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Involvement of Vesicular H^+-ATPase in Insulin-Stimulated Glucose Transport in 3T3-F442A Adipocytes

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Abstract. In secretory cells, osmotic swelling of secretory granules is proposed to be an intermediate step in exocytic fusion of the granules with the plasma membrane. For osmotic swelling of the granule, a H^+ gradient generated by vacuolar-type H^+-ATPase (V-ATPase) may be a driving force for accumulation of K^+ via its exchange with H^+, concurrent with accumulation of Cl^- and H_2O. Here, we investigated whether a similar chemiosmotic mechanism is involved in the insulin-stimulated recruitment of GLUT4 to the plasma membrane in 3T3-F442A adipocytes. Incubating cells in a hypo-osmotic medium significantly increased 2-deoxy glucose (2-DG) uptake and the plasma membrane GLUT4 content (possibly via induction of osmotic swelling of GLUT4-containing vesicles (G4V)) and also potentiated the insulin-stimulated 2-DG uptake. Promotion of the G4V membrane ionic permeability using nigericin, an electroneutral K^+/H^+ exchange ionophore, increased 2-DG uptake and the plasma membrane GLUT4 content. However, co-treatment with nigericin and insulin did not show an additive effect. Bafilomycin A_1, a diagnostically specific inhibitor of V-ATPase, inhibited insulin- and nigericin-stimulated 2-DG uptake. Immunoadsorption plus immunoblotting demonstrated that GLUT4 and V-ATPase co-localize in the same intracellular membranes. Together, these results indicate that V-ATPases in the G4V membrane may play an important role in the insulin-stimulated exocytic fusion of G4V with the plasma membrane via its participation in osmotic swelling of the vesicle.

Key words: Vacuolar-type H^+-ATPase, Chemiosmotic mechanism, Exocytosis, GLUT4, Insulin
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INSULIN stimulates glucose transport in muscle and adipose cells, primarily by promoting translocation of the insulin-sensitive glucose transporter GLUT4 from an intracellular vesicular pool to the cell surface. In the absence of insulin, GLUT4 is rapidly eliminated from the plasma membrane to which it recycles only slowly, sequestering greater than 90% of GLUT4 in the intracellular vesicle membrane. Insulin causes a large increase in the rate of exocytic insertion of GLUT4 into the plasma membrane and a smaller decrease in the rate of its retrieval from the plasma membrane; the former action accounts for a large proportion of the insulin-induced increase in the amount of GLUT4 in the plasma membrane (reviewed in [1–3]). However, the mechanism of insulin action in the exocytic pathway is not yet clear.

GLUT4 is packaged into the G4V and stored in the cytoplasm until exocytic insertion to the plasma membrane is triggered by insulin [4]. Immunoelectron microscopic studies have shown that in unstimulated cell, GLUT4 is predominantly localized in tubulovesicular elements and small vesicles, often clustered immediately beneath the plasma membrane [5, 6]. Insulin is then proposed to recruit GLUT4 to the plasma membrane perhaps via inducing fusion of these stored G4V with the plasma membrane [7]. This mechanism may be analogous to regulated exocytic fusion of secretory granules and small synaptic vesicles.

The mechanism of exocytic fusion of secretory gran-

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ules with the plasma membrane has been extensively studied in secretory cells. It has been hypothesized that osmotic swelling of secretory granules may be an intermediate step in exocytosis [8–11]. It is thought that increased ionic permeability of secretory granule membranes in response to secretagogues may cause osmotic swelling of the secretory granules, and this swelling may promote fusion of the swollen granule membrane with the plasma membrane leading to exocytosis. According to this hypothesis termed the "chemiosmotic mechanism of exocytosis [12]," the interior of the secretory granule is acidic and electrically positive with respect to the cytoplasm by an electrogenic V-ATPase. The resultant H^+ gradient might then be coupled to osmotic swelling of the secretory granule by accumulation of monovalent cations (*e.g.*, K^+) via exchange of H^+ for cytoplasmic K^+ and/or Cl^- accumulation driven by the positive electrical potential [13]. Consistent with this model is the recent observations in yeast that the homotypic vacuole fusion is dependent upon the H^+-gradients generated by a V-ATPase [14], and also on the trans-complex formation of juxtaposed its proteolipid sectors (Vo subunit) [15].

As mentioned above, there is a striking similarity between the insulin-stimulated exocytic insertion of G4V and the regulated exocytic fusion of secretory granules with the plasma membrane. In addition, it has been found that the interior of vesicular compartments maintains acidic by V-ATPases in many cell types, including non-secretory cells [13, 16, 17]. Thus, it is conceivable that the chemiosmotic mechanism may also be involved in the insulin-stimulated exocytosis of G4V in adipocytes. In this scheme, an insulin-induced increase in the G4V membrane ionic permeability would result in an influx of K^+ along with Cl^- with osmotically obliged H_2O influx leading to osmotic swelling of the vesicle. For those vesicles situated very close to the cell plasma membrane, this would lead to fusion with the plasma membrane.

In the present study, we have tested the possibility that the chemiosmotic mechanism may be involved in the insulin-stimulated exocytic fusion of G4V in 3T3-F442A adipocytes by exploring the following questions: 1) whether a hypo-osmotic medium, which is expected to induce osmotic swelling of the G4V, can stimulate glucose transport; 2) whether the promotion of an exchange of H^+ for K^+ across the vesicular membrane using nigericin [18] can stimulate glucose transport; 3) whether the V-ATPases are present in the G4V membrane; and 4) if so, whether inhibiting the V-ATPase by a specific inhibitor bafilomycin A_1 [19] exerts any effects on the insulin-stimulated glucose transport.

