Insulin treatment of rat adipocytes increases both cytosolic alkalinity and glucose transport activity. Both processes are blocked by the phosphatidylinositol 3-kinase inhibitor wortmannin. Isoproterenol pre-treatment reverses the alkalizing effects of insulin and leads to attenuation of insulin-stimulated glucose transport activity and exposure of GLUT4 to photolabeling reagents at the cell surface. These effects of isoproterenol are mimicked by acid loading and are reversed by cell-alkalinizing conditions. However, neither isoproterenol nor acid loading alters the total level of GLUT4 at the plasma membrane as revealed by Western blotting of plasma membrane fractions or immunodetection of GLUT4 in plasma membrane lawns. GLUT4 is therefore occluded from participation in glucose transport catalysis by a pH-sensitive process. To examine the kinetics of trafficking that lead to these changes in cell surface GLUT4 occlusion, we have utilized a new biotinylated photolabel, GP15. This reagent has a 70-atom spacer between the biotin and the photolabeling diazirine group, and this allows quenching of the surface signal of biotinylated GLUT4 by extracellular avidin. The rates of GLUT4 internalization are only slightly altered by isoproterenol or acidification, mainly due to reduced recycling over long internalization times. By contrast, insulin stimulation of GLUT4 exocytosis is slowed by isoproterenol or acidification pre-treatments. Biphasic time courses are evident, with an initial burst of exposure at the cell surface followed by a slow phase. It is hypothesized that the burst kinetics are a consequence of a two-phase fusion reaction that is rapid in the presence of insulin but slowed by cytosolic acidification.

Time-course studies (1–3) and experiments in which the stimulatory effects of insulin have been attenuated by isoproterenol treatment (4, 5) have demonstrated the appearance of GLUT4 at the cell surface of adipocytes prior to the full stimulation of glucose transport activity. It has been concluded from these studies that a proportion of GLUT4 vesicles can dock at the plasma membrane but that these vesicles only slowly fuse. Consequently, some of the GLUT4 appears to be plasma membrane-located when studied using techniques such as Western blotting of membrane fractions (4) and confocal microscopy (3) but is not accessible to glucose transport substrates or to photolabeling reagents that depend on exposure of the exofacial binding site at the cell surface (4). However, inactivating occlusion of GLUT4 can also occur in the endocytic process (6), and full kinetic resolution of the site of isoproterenol action requires both measurements of GLUT4 internalization and of exocytosis. We describe here new methods for measuring the kinetics of GLUT4 trafficking in insulin- and isoproterenol-treated cells that allow further resolution of the site of action. The kinetic resolution of the site of occlusion of intermediates in GLUT4 trafficking is important, because such analysis is likely to provide insights into rate-determining steps in the insulin stimulatory pathway that lead to GLUT4 translocation and its insertion into the plasma membrane.

We have also explored whether the mechanism for the counter-regulatory effects of isoproterenol on insulin-stimulated glucose transport may be related to its opposing effects on cytosolic pH. It is known that, in addition to its effects on glucose transport activity and carbohydrate metabolism, insulin plays an important role in H+ balance. Exposure of cells to insulin causes an increase in intracellular pH in fat (7, 8), 3T3-L1 cells (9), muscle (10, 11), and liver (12). It has been suggested that insulin's effect on cytosolic alkalization is mediated either by activation of Na+/H+ exchange (13–15) or by reduction in free fatty acid (8). Conversely, isoproterenol may counter the cytosol-alkalinizing effects of insulin through its effects on lipolysis and increased fatty acid release (8). We show here that the effects of insulin on cytosolic pH and GLUT4 exocytosis are both attenuated by isoproterenol pre-treatment and by an acidification protocol involving use of the Na+/H+ exchange inhibitor cariporide (4-isopropyl-3-methylsulfonylbenzoyl-guanidine), and we conclude that cytosolic pH can mediate the extent of GLUT4 occlusion in pre-fusion complexes. These studies on a GLUT4-occluded intermediate in exocytosis therefore add to the growing evidence for regulation of membrane fusion intermediates by ΔpH− (16, 17).

