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Endofacial Competitive Inhibition of Glucose Transporter-4 Intrinsic Activity by the Mitogen-Activated Protein Kinase Inhibitor SB203580

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The translocation of glucose transporter-4 (GLUT4) to the cell surface is a complex multistep process that involves movement of GLUT4 vesicles from a reservoir compartment, and docking and fusion of the vesicles with the plasma membrane. It has recently been proposed that a p38 mitogen-activated protein kinase (MAPK)-dependent step may lead to intrinsic activation of the transporters exposed at the cell surface. In contrast to data obtained in muscle and adipocyte cell lines, we found that no insulin activation of p38 MAPK occurred in rat adipose cells. However, the p38 MAPK inhibitor SB203580 consistently inhibited transport activity after preincubation with the adipose cells. These apparently contradictory findings led us to hypothesize that the inhibitor may have a direct effect on the transport catalytic activity of GLUT4 that was independent of inhibition of the kinase. Kinetic analysis of 3-*O*-methyl-D-glucose transport activity revealed that SB203580 was a noncompetitive inhibitor of zero-trans (substrate outside but not inside) transport, but was a competitive inhibitor of equilibrium-exchange (substrate inside and outside) transport. This pattern of inhibition of GLUT4 was also observed with cytochalasin B. The pattern of inhibition is consistent with interaction at the endofacial surface, but not the exofacial surface of the transporter. Occupation of the endofacial substrate site reduces maximum velocity under zero-trans conditions, because return of the substrate site to the outside is blocked, and no substrate is present inside to displace the inhibitor. Under equilibrium-exchange conditions, internal substrate competitively displaces the inhibitor, and the transport K_m is increased. (*Endocrinology* 146: 1713–1717, 2005)

TRANSLOCATION OF GLUCOSE transporter-4 (GLUT4) to the cell surface is a complex multistep process that involves movement of GLUT4 vesicles from a reservoir compartment, followed by docking and fusion of the vesicles with the plasma membrane (1, 2). Whether the transport activity of the inserted transporters is then further activated has always been controversial (3) and is currently being examined in several laboratories using new approaches (4, 5). Historically much of the debate and controversy has centered on the involvement of insulin-activated p38 MAPK (6, 7). This kinase has been implicated in the stimulation of GLUT4 intrinsic catalytic activity in cell types that include muscle and adipocyte cell lines and tissues. In addition, recent studies have suggested that stimulations of glucose transport that occur downstream of activated AMPK are mediated by p38 MAPK (8). Other studies have suggested that p38 MAPK is involved in arsenite- (9) and lipoic acid (10) -stimulated glucose transport.

Many of the studies that suggested a role for p38 MAPK have been based on inhibition of transport activity by the kinase inhibitor SB203580 (6–9, 11, 12). This compound reduces the maximum velocity (V_{max}) for uptake under zero-trans (substrate outside but not inside) conditions, requires preincubation with the cells, and is not easily washed out of the cells after the preincubation. Because it does not alter the translocation of epitope-tagged GLUT4, the data have collectively led to the postulate that this inhibitor does not competitively interact with GLUT4 at its substrate-binding site, but, rather, that a kinase-mediated inhibition occurs (6, 12).

We have examined this issue in rat adipose cells, because many previous studies have focused on cell lines that may have a greater cross-talk between signaling pathways than occurs in native cell types. Although initial studies indicated that insulin does not activate p38 MAPK in rat adipose cells, we confirmed that the p38 MAPK inhibitor does inhibit glucose transport in this system. These apparently contradictory findings led us to hypothesize that the inhibitor may have a direct effect on the transport catalytic activity of GLUT4 that is independent of inhibition of the kinase. We therefore reexamined the pattern of transport inhibition by SB203580 and developed a kinetic test for the possibility that the inhibitor combines with the endofacial surface, but not the exofacial surface, of the transporter.

Materials and Methods

Cell preparation and treatment

Adipose cells from epididymal fat pads of male Wistar rats, weighing 180–200 g, were prepared by collagenase digestion as described previously (13, 14). Cells were kept at 37 C before experiments in Krebs-

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Abbreviations: AMPK, AMP-activated protein kinase; CREB, cAMP response element-binding protein; DMSO, dimethylsulfoxide; GLUT4, glucose transporter 4; HES, HEPES-EDTA-sucrose; JNK, c-Jun N-terminal kinase; KRH, Krebs-Ringer-HEPES; SAPK, stress-activated protein kinase; V_{max}, maximum velocity.

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Ringer-HEPES (KRH) buffer (25 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, and 1.8 mM $CaCl_2$, pH 7.4) with 1% BSA and at 40% cytocrit. Cells were stimulated with insulin at the concentrations indicated in the figure legends. SB203580 was used at a final concentration of 10 μM and was preincubated with adipose cells for 15–20 min before insulin addition. Inhibitory reagents were dissolved in dimethylsulfoxide (DMSO) to produce a final DMSO concentration of 0.1% (vol/vol). The same concentration of DMSO was added to control incubations.