Materials and Methods

Materials

Bovine calf serum (BCS), fetal calf serum (FCS) and DMEM were purchased from Hyclone (Logan, UT), antibiotics were from GIBCO (Grand Island, NY), and 2-deoxy-D-[1-^{3}H]glucose was from Amersham Corp. (Arlington Heights, IL). Bafilomycin A_1 was from Wako (Japan) and protein G-sepharose was from Amersham Biosciences (Uppsala, Sweden). Nigericin, insulin, phloretin, cytochalasin B and other standard chemicals were from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-GLUT4 antibody 1F-8 was from Biogenesis Ltd. (UK) and polyclonal anti-GLUT1 antibody H-43 was from Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Monoclonal antibodies against integrin β_1 and Hsp 72/73 were from BD Transduction Laboratories (Lexington, KY) and Oncogene Research Products (Cambridge, MA), respectively. The polyclonal anti-GLUT4 antiserum was prepared by immunizing rats with a synthetic peptide corresponding to the 12 C-terminal-most amino acids (courtesy of Dr. B. H. Jhun at Pusan Univ., Korea). The polyclonal affinity-purified rabbit antibodies against the C-terminus of GLUT4 [20] and the polyclonal antisera against the 57 and 72 kDa subunit of V-ATPase [21] were prepared as previously described.

Cell Culture

3T3-F442A murine fibroblasts (provided by Dr. H. Green at Harvard Medical School, Boston, MA) were maintained in DMEM supplemented with 10% BCS. This cell line is known to differentiate spontaneously (without supplementation of insulin and dexamethasone) into adipocytes [22–24]. Thus, differentiation was induced 24–48 h after confluence by altering the medium to DMEM supplemented with 10% FCS. The cells were used for experiments 10–12 days after induction of differentiation, when 90–95% of the cells had differentiated into adipocytes.

Cell Treatment and Measurement of 2-DG Uptake

After overnight serum-deprivation, 3T3-F442A adipocytes grown in 24-well plates were incubated in the standard Krebs-Ringer bicarbonate buffer (KRB: 118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4 and 25 mM $NaHCO_3$, pH 7.4) containing 0.5 mM glucose and 2% BSA at 37°C in humidified air containing 5% CO_2. Insulin (100 nM) was added 30 min prior to 2-DG uptake assay. Measurement of 2-DG uptake was performed as previously described [25, 26].

To investigate osmotic effects on basal and insulin-stimulated 2-DG uptake, hypo-, iso- and hyper-osmotic modified media were prepared by decreasing the concentration of NaCl in the standard KRB to 59 mM, then supplementing the media with no sucrose (hypo-osmotic), 118 mM sucrose (iso-osmotic) and 384 mM sucrose (hyper-osmotic). The osmolality of the hypo-, iso- and hyper-osmotic media, which was determined by freezing point depression with an Osmette A automatic osmometer (Precision Systems Inc., Natick, MA), was 174 ± 6 mOsm/kg H_2O, 304 ± 5 mOsm/kg H_2O and 578 ± 29 mOsm/kg H_2O, respectively (n = 7 for each).

The effect of nigericin (1–100 μM), an electroneutral K^+/H^+ exchange ionophore [18], on 2-DG uptake was examined in the standard KRB and the intracellular fluid-like K^+-rich KRB, which was identical to the standard KRB except that the concentration of Na^+ was lowered to 10 mM, while that of K^+ was raised to 135 mM (pH 7.0), for reduction of the K^+ and H^+ gradient across the plasma membrane. Nigericin, alone or with insulin, was added to the media 30 min prior to the measurement of 2-DG uptake. In some experiments, cells were pretreated with bafilomycin A_1 (10 or 50 μM), a specific inhibitor of V-ATPase [19], for 30 min before the addition of insulin or nigericin.

Subcellular Membrane Fractionation

Cells grown in 15-cm dishes were homogenized and subjected to subcellular membrane fractionation as described previously [25], yielding fractions of plasma membrane (PM), high-speed pellet (HSP; also referred to as the low density microsomal fraction) and high density microsome (HDM). Quality of these membrane fractions was evaluated by the presence of marker proteins for each membrane fraction. Western blots of the PM showed a high content of β_1-integrin, a plasma membrane resident protein. In the HSP, β_1-integrin was not detectable, indicating that this fraction was relatively devoid of plasma membrane. Conversely, the intracellular membrane marker HSP 72/73 was enriched in the HSP, but almost undetectable in the PM (data not shown).

Immuno-isolation of GLUT4 vesicles

Freshly prepared HSP (200 μg of protein) was precleared by incubation with 2 μg of normal rat IgG and 100 μl of protein G-sepharose beads for 1 h at 4°C. The sample was centrifuged at 1,500 rpm for 30 sec. The supernatant was mixed with 40 μl of rat polyclonal GLUT4 antiserum and the mixture (1 ml of final volume) was incubated overnight at 4°C with gentle mixing. The sample was then layered onto a 0.4 M sucrose cushion underlain with 1.5 M sucrose in HESN buffer (255 mM sucrose, 100 mM NaCl, 1 mM EDTA, 20 mM HEPES; pH 7.4) and centrifuged at 100,000×g for 4 h. The membranes (100 μl) were collected from the interface, and mixed with 100 μl of a 20% protein G-sepharose suspension which had been preincubated with BSA. The membranes and beads were incubated overnight at 4°C with gentle mixing and centrifuged at 10,000×g for 10 sec. The supernatant containing unbound membranes was removed, centrifuged at 200,000 × g for 2 h, and the resulting pellet was solubilized in Laemmli sample buffer. The pellet containing the membrane-bead complexes was resuspended in HESN buffer, layered on a 0.7 M sucrose cushion and centrifuged at 10,000×g for 3 min. The cushion was aspirated and sample buffer was added to the pellet to solubilize the bound membranes.