**Experimental Procedures**

*Preparation and Treatments of Rat Adipocytes—Adipocytes from male Wistar rats weighing 180–200 g were prepared by collagenase digestion of the epididymal fat pads, as described earlier (18, 19). Cells were kept at 37 °C before experiments in Krebs-Ringers-HEPES (KRH) buffer (25 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 5.5 mM glucose, pH 7.4) with 3.5% bovine serum albumin (BSA), bovine serum albumin (Meso, 4-morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; t-SNARE, target soluble NSF attachment protein receptors; TIR, transferrin receptors; NHE1, Na+/H+ exchanger isoform 1.*

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buffers at 37°C which were irradiated for 1 min in a Rayonet RPR-100 photoreactor. Washing twice at 18°C and once with 1 ml of PBS buffer only. Bound biotinylated GLUT4 was thens four times with 1 ml of PBS buffer containing 0.1% (w/v) Thesit, incubated in Alexa Fluor 488 (Molecular Probes) goat anti-rabbit secondary antibody (diluted 1:250) in PBS for 90 min at room temperature, washed three times in PBS, embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and mounted on glass slides. GLUT4 distribution was examined using a Zeiss LSM-510 inverted laser-scanning confocal microscope. Images were collected using a 63x/NA1.4 plan-apochromatic oil-immersion objective. The fluorophore was excited at 488 nm with an argon laser, and fluorescence emission was collected using a band-pass filter (505-550 nm). For quantitative measurements of GLUT4 immunoreactivity associated with plasma membrane lawns, images in the x-y plane adjacent to the coverslip were collected using the eight-line average mode and 1024×1024-pixel image resolution. Data sets of emission fluorescence intensities were analyzed using Zeiss LSM-510 software. The amount of GLUT4 was estimated by sampling mean pixel values from within one circle per lawn of 102.31-μm² area (2537 pixels).

RESULTS

Cytosolic pH Balance in Rat Adipocytes—Carboxy-SNARF-1 AM has been used extensively for examination of intracellular pH in several cell types (22). Here we have used this reagent to determine whether the known effects of insulin on alkalization are reversed by pre-treatment with isoproterenol or by a cytosol acidification procedure involving NH₄Cl pre-loading of cells combined with treatment with cariporide (an inhibitor of Na⁺/H⁺ exchange). The latter inhibitor prevents efflux of hydrogen ions generated from NH₄Cl within the cell. Cariporide is more specific for the Na⁺/H⁺ exchanger isoform 1 (NHE1) than amiloride (23). Effects of amiloride on insulin-stimulated glucose transport have been previously reported (24). However, because this reagent has nonspecific effects directly on the glucose-binding site of GLUT4,2 relating effects on transport to GLUT4 translocation are problematic. By contrast, cariporide provides a specific tool for examination of whether perturbations in the cellular pH can alter glucose transport and GLUT4 translocation to the cell surface.

Insulin treatment of rat adipocytes increased intracellular pH by ~0.5 pH units compared with basal cells (p < 0.005, Fig. 1). This was seen as a decrease in emission at 580 nm and an increase in emission at 650 nm with a resultant change in the merged fluorescence emissions from a greenish yellow color to a reddish-brown color (Fig. 1A). The ratio of these two emissions was calibrated with equilibrated pH standard solutions (Fig. 1B). The pH changes were evident in the thin cytoplasmic ring around the cell (lower panels) and in the thicker area of cytoplasm on either side of the nucleus (upper panels). In most experiments it was found to be more convenient to measure the pH in the cytoplasmic rings, because taking measurements from these areas did not depend on the orientation of the cell under the microscope.