Activation of kinases

After insulin treatment, cells were washed in 20 ml KRH buffer containing 20 nM insulin where appropriate, but without BSA. Excess buffer was removed, and cells were transferred to a 1.5-ml microfuge tube. Cells were allowed to float for approximately 15 sec, and 200 μl packed cells from the top of the tube were removed with a pipette. Cells were then lysed directly in 200 μl sodium dodecyl sulfate sample buffer containing 1 mM dithiothreitol, 1 mM Na_3VO_4, 20 mM sodium fluoride, 2 mM sodium molybdate, 100 nM okadaic acid, 7.5% (vol/vol) β-mercaptoethanol, and protease inhibitors and heated for 15 min at 65 C. Lysates were centrifuged at 1000 $\times$ *g* for 1 min, and supernatants were analyzed by SDS-PAGE and transferred to nitrocellulose for Western blotting with the primary antibodies, all at 1:1000 dilution of the supplied material. Insulin receptor β-subunit antibody and phospho-cAMP response element-binding protein (phospho-CREB) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). P38 MAPK, phospho-p38 MAPK, phospho-p44/42 extracellular signal-regulated kinase-MAPK, phospho-stress-activated protein kinase (phospho-SAPK)/c-Jun N-terminal kinase (JNK)-MAPK, phospho-Akt (Thr^{308}), and phospho-Akt (Ser^{473}) were purchased from Cell Signaling Technology (Cambridge, MA). All antibodies were supplied as purified rabbit polyclonals. Gel loading was checked by blotting with either the p38 MAPK or the β-subunit of the insulin receptor antibodies.

Glucose transport activity assays

3-*O*-Methyl-D-glucose uptake was measured under zero-trans and equilibrium exchange conditions as previously described (13, 15, 16). In brief, for the zero-trans experiments, 10-μl aliquots containing 0.2 μCi 3-*O*-methyl-[^{14}C]D-glucose were mixed with nonradioactive substrate (to give the final substrate concentrations indicated in the figure legends) before the addition of a 50-μl aliquot of the adipose cell suspension at 40% cytocrit. For the equilibrium exchange experiments, the nonradioactive substrate was preincubated with the cells in 50-μl aliquots for 20 min before addition to a 10-μl aliquot containing the radioactive 3-*O*-methyl-D-glucose and sufficient substrate to maintain the equilibrated concentrations. Uptake was stopped at 3 sec by the addition of 3 ml 0.3 mM phloretin in albumin-free KRH buffer. Pulses were timed using an electronic metronome. Zero time values were obtained by adding phloretin before mixing the cells with substrate. Uptake was calculated as a fraction (*f*) of the maximum radioactivity associated with the cells at equilibrium for 20 min and was converted to rate constant v/[S] using the equation $v/[S] = -\ln(1 - f)/t$. Reciprocals of the rate constants [S]/v (determined in triplicate for each separate experiment) were then plotted against the substrate concentration [S] to obtain K_m and V_{max}.

Results

Insulin treatment does not activate p38 MAPK in rat adipocytes

MAPK activation in rat adipose cells was determined by examining the extent of phosphorylation at its regulatory Thr-X-Tyr motif in the activation loop of the kinase domain. Insulin at concentrations of up to 200 nM did not lead to changes in the level of phosphorylation of either p38 MAPK or its downstream substrate CREB (Fig. 1A). Under the same conditions, concentrations of insulin as low as 0.1–0.5 nM insulin led to extensive activation of the phosphorylation of Akt at threonine 308 and an 11.7 ± 1.2-fold (n = 3) increase over basal levels at 20 nM insulin (Fig. 1A). Treatment with