SDS-PAGE and Immunoblotting

Aliquots of proteins from the subcellular membrane fractions were solubilized in Laemmli sample buffer. Separation of proteins by SDS-PAGE and subsequent immunoblotting were performed as described previously [25]. The resulting signals from the Western blot were quantified using a video densitometer (Model 620, Bio-Rad) in the reflective mode.

Statistical Analysis

The observed values are presented as mean ± S.E.

Student's paired or unpaired *t*-tests were performed as indicated for pair wise group comparisons, and ANOVA followed by the Bonferroni test was used when more than two groups were compared. Differences were considered significant at $p<0.05$.

Results

Effects of Incubation Media Osmolality on Basal and Insulin-Stimulated 2-DG Uptake

To test whether the osmotic swelling of G4V could cause fusion of G4V with the plasma membrane and thereby increase glucose transport, we incubated cells in iso-, hypo- or hyper-osmotic media. As shown in Fig. 1A, reduction of media osmolality from 300 to 170 mOsm/kg H_2O significantly increased 2-DG uptake (by 110%), from 0.16 ± 0.02 to 0.33 ± 0.03 nmol/3 min/well (n = 7; $p<0.01$); increasing osmolality to 580 mOsm/kg H_2O also significantly raised 2-DG uptake (by 40%), to 0.23 ± 0.03 nmol/3 min/well (n = 7; $p<0.05$). Addition of insulin increased 2-DG uptake to 2.7 ± 0.1 times the basal level in the iso-osmotic medium (n = 7; $p<0.01$). The stimulatory effect of insulin on 2-DG uptake was greatly enhanced in the hypo-osmotic medium: insulin increased 2-DG uptake to 3.7 ± 0.3 times the basal level (n = 7; $p<0.01$). Thus, the absolute level of insulin-stimulated 2-DG uptake was noticeably greater in the hypo-osmotic medium than in the iso-osmotic medium (1.22 ± 0.09 *vs*. 0.42 ± 0.02 nmol/3 min/well; n = 7; $p<0.01$). In contrast, addition of insulin stimulated 2-DG uptake to only 1.2 ± 0.1 times the basal level in the hyper-osmotic medium (n = 7; $p>0.05$), indicating that the effect of insulin is significantly suppressed in the hyper-osmotic medium as compared to that in the iso-osmotic medium (2.7 ± 0.1 times; $p<0.01$).

At least two distinct glucose transporters, GLUT4 and GLUT1, were present in these spontaneously (without insulin and dexamethasone) differentiated 3T3-F442A adipocytes (Fig. 1B & C). Immunoblot-

