate interference by the heavy chain of the immunoprecipitating polyclonal antibody (~ 55 kDa). As illustrated, significant amounts of vesicles containing GLUT4 were found immunoadsorbed to the beads only when anti-GLUT4 antibodies were used, and not when non-immune serum was employed (cf. first and third lanes in Figure 1). The immunoprecipitation of GLUT4-containing vesicles was not complete, however, probably due to steric inaccessibility of the antigenic C-terminus to the antibody in a fraction of the vesicles, or of some antibody-bearing vesicles to the Protein A-treated beads. Nonetheless, more than half of the GLUT4 protein in the intracellular organelle was immunoprecipitated: in three experiments $52 \pm 8\%$ of the immunoreactive GLUT4 was recovered in the pellet. The immunoprecipitated vesicles were tested for the presence of V-type ATPases by immunoblotting using monoclonal antibody E11. The isolation and characterization of this antibody, which recognizes the 31 kDa subunit of the ATPase, has been described in detail by Hemken et al. (1992). This subunit is critical for the biochemical and functional integrity of the V-type H⁺-ATPase (Foury, 1990). As illustrated in Figure 1(b), E11 effectively recognizes two polypeptides in adipocyte LDM. The existence of heterogeneity of the ~ 31 kDa subunit of the ATPase in epithelial cells and the ability of E11 to recognize these putative isoforms was recently described (Hemken et al., 1992). The present results suggest that different isoforms also exist in adipocyte endomembranes. More importantly, reactivity with the E11 antibody was detected only in the supernatant, but not in the pellet of samples immunoadsorbed with anti-GLUT4 antibody. In three experiments, less than 2% of the E11 immunoreactive material was found in the pellet. This suggests that V-type H⁺-ATPases are not resident proteins of vesicles which are immunoprecipitable with anti-GLUT-4 antibody. As expected, similar results (not shown) were obtained when non-immune serum was used for immunoadsorption. Some polypeptides of vacuolar ATPases have been reported to dissociate from the membrane under certain incubation conditions (Moriyama and Nelson, 1989). It was therefore conceivable that such dissociation could account for our failure to detect the 31 kDa subunit in the immunoprecipitated pellet. However, the immunoreactive 31 kDa band remained attached to the LDM membrane when the supernatants of the immunoprecipitation were sedimented. Therefore, dis-

Table 3 Effect of dissipation of pH on glucose uptake

Cells were pretreated in the absence of BSA with 25 μ M monensin (Mon) and/or 0.2 mM Clq as indicated in the first column and then incubated with or without 100 nM insulin for 30 min with BSA. D-Glucose uptake was measured as described in the Materials and methods section. Data are means $\pm$ S.E.M. of 4 experiments.

Pretreatment	Insulin treatment (30 min)	D-Glucose uptake (pmol/30 min per 10 ⁶ cells)
—	—	7.80 $\pm$ 0.95
—	+	44.80 $\pm$ 3.45
30 min Clq	—	7.05 $\pm$ 1.20
30 min Mon	—	11.65 $\pm$ 4.00
30 min Clq	+	11.55 $\pm$ 0.80
5 min Mon	+	30.15 $\pm$ 2.50
5 min Mon $\rightarrow$ 30 min Clq	+	8.95 $\pm$ 1.00

sociation is unlikely to account for the absence of 31 kDa subunits in the precipitate, suggesting instead that the immunoprecipitated GLUT4-containing organelles are devoid of this subunit of the V-type H⁺ ATPases. Because this subunit is required for pump activity of the V-type H⁺ ATPase complex and is thought to anchor and hold together the subunits of the H⁺ pump (Foury, 1990; Sabolic et al., 1992; Ho et al., 1993), we surmise that the immunoprecipitated GLUT4-containing organelles are devoid of H⁺-pump activity.