2 G. D. Holman and W. D. Rees, unpublished observations.
Wortmannin treatment of adipocytes partially blocked the insulin stimulation of cytosolic acidification ($p < 0.05$) and maintained cytosolic pH at just above basal values. The isoproterenol and cytosol acidification pre-treatments (cariporide maintained cytosolic pH at just above basal values. The isoproterenol with 100 nM wortmannin ($\text{Ins} + \text{CyA}$) pre-treatments were irradiated in basal and insulin-treated cells ($\text{Ins}$). Similar pH changes were also observed in the area between the fat droplet and the cell exterior was examined in individual cells (lower panels). Similar pH changes were also observed in the area of cytoplasm close to the nucleus (upper panels). Fluorescence emissions were collected in two channels at 560–615 nm and > 615 nm. The merged channel output is shown. In B the channel ratio (mean ± S.E.) has been plotted against a range of standard pH buffers that have been equilibrated with the cell cytoplasm in the presence of nigericin. In C the intracellular pH has been estimated for cells maintained either under basal conditions ($\text{Ba}$, $n = 5$), treatment with 20 nM insulin ($\text{Ins}$, $n = 5$), insulin with 100 nM wortmannin ($\text{Ins} + \text{W}$, $n = 5$), insulin treatment in the presence of 1 $\mu$M isoproterenol ($\text{Ins} + \text{Iso}$, $n = 4$) or cytosol acidification ($\text{Ins} + \text{CyA}$, $n = 4$). Results are the mean ± S.E. for the number of experiments indicated above. In each experiment, 6–8 cells were analyzed. **, $p < 0.005$; *, $p < 0.05$ compared with insulin.

Fig. 1. Isoproterenol reverses the effect of insulin on alkalization of adipocyte cytoplasm. Basal and insulin-treated cells (A) were loaded with carboxy-SNARF-1 AM. Intracellular carboxy-SNARF-1 was excited at 488 nm by a argon laser using a Zeiss LSM-510 confocal microscopy apparatus. Usually, the thin rim of cytoplasm between the fat droplet and the cell exterior was examined in individual cells (lower panels). Similar pH changes were also observed in the area of cytoplasm close to the nucleus (upper panels). Fluorescence emissions were collected in two channels at 560–615 nm and > 615 nm. The merged channel output is shown. In B the channel ratio (mean ± S.E.) has been plotted against a range of standard pH buffers that have been equilibrated with the cell cytoplasm in the presence of nigericin. In C the intracellular pH has been estimated for cells maintained either under basal conditions ($\text{Ba}$, $n = 5$), treatment with 20 nM insulin ($\text{Ins}$, $n = 5$), insulin with 100 nM wortmannin ($\text{Ins} + \text{W}$, $n = 5$), insulin treatment in the presence of 1 $\mu$M isoproterenol ($\text{Ins} + \text{Iso}$, $n = 4$) or cytosol acidification ($\text{Ins} + \text{CyA}$, $n = 4$). Results are the mean ± S.E. for the number of experiments indicated above. In each experiment, 6–8 cells were analyzed. **, $p < 0.005$; *, $p < 0.05$ compared with insulin.
Reversal of Isoproterenol Effect in Alkalinizing Chloride-free Buffer—To test the hypothesis that the effects of isoproterenol are due to cytosol acidification, cells were insulin-treated both in the presence and absence of isoproterenol and is an alkalinizing chloride-free buffer. Carboxy-SNARF measurements of intracellular pH confirmed that the chloride substitution raised intracellular pH in adipocytes (Fig. 4A). The alkalinization led to substantial reversal of the ability of isoproterenol to inhibit glucose transport activity (Fig. 4B). The insulin-stimulated transport activity was reduced by 52.3 ± 3.4% by isoproterenol in the chloride buffer but by only 68.4 ± 3.4% in the sulfate buffer (p < 0.001; four paired experiments). It is also evident that the isoproterenol-induced acidification effect is quite robust, because it totally reverses the alkalinizing effects of isoproterenol (see below).