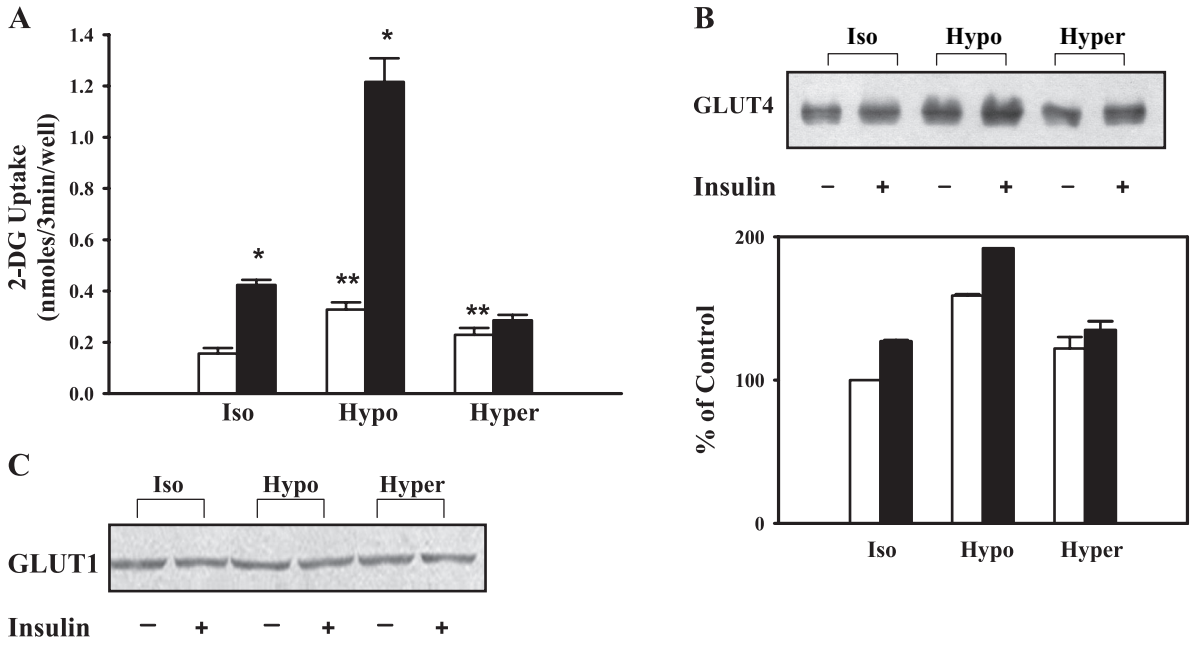

Fig. 1. Effects of the incubation media osmolality on 2-DG uptake (A) and the plasma membrane contents of GLUT4 (B) and GLUT1 (C) in 3T3-F442A adipocytes. A, Cells grown on 24 well plates were incubated in iso-, hypo-, or hyper-osmotic media with or without (basal) 100 nM insulin for 30 min before the determination of 2-DG uptake. The results represent the mean ± SE of 7 experiments, each performed in triplicate. The open and closed bars represent basal and insulin-stimulated 2-DG uptake, respectively. *$p<0.05$ *vs*. 2-DG uptake in the absence of insulin in the same medium. **$p<0.05$ *vs*. the basal 2-DG uptake in the iso-osmotic medium. B, Cells treated as indicated were fractionated into plasma membrane (PM) and 2 other intracellular membrane fractions as described under Materials and Methods. Proteins (30 μg) were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with either polyclonal anti-GLUT4 (B) or anti-GLUT1 (C) antibody. The blots are representatives of 3 independent experiments and the bar graph represents their densitometric analysis.

ting of the subcellular membrane fractionations showed that GLUT4 content in the PM is significantly increased by incubation of the cells in the hypo-osmotic medium (lane 3 *vs.* lane 1 in Fig. 1B). GLUT4 content in the HSP was correspondingly decreased by the hypo-osmotic medium (data not shown), indicating that this incubation condition induces redistribution of GLUT4 from inside of the cell to the cell surface. In addition, insulin-induced increases in the PM GLUT4 content were markedly enhanced in the hypo-osmotic medium in comparison with that observed in the iso-osmotic medium (lane 4 *vs.* lane 2 in Fig. 1B). The hyper-osmotic medium also increased GLUT4 content in the PM (lane 5 *vs.* lane 1 in Fig. 1B), but insulin did not significantly alter these levels in the hyper-osmotic medium (lane 6 *vs.* lane 5 in Fig. 1B). In agreement with our previous observation [25], GLUT1 was primarily (if not entirely) located in the PM; its content in the PM was not altered by insulin or media osmolality (Fig. 1C).

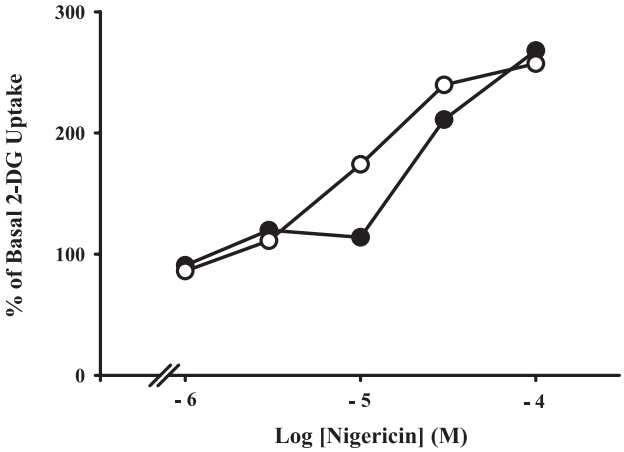

Fig. 2. Concentration-stimulation relationship of nigericin-induced 2-DG uptake. 3T3-F442A adipocytes were incubated in the standard KR (closed circles) or the K^+-rich KR (open circles) with or without (basal) varying concentrations of nigericin for 30 min. 2-DG uptake is expressed as the ratio to the basal rate. The data represent the average of 2 experiments, each measured in triplicate.

Effects of Nigericin on 2-DG Uptake

To determine whether an increase in the G4V membrane ionic permeability and the subsequent osmotic swelling of G4V (as well as other intracellular organelles) could stimulate glucose transport, we employed nigericin, a carboxylic ionophore that promotes electroneutral exchange of K^+ for H^+ along their concentration gradients across a biological membrane [18]. As shown in Fig. 2, addition of nigericin (1–100 μM) to incubation media stimulated 2-DG uptake in a concentration-dependent manner; at 100 μM (the highest concentration tested), nigericin treatment increased 2-DG uptake to nearly 3 times the basal level. To distinguish the possibilities that nigericin stimulates 2-DG uptake via its ionophoretic actions across the plasma membrane or the G4V membrane, the gradients of K^+ and H^+ across the plasma membrane were reduced by incubating cells in the intracellular fluid-like K^+-rich KR at pH 7.0. Nigericin stimulated 2-DG uptake to the same or even a greater extent in cells incubated in the K^+-rich KR (open circles in Fig. 2) than in cells incubated in the standard low-K^+ KR (closed circles in Fig. 2).

