Insulin-stimulated cytosol alkalinization facilitates optimal activation of glucose transport in cardiomyocytes

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Yang, Jing, Alison K. Gillingham, Alois Hodel, Françoise Koumanov, Brian Woodward, and Geoffrey D. Holman. Insulin-stimulated cytosol alkalinization facilitates optimal activation of glucose transport in cardiomyocytes. Am J Physiol Endocrinol Metab 283: E1299–E1307, 2002. First published August 13, 2002; 10.1152/ajpendo.00341.2002.—Abnormalities in intracellular pH regulation have been proposed to be important in type 2 diabetes and the associated cardiomyopathy and hypertension. We have therefore investigated the dependence of insulin-stimulated glucose transport on cytosolic pH in cardiomyocytes. Insulin treatment of cardiomyocytes resulted in a marked alkalinization of the cytoplasm as measured using carboxy-semi-napthorhodofluor-1. The alkalinizing effect of insulin was blocked by treatment with either cariporide (which inhibits the Na+/H+ exchanger) or by bafilomycin A1 (which inhibits H+-ATPase activity). After treatments with cariporide or bafilomycin A1, insulin stimulation of insulin receptor and insulin receptor substrate-1 phosphorylation and Akt activity were normal. In contrast, glucose transport activity and the levels of functional GLUT4 at the plasma membrane (detected using an exofacial photolabel) were reduced by ~50%. Immunocytochemical analysis revealed that insulin treatment caused a translocation of the GLUT4 from perinuclear structures and increased its co-localization with cell surface syntaxin 4. However, neither cariporide nor bafilomycin A1 treatment reduced the translocation of immunodetectable GLUT4 to the sarcolemma region of the cell. It is therefore hypothesized that insulin-stimulated cytosol alkalinization facilitates the final stages of translocation and incorporation of fully functional GLUT4 at the surface-limiting membrane.

GLUT4 translocation; cytosolic pH; cariporide; bafilomycin A1

IN ADDITION TO ITS EFFECTS on glucose transport activity and glycolysis, insulin plays an important role in H+ balance. Stimulation of cells by insulin causes an increase in intracellular pH in adipocytes (6, 30), 3T3-L1 cells (23), muscle (12, 22), and liver (32). Conversely, cytosol acidification has been implicated in pathophysiological processes related to insulin action, including hypertension and type 2 diabetes (24, 25, 36, 45). Cytosolic pH regulation is of particular importance for physiological and pathophysiological processes in cardiomyocytes because of the susceptibility of these cells to changes in pH during osmotic shock and contraction and under anoxic/ischemic conditions (1, 2). A cocktail of glucose, insulin, and potassium is known to have beneficial effects on the ischemic heart (1, 7, 19). Cardiomyopathy is associated with type 2 diabetes and contributes significantly to cardiovascular disease morbidity and mortality in diabetic patients, especially those with coexistent hypertension (45). It is therefore of importance to unravel the interdependence of cardiomyocyte pH and insulin action in these cells.

It has been suggested that part of insulin’s effect on cytosol alkalinization is mediated by activation of Na+/H+ exchange (10, 18, 41). We have examined here whether inhibition of the Na+/H+ exchanger with cariporide can antagonize the effects of insulin on cytosolic pH and glucose transport activity. To examine more widely whether other cell acidification processes may antagonize the alkalinizing effects of insulin, we have examined the effects of bafilomycin A1, an inhibitor of H+-ATPases (50). Both cariporide and bafilomycin A1 were found to markedly reduce insulin-stimulated glucose transport activity.