Effects of Isoproterenol and Cytosol Acidification on GLUT4 Internalization and Exocytosis—The low level of cell surface-exposed GLUT4 observed following cytosol acidification may be due to occlusion in internalization vesicles or in vesicles that may accumulate in exocytosis. However, when we tagged the surface GLUT4 with the biotinylated photolabel GP15 (Fig. 5) and measured the internalization rate (Fig. 6) we found only slight differences due to the acidification pre-treatments when compared with insulin treatment alone. GLUT4 leaves the cell surface more slowly in rat adipocytes (Fig. 5 and Ref. 2) than in 3T3-L1 cells (25), and this probably reflects the high levels of clathrin in the latter cells (26). The rate of internalization of the biotinylated GLUT4 was initially slightly slower following acidification, and this effect probably reflects reduced recycling (or re-exocytosis) of the tagged transporter. The effects of acidification on exocytosis were more evident when this process was directly measured (see below).

Internalized biotinylated GLUT4 only slowly recycles in the basal state. In un-stimulated cells, the GLUT4 signal was only very slowly reduced by extracellular avidin, indicating that translocation to the surface is slow (Fig. 7A). By contrast, when cells were treated with insulin, the signal from biotinylated GLUT4 very rapidly declined. This decrease in signal occurred because of complex formation with extracellular avidin, and this indicated that translocation to the surface had greatly accelerated. In contrast to the rapid decline in GLUT4 signal in cells treated with insulin alone, the decreases in GLUT4 signal following the combined treatments with insulin and isoproterenol.
The interaction with avidin is used to quench cell-surface biotinylated GLUT4 in trafficking kinetic experiments.

Time-course analysis indicated that both isoproterenol and cytosol acidification pre-treatments slowed the exocytosis of GLUT4. Single-exponential fits to these data indicated that the apparent first order rate constants were reduced by ~50% following the isoproterenol and acidification pre-treatments (Fig. 8A). However, the single-exponential fits were poor. The difference between treatments with insulin alone and the combined treatment with isoproterenol was much more evident in the samples with long times (5–20 min) than in those with short times (2 min). The biphasic nature of the time courses became much more pronounced following the pre-treatments, and double-exponential curve fits were good (Fig. 8B).

**DISCUSSION**

Regulation of Cytosolic pH and GLUT4 Occlusion by Insulin and Isoproterenol—Insulin has been found to lead to alkalinization of adipocyte cytosol. Insulin has been previously reported to increase cytosolic pH in several cell types (7–10, 12, 13). In human erythrocytes, insulin stimulates cytosol alkalinization via a wortmannin-sensitive activation of NHE1 (13). We report here that the alkalinization induced by insulin treatment of adipocytes is wortmannin-sensitive. Therefore, insulin-stimulatory effects on both cytosol alkalinization and glucose transport in adipocytes occur downstream of phosphatidylinositol 3-kinase activity.

The effects of adrenergic agonists on cytosolic pH are more varied, and, in some cell types, α-adrenergic agonists have been found to increase Na+/H+ exchange activity (27). However, the effects of isoproterenol in adipocytes are thought to be mediated by β3 receptors (28). Civelek et al. (8) reported that isoproterenol alone was capable of lowering cytosolic pH below...
that of untreated (basal) cells, but the combined effects of insulin and isoproterenol were not reported in their study. Here we find that isoproterenol can reverse the effects of insulin on cytosolic pH and that this effect is mimicked by a cytosol alkalinization involving the combined use of NH4Cl and the Na+/H+ exchange inhibitor, cariporide. We also find that both the pH and transport changes induced by isoproterenol are reversed in an alkalinizing chloride-free buffer. This reversal effect provides support for the hypothesis that the isoproterenol attenuation of the insulin response on glucose transport is linked to pH. However, the acidifying effect of isoproterenol is quite strong; it abolishes the alkalinizing effect of insulin and is not easily reversed.

It has been postulated by Civelek et al. (8) that the cytosolic acidification effects induced by isoproterenol treatment are due to lipolytic release of free fatty acid. Insulin and isoproterenol produce antagonistic effects on lipolysis, but it is also possible that insulin and isoproterenol alter cytosolic pH through different mechanisms. For example, insulin may lead to cytosolic alkalization via its effects on sodium/hydrogen exchangers whereas isoproterenol may antagonize this effect by stimulating lipolysis with subsequent release of acidifying fatty acids. Antagonism of insulin action by isoproterenol has also been proposed to occur at the level of insulin receptor phosphorylation (29) and trimeric G-protein activity (30). It is also likely that many physiological and pathophysiological processes, either separately or in combination, lead to antagonism of insulin's alkalinizing effects. The alkalinizing effects of insulin may be required for full activation of glucose transport, and, therefore, pathophysiological processes that acidify cytoplasm may lead to resistance in insulin-stimulated glucose transport.