The magnitude of the effects of nigericin (100 μM) on 2-DG uptake was approximately one half of the maximal effect of insulin ($n = 5$; $p<0.01$; Fig. 3A). However, the effect of simultaneous addition of nigericin and insulin was not significantly different from that of insulin alone ($n = 5$; $p>0.9$). As shown in Fig. 3B, insulin significantly increased the GLUT4 content in the PM by 2.58 ± 0.32 times ($n = 3$; $p<0.01$), with a concomitant reduction of the GLUT4 level in the HSP. Nigericin also resulted in recruitment of GLUT4 to the plasma membrane, but to a lesser degree (2.05 ± 0.11 times). Another glucose transporter, GLUT1, was mostly located in the PM in unstimulated cells, and its content in the PM was not affected by insulin or nigericin (Fig. 1C & 3C). Thus, in these cells, increases in glucose transport by these agents appear to mainly result from the increased GLUT4 in the PM without (or with only a minor) contribution from GLUT1.

Effects of Bafilomycin A_1

To determine whether the V-ATPase is involved in the stimulation of glucose transport, we utilized bafilomycin A_1, a diagnostically specific inhibitor of this enzyme [19]. Preincubation of the cells with bafilomycin A_1 inhibited both insulin- and nigericin-stimulated 2-DG uptakes in a concentration-dependent manner (Fig. 4). Bafilomycin A_1 reduced the insulin effect by approximately 45% and 80% ($n = 6$; $p<0.01$) and the nigericin effect by about 60% and 90% ($n = 6$; $p<0.01$) at 10 and 50 μM, respectively. Moreover, we have ob-

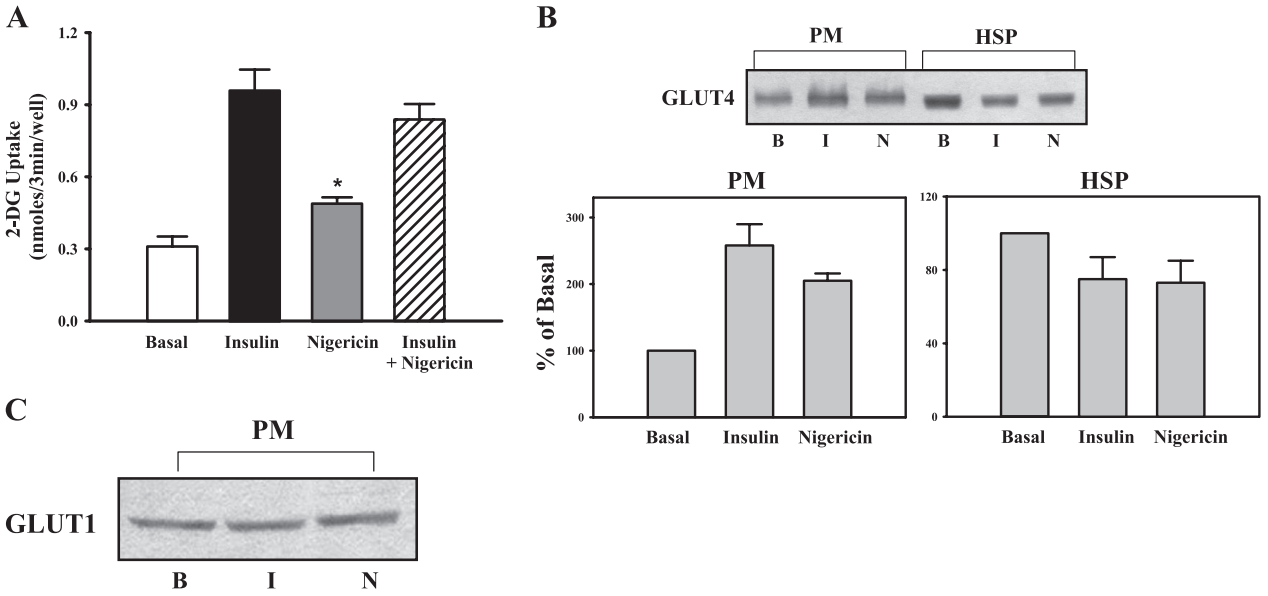

Fig. 3. The effects of insulin and nigericin alone or in combination. A, 3T3-F442A adipocytes cultured on 24-well plates were incubated with or without 100 nM insulin, with or without 100 μM nigericin, or co-treated with both for 30 min prior to the measurement of 2-DG uptake rate. The data represent an average of 5 experiments, each measured in triplicate. B & C, 3T3-F442A adipocytes were incubated with or without (basal; B) insulin (I) or nigericin (N) for 30 min. Cells were homogenized and subjected to subcellular membrane fractionation to generate fractions enriched in the plasma membrane (PM) and the high-speed pellet (HSP) fractions. Proteins (30 μg) were resolved by SDS/PAGE and immunoblotted with polyclonal anti-GLUT4 antibody (B) or anti-GLUT1 antibody (C). The blots are representative of 3 independent experiments. Immunoblot intensities were quantitated by densitometry and expressed in arbitrary units with the basal intensity set at 100%. *$p<0.05$ *vs.* basal level.

served, in 3T3-L1 adipocytes expressing GLUT4 with a Myc epitope in its first exofacial loop, that bafilomycin A_1 inhibits insulin-stimulated GLUT4 translocation to the plasma membrane (unpublished data). These results imply that the stimulatory effects of insulin and nigericin on glucose transport are at least in part dependent upon the V-ATPase. In addition, these results also suggest a possible association between this enzyme and the G4V.