Insulin stimulation of glucose transport activity is a multistep process involving a convergence of signaling steps on the process of GLUT4 vesicle translocation from its intracellular storage compartment and subsequent docking and fusion of these vesicles with the plasma membrane (17, 40, 44). To measure GLUT4 translocation, subcellular fractionation techniques are often used. However, this technique was found to be unreliable in cardiomyocytes, particularly when only small samples of cell material were available. We have therefore used an immunocytochemical approach to examine changes in insulin-stimulated GLUT4 translocation. Intracellular GLUT4 has been localized to tubulovesicular structures by confocal microscopy in fat cells, skeletal muscle, and heart tissue (28, 29, 34), and this technique therefore provides us a means of examining the effects of pH perturbation on GLUT4 translocation in small-scale preparations of cardiomyocytes. To examine the extent to which treatments that induce cytosol acidification lead to altered cell...
surface exposure of translocated GLUT4, we have used a photolabeling technique. This utilizes an impermeant photoaffinity label (26). The studies described suggest that the cytosol acidification effects of carbopiride and bafilomycin A1 antagonize the final stages of insertion of fully functional GLUT4 at the sarcolemma membrane.

MATERIALS AND METHODS

Cardiomyocyte isolation. Cardiomyocytes from adult male Wistar rats (260–280 g) were prepared by collagenase digestion (type II, from Worthington Biochemical) by use of a method adapted from those previously described (9, 14) with the inclusion of 20 mM inosine in the final cell suspension. Cell suspensions were adjusted to ~10% cytocrit in Krebs-Ringer-HEPES (KRH) buffer (in mM: 6 KCl, 0.2 Na2HPO4, 1.4 MgSO4, 1 CaCl2, 128 NaCl, 10 HEPES, 20 inosine, pH 7.4) with 2% fatty acid-free BSA (Roche Molecular Biochemicals). Freshly isolated rat cardiomyocytes in 2% BSA-KRH buffer were maintained for 10 min at 37°C (with continuous gassing with O2) in the presence and absence of carbopiride (20 μM) or bafilomycin A1 (100 nM). Cells were then stimulated with 30 nM insulin, where appropriate, for 30 min at 37°C. In some experiments, cells were prepared in KRH buffer and transferred to a Krebs-Henseleit-bicarbonate (KHB) buffer (in mM: 4.7 KCl, 1.2 KH2PO4, 25 NaHCO3, 118.5 NaCl, 2.5 CaCl2, 1.2 MgSO4, 5 HEPES) and continuously gassed with 95% O2-5% CO2 throughout the treatments.

Confocal microscopic measurement of cardiomyocyte pH. For measurements of cytosolic pH, 0.5 ml per condition of 10% cytocrit cell suspensions was loaded with 10 μM of 5/6-carboxy-semi-naphthodiolfluor-1 (carboxy-SNARF-1). This was added as the acetoxyethyl ester (Molecular Probes) for 30 min at 37°C after the preincubation period. Intracellular pH was measured essentially as described (49). Three to six cells from each experiment were analyzed using a Zeiss LSM-510 inverted confocal microscopy. Carboxy-SNARF-1 was excited at 488 nm, and the mean of the ratios of the fluorescence emission intensities at >615 nm and 560–615 nm was compared with an in situ calibration curve obtained by use of the ionophore nigericin (49).

Insulin signaling. For determination of the extent of phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), 1-ml aliquots of cardiomyocytes (10% cytocrit) were centrifuged and the pellets lysed in a buffer containing 1% Triton X-100 and 10% glycerol in 50 mM HEPES, pH 7.0, 150 mM NaCl, and 1 mM EDTA, with the phosphatase inhibitors 100 mM NaF, 10 mM Na2O4, 1 mM Na3VO4, and the protease inhibitors antipain, pepstatin A, and leupeptin (each at 1 μg/ml) and 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride. Lysates were subjected to precipitation using anti-phosphotyrosine agarose (Sigma), and then the precipitates were resolved by SDS-PAGE and Western blotted using anti-phospho-Ser473-Akt (New England Biolabs). In each case, signals were detected by enhanced chemiluminescence (ECL) and quantified using an Optichem Detector with associated software (Ultra Violet Products).