The effects of acidification of cell pH described here were accompanied by reductions in glucose transport activity and the exposure of GLUT4 at the cell surface as detected by photolabeling. By contrast, Western blotting of plasma membrane fractions and confocal microscopy of plasma membrane lawns have revealed that GLUT4 is present in the plasma membrane at similar levels with and without the acidification. These results indicate that both isoproterenol and cytosol acidification induce a dissociation of insulin-stimulated glucose transport and GLUT4 translocation to the cell surface. At steady state, a proportion (approximately half of the transporters) appears to be present in the surface membrane but occluded from participation in transport and are not exposed to photolabel (4). This effect is not due to a change in the affinity for the photolabel as direct measurements of the half-maximal affinity constant (K_d) for the photolabel show that this is unaltered. Instead, it is a kinetic effect related to a reduced rate of exposure of the GLUT4 to the cell surface, which we have shown is reduced by ~50% following these treatments. Importantly, the measurements of the exocytosis rates reported here are not dependent on the affinity of GLUT4 for photolabel, because labeling is carried out on a single population of cells prior to incubation with isoproterenol or the cytosol acidification treatment.

The lag period between translocation of transporters to the cell surface and the increase in transport activity has been attributed to a population of glucose transporters in occluded precursor/fusion-incompetent states or bound to regulatory proteins, which prevent the full exposure of the transporter to the extracellular environment (1–3). The effects of cytosolic acidification described here indicate that the exposure of functional surface transporters is a pH-modulated step.

Role of pH in Membrane Protein Trafficking—Cytosolic acidification pretreatments slightly slow internalization (by ~7% over the initial internalization time period) of GLUT4 compared with insulin treatment alone. This slowing effect is likely to be due to a pH-dependent reduction in clathrin-mediated internalization. However, no acceleration of internalization is observed, so it seems unlikely that the GLUT4 occluded at the plasma membrane is present in internalization intermediates such as clathrin-associated vesicles.

We consider here two mechanisms by which the electrochemical gradient for H^+ (ΔμH^+) may facilitate the translocation of GLUT4 to the cell surface and functional incorporation into this limiting membrane (that is, budding and fusion mechanisms). Several studies suggest the involvement of endosome acidification in receptor tail recognition and in control of recycling via interactions with internalization motifs (31, 32). Analysis of TIR recycling (33, 34) has revealed that reduced endosome acidification results in normal accumulation of TIRs in recycling endosomes but to a reduced rate of exit of TIRs from the recycling compartment to the plasma membrane. In addition, it has been found that the distribution of ADP ribosylation factor (ARF) to the cytoplasmic face of the trans-Golgi network, where it is required for vesicle coating and budding of secretory vesicles, is a pH-dependent process (35). GLUT4 contains targeting motifs at both the N- and C-terminal domains. These domains include acidic motifs that may act to fine-tune the recognition by adaptor complexes of motifs based on hydrophobic residues, phenylalanine at the N terminus and di-leucine at the C terminus (36–39). It seems likely that these acidic domains may be recognized by vesicle-coating proteins (36, 38). We therefore hypothesize that the recognition of these domains may be dependent on the pH around the GLUT4 vesicles and tubulo-vesicular structures and may influence vesicle coating, budding, and release steps. The alkalinization of the cytoplasm...
that results from insulin activation may result in release of more GLUT4 vesicles.

There may be a balance between pH effects at the budding and fusion steps in trafficking. For example, an increase in \( \Delta \mu H^- \) (either by increased endosomes acidification or increased cytosol alkalization) may increase release of vesicles from their storage compartment and increase vesicle fusion at the surface membrane. The net result would then be dependent on both these steps. The balance may vary in different cell types with different receptor types and intracellular pH buffering mechanisms.