Presence of V-ATPase on G4V

We then used two different approaches to assess whether V-ATPases are located in the G4V membrane. First, homogenates and the subcellular membrane fractions from 3T3-F442A adipocytes were immunoblotted with antiserum against the 72 kDa subunit of V-ATPase. As shown in Fig. 5, the enzyme was identified in homogenates, enriched in HSP and HDM fractions, but not detectable in the PM. The 57 kDa subunit of the enzyme showed a similar pattern of cellular distribution (data not shown). These findings are

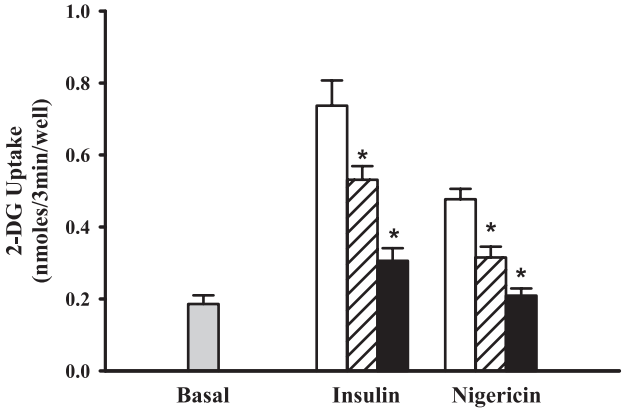

Fig. 4. Effects of bafilomycin A_1 on insulin- and nigericin-stimulated 2-DG uptake. 3T3-F442A adipocytes were pre-treated with 0 (open bar), 10 (hatched bar) or 50 μM (solid bar) of bafilomycin A_1 for 30 min then incubated with or without (basal) 100 nM insulin or 100 μM nigericin for 30 min prior to measurement of the 2-DG uptake rate. The data represent an average of 6 experiments, each measured in triplicate. *$p<0.01$ *vs.* 0 μM bafilomycin A_1.

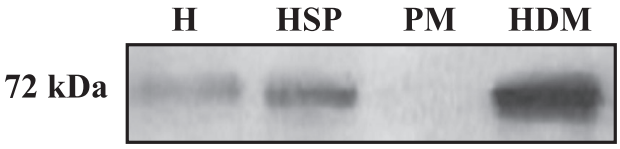

Fig. 5. Identification of V-ATPases in homogenates and subcellular membrane fractions of 3T3-F442A adipocytes. Cells were subjected to the subcellular membrane fractionation as described under Materials and Methods. Fractionated proteins (25 μg) were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with antiserum against the 72 kDa subunit of the V-ATPase. H, HSP, PM, and HDM represent homogenates, high-speed pellets, plasma membranes, and high density microsomes, respectively. The result shown is representative of 3 independent experiments with similar results.

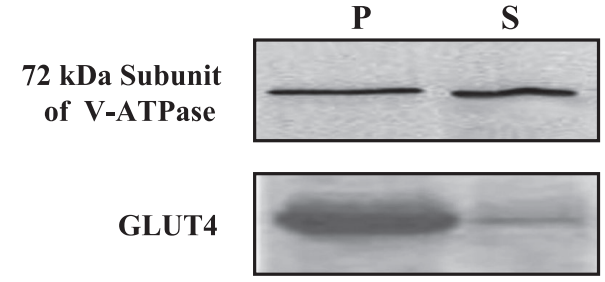

Fig. 6. Presence of V-ATPases in the G4V membrane. The HSP fractionated from 3T3-F442A adipocytes was immunoadsorbed using polyclonal antiserum against the C-terminus of GLUT4. The resulting pellet (P) and supernatant (S) were solubilized, electrophoresed, and immunoblotted using polyclonal antibodies against the 72 kDa subunit of V-ATPase and monoclonal anti-GLUT4 antibody 1F-8. The result shown is representative of 3 independent experiments with similar results.

consistent with the notion that in most mammalian cells, V-ATPases reside largely in the intracellular organelle membranes and are rare in the plasma membrane [13, 17]. Next, we tested for a direct association between V-ATPase and G4V, by pre-clearing the HSP by incubation with rat IgG and then immunoadsorbing the HSP with a polyclonal antiserum raised against the C-terminus of GLUT4 (Fig. 6). Immunoblotting with the anti-GLUT4 antibody showed that GLUT4 is enriched in the pellet and is scarce in the supernatant following immunoadsorption, demonstrating the effectiveness of the immunoadsorption procedure. The anti-GLUT4 monoclonal antibody, 1F-8, was used for blotting to avoid interference by the heavy chain of the immunoadsorbing polyclonal antibody, which has a MW of about 55 kDa. The 72 kDa subunit of V-ATPase was identified in the pellet as well as in the supernatant. This observation clearly demonstrates colocalization of V-ATPase and GLUT4 in the same intracellular organelle membrane, which agrees with the notion that most of the intracellular organelle membranes are endowed with V-ATPases [27].

Discussion

Insulin stimulates glucose transport in adipocytes primarily by recruitment of GLUT4 to the plasma membrane in a manner similar to regulated exocytosis of secretory granules. In the present study, we have investigated the possibility that the chemiosmotic mechanism [8–11], which has been shown to mediate the exocytic fusion of secretory granules stimulated by secretagogues [12, 28–30], may be involved in the insulin-stimulated fusion of G4V with the plasma membrane in 3T3-F442A adipocytes.