Glucose transport activity. Nine hundred-microliter aliquots of cardiomyocyte suspensions at 10% cytocrit in 2% BSA-KRH buffer were maintained at 37°C and continuously gassed with O2. The transport assay was initiated by the addition of 2-deoxy-D-glucose [100 μM final concentration, containing 0.5 μCi of 2-deoxy-D-[3H]glucose (Amersham Pharmacia Biotech)]. Where indicated, the KRH buffer was replaced by KHB buffer. Sugar uptake was terminated after 10 min by transferring the cell suspension to microfuge tubes containing 400 μM phloretin in KRH buffer. Background activity was determined by addition of cells to tubes that contained 2-deoxy-D-glucose premixed with 400 μM phloretin. The samples were quickly mixed and immediately centrifuged at 3,500 g for 1 min. The supernatants were removed, and the cells were washed three times with 1 ml of KRH buffer containing 400 μM phloretin. Cells were lysed with 1 ml of ice-cold 0.1 M NaOH, and aliquots were taken for determination of radioactivity and protein levels.

RESULTS

Insulin-induced alkalinization in cardiomyocytes and reversal by cariporide and baflomycin A1. To measure insulin-induced changes in cytosolic pH, we used carboxy-SNARF-1 (Fig. 1A). The fluorescence signal of this reagent changes to longer wavelengths as intracellular pH rises. Such changes were quantified using a calibration curve in which nigericin was used to equilibrate cytosolic pH with a range of pH standard solutions. There was a significant rise in red fluorescence (Fig. 1A) and associated cytosolic pH (Fig. 1B) when cardiomyocytes were treated with insulin. Inhibition of sodium/proton exchange by use of cariporide blocked this effect and maintained intracellular pH at basal levels (or slightly below). Likewise, baflomycin A1 was found to block the alkalinizing effects of insulin on cytosolic pH.

Effects of cariporide and baflomycin A1 on insulin signaling. To examine the possibility that the pharmacological treatments might alter GLUT4 activity through a reduction in insulin-signaling activity, we examined the extents of phosphorylation of the insulin receptor, IRS-1, and Akt (Fig. 2A and quantified in B). Data are from 3 separate experiments for IR and IRS-1 and 3 experiments for Akt.

Glucose transport in cardiomyocytes treated with cariporide or with baflomycin A1. To determine whether inhibition of Na⁺/H⁺ exchange and/or H⁺-ATPase activities affects glucose transport activity, the uptake of 2-deoxy-d-glucose was analyzed. Treatment with cariporide slightly increased basal transport activity. In agreement with previous findings (8, 13), insulin increased glucose transport activity nearly fivefold above the rates measured in basal cells (Fig. 3A). Treatment with 20 μM cariporide before the addition of insulin resulted in a marked inhibition of insulin-stimulated glucose transport by 40–50% (Fig. 3). Treatment with 0.1 μM baflomycin A1 before the addition of insulin also resulted in a marked inhibition of insulin-stimulated glucose transport by nearly 60% (Fig. 3A). The combined effect of cariporide and baflomycin A1 was no greater than with either reagent alone. Similar effects of cariporide and baflomycin A1 were obtained when a bicarbonate-containing buffer was used (Fig. 3B) instead of a bicarbonate-free buffer (Fig. 3A). How-
ever, it was found that the insulin-stimulated transport activity was somewhat smaller in the bicarbonate buffer when this response was directly compared with that in the bicarbonate-free buffer. We have used a bicarbonate-free buffer for most of the experiments described here because it stabilizes, but amplifies, intracellular pH changes when compared with the more physiological bicarbonate-containing buffer systems.