Because H⁺ pumping by V-type ATPases is the sole reported mechanism of intravesicular acidification, the ostensible absence of pumps from the immunoprecipitable GLUT4 bearing organelle suggests two explanations for the effects of Clq described in Table 2. First, it is possible that a separate compartment, endowed with pumps, is essential for the translocation of GLUT4 molecules to the PM. Alternatively, the effects of Clq may be due to a mechanism other than dissipation of intravesicular acidification. To test the latter hypothesis, the effectiveness of Clq to collapse pH gradients across endomembranes was tested. Adipocytes were incubated with Acridine Orange and its accumulation in vesicular compartments was monitored by fluorescence microscopy. The existence of numerous acidic organelles was verified by the appearance of bright punctate orange fluorescence. Treatment with Clq under the conditions used for the experiments of Table 2 completely eliminated the accumulation of the fluorophore in endomembrane organelles (results not shown). Similar results were obtained with monensin, an ionophore that catalyses the exchange of H⁺ for alkali cations and is known to collapse pH gradients across endomembranes (Mollenhauer et al., 1990). Interestingly, 200 nM Clq was able to discharge the acidic gradient of Acridine Orange accumulated in intracellular acidic compartments both in the presence and in the absence of BSA. A similar observation was made in 3T3-L1 adipocytes. This suggests that the BSA inhibitory effect on Clq action was not due to insufficient partitioning of Clq into acidic organelles. It is conceivable that the concentration of Clq required for inhibition of insulin stimulation of glucose transport is higher than that needed to counteract the accumulation of Acridine Orange in acidic compartments.

Although Clq was seemingly able to dissipate the intravesicular acidification, this is not necessarily the mechanism whereby the weak base impairs stimulation of hexose uptake. The observation that both monensin and Clq effectively precluded accumulation of Acridine Orange provided a means of testing whether the

Figure 2 Effects of Clq and monensin on insulin-induced GLUT4 translocation

Where indicated, adipocytes were preincubated for 5 min with 25 μ M monensin (Mon) and then for 30 min with 0.2 mM Clq in the absence of BSA. The cells were then treated without (Bas) or with 100 nM insulin (Ins), as indicated in the presence of 5% BSA (protocol 3 in the Materials and methods section). The reaction was stopped, and the cells were homogenized and fractionated, and samples of PM and LDM were analysed by electrophoresis as described in the text. The samples were finally immunoblotted with polyclonal anti-GLUT4 antibody. A typical autoradiogram is shown in (a), and averages of densitometry scans, normalized to the untreated control (Bas), are presented in (b). Data in (b) are means $\pm$ S.E.M. of 4 experiments.

acidotropic properties of Clq mediate its inhibitory action on D-glucose uptake. Experiments testing the effects of monensin on hexose uptake are presented in Table 3. The ionophore was added at a final concentration of 25 μ M, which is comparable with or greater than that used by others to dissipate pH gradients across endomembranes (for review, see Mollenhauer et al., 1990) and suffices to prevent Acridine Orange accumulation in our experiments (see above). Under these conditions, monensin had no significant effect on the basal rate of sugar uptake, and only partly decreased the insulin-induced stimulation. Under comparable conditions, Clq essentially eliminated the stimulation (Table 3). Similar results were observed with 3T3-L1 adipocytes, where 50 μ M monensin did not prevent stimulation of glucose transport by insulin, whereas chloroquine inhibited this hormonal response in a dose-dependent manner (results not shown). The effect of Clq on glucose transport was closely mirrored by the mobilization of GLUT4 from the LDM to the PM (Figure 2). Whereas Clq abolished the migration of glucose transporters, monensin had little effect. This was apparent from the appearance of GLUT4 molecules in the PM, as well as from their disappearance from the LDM. Neither Clq nor monensin affected the distribution of GLUT4 in cells not treated with insulin (results not shown).