Diverse microscopy approaches have localized GLUT4 to tubulovesicular structures in the perinuclear region and in distinct foci throughout the cytoplasm, often in close proximity with the plasma membrane [5, 31]. We first tested whether osmotic swelling of the G4V could draw the vesicles closer to the plasma membrane, leading to fusion of the vesicle membrane with the plasma membrane and subsequent insertion of GLUT4 into the plasma membrane. Incubating the cells in hypo-osmotic medium, which presumptively induces nonselective swelling of the G4V (as well as other intracellular vesicles) and the cells [8, 32], significantly increased 2-DG uptake (Fig. 1A) and GLUT4 content in the plasma membrane (Fig. 1B), suggesting that swelling of the G4V may lead to recruitment of GLUT4 to the plasma membrane. In addition, the stimulatory effects of insulin on 2-DG uptake and on GLUT4 recruitment to the plasma membrane were greatly enhanced in cells incubated in the hypo-osmotic medium in comparison with those incubated in the iso-osmotic medium. Conversely, these effects were almost completely blocked in the hyper-osmotic medium. These findings are consistent with the hypothesis that osmotic swelling of G4V is involved in the insulin-induced exocytosis of the vesicles. Insulin-induced fusion of G4V to the plasma membrane might be facilitated when the vesicles were enlarged in the hypo-

osmotic medium, but inhibited when swelling of the G4V was blocked in the hyper-osmotic medium. Insulin treatment has been shown to change the physicochemical properties of the G4V in adipocytes as well as in muscle cells: insulin increases their sedimentation coefficients by 15~20S and decreases buoyant densities from 1.13 to 1.11 g/cm [33]. These changes can be considered to represent the G4V swelling, providing further support for our hypothesis.

Consistent with the previous reports [34, 35], we found that hyper-osmotic stress inhibits the insulin-stimulated 2-DG uptake, though it increases the basal 2-DG uptake (Fig. 1). According to the chemiosmotic model, the G4V will shrink under hyper-osmotic conditions, with the possible consequence that the insulin-induced swelling might not be sufficient to induce fusion-facilitating levels of stretching. However, inhibition of the insulin-stimulated glucose transport by the hyper-osmotic milieu may not be solely attributed to shrinkage of the G4V. It is possible that other mechanisms (reviewed in [36]), including increased serine phosphorylation of IRS-1 and/or inhibition of insulin-induced stimulation of protein kinase B activity, may also contribute to the hyper-osmotic stress-induced inhibition of insulin action on glucose transport.

We then employed nigericin, an electroneutral K^+/H^+ exchange ionophore [18], to determine whether an increase in ionic permeability of the G4V membrane (as well as other intracellular organelles) causing osmotic swelling of the G4V could stimulate glucose transport. Given a high concentration of H^+ within the G4V (generated and maintained by V-ATPase) and a high concentration of K^+ in the cytoplasm, nigericin would promote inward movement of K^+ coupled with outward movement of H^+. Increased vesicular interior pH would, in turn, lead to increased H^+ transport by relieving the pH gradient-limited V-ATPase activity [13]. The V-ATPase would continue to transport H^+ until a new equilibrium was reached at a greater vesicular osmolality, owing to the accumulation of K^+. The V-ATPase is electrogenic and the H^+ transport would also be limited by transvesicular electrical potential, unless membrane-permeant anions were available to maintain electrical neutrality for continuous H^+ transport. Accordingly, in the presence of permeant anions such as Cl^-, nigericin would cause a net intravesicular accumulation of KCl. The increase in intra-vesicular osmotic activity would provide a driving force for influx of water into the G4V, thereby causing it to swell and perhaps promoting exocytic fusion of G4V with the plasma membrane. As predicted on the basis of this chemiosmotic mechanism, nigericin (1–100 μM) increased 2-DG uptake in a concentration-dependent manner (Fig. 2). This increase in glucose transport appears to be mainly due to the recruitment of G4V to the plasma membrane, as evidenced by an increase in GLUT4 content in the PM with a corresponding reduction of GLUT4 in the HSP (Fig. 3). These effects of nigericin are most likely from its ionophoretic action on intracellular membranes rather than that on plasma membranes based on the finding that nigericin stimulates 2-DG uptake to the same or even greater extent in the K^+-rich KR than in the standard low-K^+ KR (Fig. 2).

In our study, the stimulatory effects of nigericin on 2-DG uptake and GLUT4 recruitment to the plasma membrane were smaller than those of insulin, and the effects of simultaneous administration of the two substances did not differ from those of insulin alone (Fig. 3). Accordingly, we can speculate that the chemiosmotic mechanism may be involved in both nigericin- and insulin-stimulated glucose transports; however, there may be (an) additional insulin-specific mechanism(s). On the other hand, it was previously reported that pretreatment with nigericin reduces the insulin-stimulated 2-DG uptake and GLUT4 recruitment in 3T3-L1 adipocytes [24]. Although its ionophoretic action induces fusion of the juxtaposed vesicles to the plasma membrane, the pretreatment with nigericin still can inhibit other steps involved in the insulin-stimulated GLUT4 recruitment such as insulin signaling pathway, budding and/or translocation of the G4V. Thus, the previous report [24] is not necessarily opposed to our hypothesis.