To test directly whether lowering of cytosolic pH can lead to an attenuation of insulin-stimulated glucose transport that is comparable with that observed after cariporide and baflomycin A1 treatments, we equilibrated internal and external pH by incubation with the ionophore nigericin. We compared pH values of 7.4 and 6.8, because the latter was comparable with the low intracellular pH observed after cariporide treatment (Fig. 1B). Treatment with nigericin at both pH 7.4 and 6.8 led to an increase in basal glucose transport activity. The insulin-stimulated glucose transport activity was markedly reduced after nigericin-induced equilibration of the cytosol pH with the pH 6.8 but not the pH 7.4 buffers. The transport activity was reduced by 80% and to a level that approached that of the elevated basal activity (Fig. 4). Neither lowering of the extracellular pH without nigericin nor incubation of the cardiomyocytes with nigericin at pH 7.2 for several hours has recently been reported to reduce insulin-stimulated glucose transport activity (5). The pH-lowering effect of nigericin was much more immediate and marked when it was used to equilibrate the pH 6.8 buffer. The large inhibitory effect observed after direct cytosol acidification suggests that the indirect pharmacological pH-lowering effects of cariporide and baflomycin A1 (Fig. 1) are sufficient to produce the observed reductions in insulin-stimulated glucose transport activity and that intracellular pH lowering is the basis of their action on this activity.

**Effects of cariporide and baflomycin A1 on cell surface levels of GLUT4.** To determine whether there was a correlation between the prevention of insulin-induced cytosolic alkalinization produced by cariporide and baflomycin A1 and the effects of these reagents on insulin-stimulated exposure of GLUT4 at the cell surface, the cell-impermeant photolabel Bio-LC-ATB-BMPA was employed. This photolabel tags transporters accessible at the cell surface with biotin. Cells were treated with cariporide or with baflomycin A1 in the presence or absence of insulin and then irradiated in the presence of the photolabel. Biotin-tagged transporters were separated from untagged GLUT4 on

![Fig. 3. Reduction in glucose transport activity of insulin-treated cardiomyocytes treated with cariporide and baflomycin A1. A: 2-deoxy-D-glucose uptake was determined in cardiomyocytes in the basal state or in cells treated with 30 nM insulin for 30 min at 37°C in Krebs-Ringer-HEPES (KRH) buffer. Where indicated, the cardiomyocytes were treated with cariporide (20 μM) or baflomycin A1 (100 nM) for 10 min before insulin stimulation. Data are presented as percent transport activity in the insulin-stimulated cells. Data are from 3–8 experiments, P values vs. In. B: treatments were carried out in Krebs-Henseleit-bicarbonate (KHB) buffer. Data are from 3 separate experiments in which transport activity was directly compared with that occurring in insulin-stimulated cells in KRH buffer (final bar). P values vs. In (with bicarbonate).](image)

![Fig. 4. Direct cytosol acidification leads to reduced insulin-stimulated glucose transport activity. Nigericin (Ng) at 5 μg/ml was added to cardiomyocytes for 10 min to equilibrate external KRH buffers at pH 7.4 and 6.8 with intracellular solution. The cells were then maintained in the basal (Ba) state or treated with 30 nM insulin for a further 20 min. P values vs. In + Ng (pH 7.4). Data are from 3 separate experiments.](image)

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streptavidin beads and Western blotted with antibodies against GLUT4 (Fig. 5A). GLUT4 at the cell surface increased following insulin stimulation nearly fivefold over basal levels (Fig. 5B). However, in the presence of cariporide or bafilomycin A1, this effect was attenuated by ~50%. There was a small increase in the cell surface labeling of basal cells after the treatment with cariporide. No change in the total amount of GLUT4 was detected in detergent-solubilized supernatants, indicating that 1) an equal amount of protein had been incubated with the streptavidin beads and 2) neither cariporide nor bafilomycin A1 has a significant effect on the total amount of GLUT4 in cardiomyocytes.

Distribution of GLUT4 revealed by confocal microscopy. Several studies have indicated that insulin may regulate GLUT4 translocation at both the level of release from its storage compartment and the level of the plasma membrane where translocated vesicles dock and fuse (17, 40, 44). To examine whether the effects of cytosolic acidification were mediated at either of these sites, the subcellular distribution of GLUT4 was analyzed by confocal microscopy.