The experiments in Table 3 and Figure 2 suggest that Clq inhibits insulin-stimulation of glucose transport by a mechanism other than dissipation of endomembrane acidification. Additional experiments were performed to test whether accumulation of the drug in acidic compartments was essential for its action. Cells were first preincubated with monensin to alkalize the endomembrane compartments, and then Clq was added. The inhibitory effects of the weak base on sugar transport (Table 3) and GLUT4 translocation (Figure 2) were unaffected by monensin. Thus the ability of Clq to collapse endomembrane pH gradients is not sufficient to inhibit glucose-transport stimulation by insulin, and moreover, Clq does not seem to require an intact pH gradient across intracellular organelles to exert its inhibitory effect on glucose-transporter translocation. These conclusions are consistent with the absence of V-type H⁺ pumps in the GLUT4-bearing vesicles.

In summary, two findings emerged from the present studies. First, the immunoprecipitated endomembrane vesicular compartment containing GLUT4 is devoid of V-type H⁺ATPases. It is conceivable that H⁺ATPases not containing the 31 kDa polypeptide recognized by antibody E11 are present in this compartment. However, the 31 kDa subunit appears to be a ubiquitous constituent of V-type H⁺ATPases (Hemken et al., 1992), and the E11 antibody was selected because of its comparatively wide specificity. Whereas other monoclonal antibodies recognize only some of the isoforms of this subunit, antibody E11 reacts with the 31 kDa forms as well as with those of slightly larger molecular mass (Hemken et al., 1992; and Figure 1). It is also possible that, because the immunoadsorption protocol is not quantitative, a small subpopulation of GLUT4 vesicles without pumps was selected, whereas others with pumps were not precipitated. We regard this possibility as unlikely, since over 50% of the GLUT4 vesicles were immunoprecipitated; given that ~50% of the GLUT4 in the LDM is translocated upon stimulation with insulin, this fraction must include at least some of the vesicles devoid of H⁺ATPases.

The second finding is that Clq blocked the insulin-dependent translocation of GLUT4 in insulin-stimulated adipocytes, apparently through an effect independent of its protonophoric action. The action of this drug was noted only in the absence of BSA, which when present in a molar excess over Clq (0.74 mM versus 0.2 mM) may bind and prevent the weak base from altering glucose transport. The prevailing free concentration of Clq may suffice to collapse endomembrane pH gradients in vesicles endowed with H⁺ pumps, but may be too low to affect migration of the insulin-responsive vesicles containing glucose transporters. This would be in line with the results presented in this study and with those of Oka et al. (1987), who observed lack of inhibition of insulin-stimulation of glucose transport or transporter recruitment in Clq-treated adipocytes in the presence of BSA, although recruitment of vesicles containing IGF-II (insulin-like growth factor-II) receptors was prevented. We surmise that IGF-II-receptor-containing vesicles do have H⁺ pumps, and this ATPase activity is important for their intracellular migration. This would be in agreement with reports that GLUT4 glucose transporters and IGF-II receptors reside in different intracellular vesicles in rat adipocytes, although both populations respond to insulin (Zorzano et al., 1989), albeit to different quantitative extents (Jhun et al., 1992). The present work substantiates the view that dissipation of intravesicular acidity by Clq does not appear to be responsible for its inhibitory effect on GLUT4 translocation and function. This conclusion was reached on the basis of the observation that monensin, at concentrations that dissipate endomembrane acidification (as estimated by Acridine Orange accumulation), did not have a

comparable inhibitory effect. Consistent with this view, treatment of adipocytes with 30 mM NH₄Cl also failed to prevent the stimulation of hexose uptake by insulin (Oka et al., 1987). We also found that pretreatment with monensin did not preclude the effect of Clq, suggesting that the existence of acidic compartments is not a prerequisite for the inhibitory action of the weak base. Consistent with these observations, it has been reported that endosomal fusion *in vitro* is not altered by disruption of the vesicular pH gradient by H⁺ ionophores (Braell, 1987; Diaz et al., 1988), supporting the notion that membrane fusion in intact cells may not depend on the intravesicular acidity of endosome-like organelles.

Together, these findings suggest a novel mechanism of inhibition by Clq. Fractionation and immunoblotting experiments indicate that the primary site of action is the event(s) responsible for translocation of GLUT4 to the PM. Because little is known about the translocation mechanism, Clq may provide a useful tool to unravel the signalling or fusion steps involved.

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