Our hypothesis is based on the assumption that electrogenic V-ATPases are present in the G4V membrane. Most endomembrane compartments including lysosomes, trans-Glogi networks, endosomes, and secretory granules are endowed with V-ATPases [13, 17]; the acidification of vesicles plays important roles in the vesicular transport [13, 16, 37]. These notions strongly suggest the occurrence of V-ATPases in the G4V membrane. However, a previous report have failed to detect V-ATPases in the GLUT4-bearing vesicles isolated from rat adipocytes [38]. To clarify this issue, we isolated G4V via immunoadsorption and performed immunoblotting with antiserum against the 72 kDa subunit of V-ATPase. The result in Fig. 6 clearly dem-

onstrates that V-ATPases are resident proteins of vesicles which are isolated by immunoadsortpion with anti-GLUT4 antibody. In addition, our current immunocytochemistry studies in 3T3-L1 adipocytes using antibodies against GLUT4 and the 56 kDa B2 subunit of V-ATPase, shows the results equivalent to this (unpublished data). At present, we have no clear explanation for the discrepancy between our results and the previous report [38]. Nonetheless, the coexistence of GLTU4 and V-ATPase demonstrated with two different approaches provides clear evidence that V-ATPases reside in the G4V membrane of cultured adipocytes.

Importantly, along with the coexistence of V-ATPase and GLUT4 in the same vesicle membrane (Fig. 6), the present study demonstrates that pre-treatment of the cells with bafilomycin A_1, a diagnostically specific inhibitor of V-ATPase, significantly inhibits insulin- as well as nigericin-stimulated 2-DG uptakes in a concentration-dependent manner (Fig. 4). We also found that bafilomycin A_1 inhibits the insulin-stimulated recruitment of GLUT4 to the plasma membrane in 3T3-L1 adipocytes over-expressing the GLTU4 containing a Myc epitope in its first exofacial loop and EGFP at the carboxy terminus (unpublished data). These observations strongly suggest that the effect of insulin on GLUT4 recruitment is at least in part dependent upon V-ATPase, and support our hypothesis that the electrochemical gradient of H^+ generated by V-ATPase may provide a driving force for swelling of G4V, leading to exocytic fusion of G4V with the plasma membrane. Consistent with these, Yang *et al.* [39] reported that bafilomycin A_1 inhibits insulin-stimulated glucose uptake and cell surface biotinylation of GLUT4 without impeding the translocation of GLUT4 to the sarcolemma in cardiomyocytes. The data, thus, indicate that bafilomycin A_1 inhibits incorporation of GLUT4 into the plasma membrane.

It can be also suspected that bafilomycin A_1 may reduce insulin-stimulated glucose transport (as shown in Fig. 4) via mechanisms other than disruption of chemiosmotic swelling of the G4V. In cardiomyocytes, Yang *et al.* [39] demonstrated that bafilomycin A_1 blocks insulin-induced alkalinization of the cytosol as well as glucose transport. However, in adipocytes, it was observed not only that simple lowering of cytosolic pH has no effect on insulin-stimulated glucose transport [24] but also that bafilomycin A_1 does not induce any noticeable effect on the insulin-induced alkalinization under our experimental conditions of employing bicarbonate buffer (unpublished data). Thus, in our experiments, it is very unlikely that bafilmycin A_1 inhibits the insulin-stimulated 2-DG uptake by perturbing the alkalinizing effect of insulin. Another possibility is that bafilomycin A_1 may inhibit insulin signaling cascades as it did in rat hepatocytes [40]. Then again, Malikova *et al.* [41] demonstrated that bafilomycin A_1 and concanamycin A, another specific inhibitor of V-ATPase, do not affect insulin signal transduction in adipocytes even though they inhibit insulin-stimulated glucose transport. The authors, on the other hand, found that these inhibitors block the formation of small insulin-responsive vesicles on donor intracellular membranes. Possibly, the inhibitors of V-ATPse may block the formation of insulin-responsive G4V in addition to its fusion with the plasma membrane.

Collectively, our results support possible involvement of the chemiosmotic mechanism in the insulin-stimulated exocytic fusion of the G4V. According to this hypothesis, V-ATPases in the G4V membrane actively pump H^+ into the G4V lumen, generating an interior positive membrane potential and H^+ concentration gradient. Since these electrical and chemical gradients prevent continuous translocation of H^+ into the vesicle lumen, the gradients need to be dissipated by influx of external anions or efflux of internal cations. Hence, it is envisaged that anion (*e.g.*, Cl^-) permeability and H^+-cation (*e.g.*, K^+) exchange across the G4V membrane may limit V-ATPase activity in the basal state. During insulin stimulation, exchange of H^+ for K^+ across the G4V membrane and/or the V-ATPase might be activated such that intravesicular H^+ is exchanged for cytoplasmic K^+. This may, then, lead to accumulation of KCl associated with an osmotic water influx, causing swelling of the G4V; further swelling will result in fusion of the G4V with the plasma membrane. Thus, one may postulate that the H^+ gradient-dependent osmotic swelling of G4V would lead to its close juxtaposition to the plasma membrane and the resultant exocytic fusion following expansion of fusion pore [42]. In support of this hypothesis, studies in yeast showed that V-ATPases play an important role in homotypic vacuole-to-vacuole fusion: the H^+-gradient generated by V-ATPase is required for trans-SNARE complex formation [14] and trans-complex formation of juxtaposed V_o subunits of V-ATPase occurs downstream from trans-SNARE pairing [15]. Thus, the V-ATPase-mediated chemiosmotic mechanism of membrane fusion could be evolutionally conserved from

yeast to mammalian species.

In conclusion, the present study demonstrates that the V-ATPases exist in the G4V membrane and play an important role in the insulin-stimulated GLUT4 recruitment via chemiosmotic mechanism. However, the underlying mechanism(s) by which insulin activates V-ATPase, H^+/monovalent cation exchange and/or anion conductance leading to G4V swelling is yet unknown and will be a priority of our future research.

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