Syntaxin 4 is primarily localized at the cell surface in insulin-responsive tissues, including muscle (21). Therefore, to analyze the effects on translocation to the cell surface, we carried out co-localization studies in which both GLUT4 and syntaxin 4 were examined. Secondary antibodies coupled to either Alexa Fluor 568 anti-mouse IgG (4 μg/ml) or Alexa Fluor 488 anti-rabbit IgG (4 μg/ml) were used to detect GLUT4 and syntaxin 4 antibodies as red and green signals, respectively (Fig. 6A). There was a high concentration of immunodetectable GLUT4 in the perinuclear area and between the two cell nuclei (in cases where 2 nuclei per cell were observed). GLUT4 was also found in lines that radiated out laterally to the cell surface. The GLUT4 in these locations is likely to be associated with the T-tubule system that lies between the myosin fibers.

After insulin treatment, there was a loss of immunodetectable GLUT4 around the nuclei. There was a concomitant increase in the extent of co-localization of GLUT4 with syntaxin 4 at the sacrolemma surface. Any changes in the distribution of GLUT4 in the T-tubules were difficult to resolve using this technique. Treatment of basal cells with cariporide led to a slight increase in sacrolemma-associated GLUT4 signal (Fig. 6A), but this increase was not as extensive as that which follows an insulin treatment. Neither cariporide nor bafilomycin A1 appeared to decrease the extent of GLUT4 co-localization with syntaxin 4. A distinct rim of GLUT4 was found around the cell circumference in insulin-stimulated cells treated with these reagents. The changes in GLUT4 distribution that occurred were quantified by pixel sampling along radial lines from the perinuclear region out to the sacrolemma membrane (~150 samples at 0.14-μm intervals). From this, we calculated the percentage of the total GLUT4 (along the sampled gradient of distribution) that co-localized with syntaxin 4 at the sarcolemma membrane. This approach was limited in that it gave no information on any changes in GLUT4 at the T-tubules. However, this analysis took into account the reduction of GLUT4 in the internal compartments as well as the increase in the sarcolemma membrane. It indicated that the insulin-stimulated change in GLUT4 distribution (translocation) was unaltered by either cariporide or bafilomycin A1 (Fig. 6B).

DISCUSSION

We have found that insulin treatment can lead to alkalization of cardiomyocyte cytosol and that this effect can be blocked by treatment with either cariporide or bafilomycin A1. The neutralization effects induced by cariporide or bafilomycin A1 were accompanied by reductions in glucose transport activity and exposure of GLUT4 at the cell surface as detected by photolabeling. In contrast, confocal microscopy revealed that the effects of insulin on release of GLUT4 from its storage compartment were not reversed by these treatments. Instead, the gross distribution of GLUT4 appeared to be indistinguishable from that observed with insulin treatment alone, and in both cases an intense fluorescent signal from GLUT4 was evident around the cell periphery. Therefore, approxi-

![Image](http://ajpendo.physiology.org/)
Fig. 6. Neither cariporide nor baflomycin A1 treatments lead to a decrease in the co-localization of GLUT4 with syntaxin 4 at the cell surface of cardiomyocytes. A: immunocytochemical signals from Alexa Fluor 568 second-antibody-tagged GLUT4 and Alexa Fluor 488 second-antibody-tagged syntaxin 4 were determined in cardiomyocytes in the basal state (B) or after treatment with 30 nM insulin (In) in the presence of either 20 μM cariporide (Cr) or 100 nM baflomycin A1 (Bf). Insulin-stimulated GLUT4 translocation was seen as a decrease in the proportion of GLUT4 in the peri- and intranuclear areas and increase in the proportion that formed a rim of fluorescence around the cell periphery. B: for quantification of these changes in GLUT4 translocation, the intensities of the GLUT4 and syntaxin 4 fluorescence were sampled along radial lines from the perinuclear area to the sarcolemma surface. PM, plasma membrane. The proportion of GLUT4 that co-localized with syntaxin 4 at the sarcolemma surface was calculated as percent total GLUT4 sampled. Results shown are representative of 3–8 cells selected at random per condition per experiment and of 2 independent experiments. All cells were viewed in approximately the same focal plane. Measure bar, 10 μm.
Cariporide treatment of basal cells slightly increased transport activity and the levels of photolabeled and immunodetectable GLUT4 at the cell surface. However, no consistent increase in basal transport or GLUT4 translocation was found following bafilomycin A1 treatment. Treatment of 3T3-L1 cells with the H^+-ATPase inhibitor bafilomycin A1 has been reported to induce the translocation of GLUT4 to the cell surface in the basal state (4). In this 3T3-L1 cell study, GLUT4 translocation was analyzed by Western blotting of membrane fractions, and any effect of bafilomycin A1 on transport activity was not reported. Any effects of cariporide or bafilomycin A1 on basal cardiomyocytes were slight compared with the stimulatory effect of insulin. More importantly, these treatments resulted in a substantial inhibition of insulin-stimulated glucose transport activity. Although somewhat contrasting effects are observed in 3T3-L1 adipocytes and in cardiomyocytes, the data collectively suggest that pH-dependent trafficking steps occur in both cell types. There may be a balance between pH effects at sequential steps in trafficking. For example, acidification may increase GLUT4 vesicle budding and release from its perinuclear storage compartment, with a consequent increase in basal GLUT4 at the cell surface, but this acidification may reduce GLUT4 vesicle fusion at the surface membrane. The net result would then be dependent on both of these steps. The balance may vary in different cells with different intracellular pH-buffering mechanisms.