**Role of \( \Delta \mu H^- \) in Membrane Fusion Catalysis**—A more important site for pH regulation of GLUT4 trafficking (in relation to the present study in which the insulin effect on translocation is antagonized) appears to be at the vesicle fusion step at the plasma membrane. Perturbation at this step could account for the low transport activity and reduced exposure of GLUT4 to the impermeant photolabeling reagent but retention of high levels of GLUT4 immunocytochemically localized in the vicinity of the plasma membrane. Analysis of the kinetics of exocytosis has revealed that there is an initial rapid burst of exposure of GLUT4 at the cell surface, which is then followed by a slower phase. These burst kinetics in GLUT4 exocytosis are similar to those reported for other vesicle secretory systems (40, 41). In such systems it is generally assumed that this phenomenon occurs because there are two populations of intracellular vesicles that fuse with the plasma membrane at different rates. A different aspect of this model has been recently proposed in which t-SNAREs are assumed to be present in two interconvertible forms with differing susceptibility to neurotoxin cleavage (40). These models invoke parallel processing by two populations, of either vesicles or t-SNAREs, respectively. Our studies indicate that a pH-sensitive step occurs in the plasma membrane, and consequently the possibility of parallel processing of fusion intermediates may not appropriately account for the burst kinetics observed in GLUT4 trafficking. A more appropriate model would be one in which two vesicle fusion intermediates are present in sequence rather than in parallel. This sequential processing would be similar to that occurring in an isomerizing enzyme and would also give a double-exponential curve for the exocytosis time course.

Our findings demonstrate that fusion of GLUT4 vesicles with the plasma membrane is similar to other known membrane fusion processes, which are also dependent on \( \Delta \mu H^- \). The transmembrane segment of the vacuolar \( H^+ \) ATPase has recently been shown to be a key component that is required for vesicle fusion. It has been demonstrated that this protein is involved in formation of a fusion pore that is required to complete the membrane fusion steps initially facilitated by formation of a SNARE complex (17). It has been demonstrated that esophagin granule secretion is a pH-dependent process, and a dependence of this process on the vacuolar \( H^+ \) ATPase has been postulated (42). pH-dependent fusion steps are known to be involved in virus-induced fusion involving hemagglutinin (43). The conformational change induced in hemagglutinin has been likened to those occurring in SNARE proteins (43, 44). A low intravesicular pH is required for homotypic vacuole fusion in yeast. In this system uncoupling of the membrane pH gradient reduces SNARE complex formation (16). Heterotypic fusion of vesicles at the plasma membrane occurs under conditions in which the extracellular space outside the plasma membrane cannot be readily acidified. However, it seems likely that generation of a pH gradient by cytosol alkalization may in effect accomplish the same conformation switching in SNARE availability as occurs in fusion between acidic organelles. If SNAREs are catalytic in the many rounds of fusion that are necessary to move from a basal to a fully stimulated state, transition between active and inactive SNARE conformations may occur many times, and the pH dependence of these processes will be important for efficient catalysis.

It is unlikely that the effect of insulin on alkalinating cytosolic pH effect is alone sufficient to be able to accelerate fusion. Instead, insulin-dependent SNARE-binding proteins (for example Munc18c (45) and synip (46)) are also likely to be important in stimulating the SNARE isomerization. Perturbation of calcium and calmodulin lead to accumulation of occluded vesicles and an apparent slowing of fusion of GLUT4 vesicles (47). This effect may be linked to the activity of the vacuolar \( H^+ \)-ATPase, which is postulated to be required for formation of membrane fusion pores and complexes and has been demonstrated to be a calmodulin-binding protein (17).

In conclusion, optimal trafficking of GLUT4 vesicles requires the cytosol alkalinating effects of insulin. This leads to an increase in the rate of fusion of docked vesicles with the plasma membrane. The counter-regulatory effects of isoproterenol (and possibly pathophysiological mediators of insulin resistance) occur through mechanisms that involve cytosol acidification.
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