There was no additivity in the inhibitory effects of a combination of cariporide and bafilomycin A1 on insulin-stimulated glucose transport activity. This suggests that, although they perturb intracellular pH by different mechanisms, they operate to reduce the proportion of functional GLUT4 exposed at the cell surface via a common mechanism. This common mechanism may be a cytosolic pH-dependent perturbation of the GLUT4 vesicle fusion step at the plasma membrane, which could account for the nonfunctional GLUT4 that accumulates at the cell surface. The transmembrane segment of the vacuolar H^+-ATPase has recently been shown to be a key component required for vesicle fusion in yeast (33). This protein is involved in formation of a fusion pore and completion of the membrane fusion steps initially facilitated by formation of a SNARE complex (33). In addition, pH-dependent fusion steps are known to be involved in virus-induced fusion involving hemagglutinin (46). Thus the actions of cariporide and bafilomycin A1 may be to slow the transition into the plasma membrane of GLUT4 from occluded vesicles. The postulated links between pH and the molecules involved in fusion are speculative at present but suggest that examination of the details of such links may be important for future investigation of GLUT4 trafficking.

Recently, it has been demonstrated that incubation of cardiomyocytes under normoxic conditions with an extracellular buffer of pH values below (but not above) 6.8 leads to reduced insulin signaling at the level of tyrosine phosphorylation of the insulin receptor and IRS-1 with a consequent decreased activation of Akt (1). Similar decreases in signaling occur in ischemia and after treatment with amiloride plus ouabain, conditions that decrease intracellular pH to 6.4 (1). In our studies, the blocking of insulin-induced alkalinization by cariporide or bafilomycin A1 treatments led to maintenance of cytosolic pH at basal levels of ~6.8–7.0.
Under the relatively mild conditions of cytosol acidification induced in our study, we find no reduction in early signaling steps. We find that a full translocation of immunodetectable GLUT4 to the cell periphery occurs when the insulin-induced cytosol alkalinization is neutralized, and this suggests that signaling to the initial translocation step is intact.

Our studies suggest that insulin’s cytosol-alkalinizing effect could be critical in the mechanism of cardioprotective glucose-insulin-potassium therapy (particularly in early stages of ischemia, when the therapy is considered to be most effective). Cariporide is cardioprotective during reperfusion after ischemia. This effect is attributed to inhibition of sodium influx via the Na⁺/H⁺ exchanger, with a consequent reduction in Na⁺/Ca²⁺ exchange activity and calcium overload (20, 37, 42). Cardioprotective interventions, therefore, include the cell-alkalinizing insulin therapy during ischemia and the cell-acidifying cariporide therapy during reperfusion. There is clearly a complex interplay among early signaling steps. We also thank the European Community (COST action B5) for a travel grant to A. Gillingham to visit Dr. Eckel’s laboratory.

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