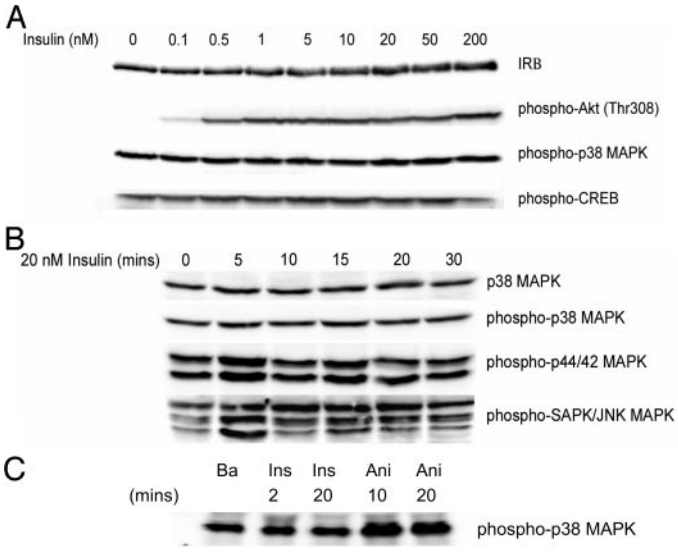

FIG. 1. Insulin stimulation of MAPKs in rat adipose cells. A, Rat adipose cells were stimulated with the indicated concentrations of insulin for 10 min and lysed, and the proteins were resolved by SDS-PAGE. Western blotting with antibodies to the β-subunit of the insulin receptor (IRB) was used as a loading control. Phospho-Akt-(Thr^{308}) levels were increased by insulin, but levels of phospho-p38 and its downstream substrate phospho-CREB were not. B, The possibility of transient stimulation of MAPKs was evaluated by treatment with 20 nM insulin for the indicated times. The total level of p38 MAPK was used as a loading control. No time-dependent increase in phospho-p38 MAPK was evident, but phospho-p44/42 MAPK and phosphor-SAPK/JNK-MAPK showed stimulated phosphorylation at 5 min that declined to basal levels over 30 min. C, Anisomycin at 37 μM leads to significant increases in p38 MAPK phosphorylation. The blots are representative of at least three separate experiments in each case.

20 nM insulin for up to 30 min did not alter the total amount of p38 MAPK or the extent of phosphorylation (Fig. 1, B and C). However, treatment with the p38 MAPK activator anisomycin at 37 μM for 20 min increased phosphorylation by 1.6 ± 0.2-fold (n = 3; Fig. 1C). Thus, although adipocytes may be partially activated by cell isolation (17), there is additional potential for p38 MAPK activation that is not used in insulin signaling. After insulin treatment, we observed a rapid and transient 1.5 ± 0.2-fold (n = 3) increase in p44/42 extracellular signal-regulated kinase-MAPK phosphorylation, but this returned to basal levels within 10 min. Phosphorylation of SAPK/JNK also peaked at 5 min after insulin treatment to a level that was 2.8 ± 0.7 times (n = 3) the basal level. This rise also returned to basal levels in 20 min.

As SB203580 has been extensively used to implicate p38 MAPK in insulin signaling, we next tested whether this reagent would selectively inhibit the phosphorylation of the MAPK substrate CREB. Surprisingly, CREB phosphorylation was unaltered by the kinase inhibitor, but this inhibitor reduced the extent of phosphorylation of p38 itself. The level of phosphorylation was reduced by 35% and 41% (n = 3) in basal and insulin-stimulated cells, respectively. This suggests that the reagent is active in adipose cells, but that it is inhibiting a kinase upstream of p38 MAPK or is reducing the autophosphorylation of this enzyme. Although Akt phosphorylation on threonine 308 was unaffected by the inhibitor, we observed a small and consistent (~6%; n = 3) reduction

in Akt phosphorylation on serine 473 (Fig. 2). These data are consistent with a reported 38% reduction in recombinant Akt enzyme activity in the presence of 10 μM SB203580 (18). This may occur because of inhibition of the putative phosphoinositide-dependent kinase 2, which is thought to phosphorylate serine 473 of Akt, by SB203580. It has been reported that PDK2 phosphorylation is sensitive to SB203580 at concentrations similar to those known to be active against p38 MAPK (19).

As previously reported (7), although the active p38 inhibitor SB203580 inhibits glucose transport activity by 40–50%, the negative control compound SB202474 does not significantly inhibit this activity. Furthermore, although anisomycin leads to increased p38 MAPK phosphorylation, it alone does not extensively activate transport activity in rat adipose cells (Fig. 2B).

The p38 MAPK inhibitor, SB203580 acts as a competitive inhibitor at the endofacial surface of GLUT4

To test the hypothesis that SB203580 acts directly on GLUT4 to inhibit its transport activity, we employed both zero-trans (no substrate inside) and equilibrium exchange (substrate inside and outside) transport protocols. Because it had previously been shown (6, 7, 20) that SB203580 does not inhibit glucose transport when applied directly with the substrate to the outer surface, we hypothesized that it may interact at the internal or endofacial surface of the system. If this were the case, then the competitive nature of the interaction would only be revealed in the equilibrium exchange experiment, where competing substrate is present at the putative inhibitor site. In human erythrocytes, cytochalasin B is known to competitively inhibit only at the inside site. Basketter and Widdas (21) showed that kinetically this interaction produces a noncompetitive mode of inhibition in zero-trans entry experiments, but a competitive mode of inhibition in equilibrium exchange transport experiments. The erythrocyte transport activity is now known to be due to GLUT1, which is a kinetically asymmetric system. We therefore thought it important to test whether cytochalasin B would behave in the same way when inhibiting the adipocyte GLUT4 system, which is kinetically symmetric (13). An endofacial interaction of cytochalasin B with GLUT4 was demonstrated (Fig. 3, C and D). The inhibitor reduced the V_{max} for zero-trans 3-*O*-methyl-D-glucose transport, but did not alter the K_m (Fig. 4). By contrast, in the equilibrium exchange experiment, cytochalasin B increased the K_m without altering the V_{max}. SB203580 produced the same pattern of transport inhibition. It acted as a noncompetitive inhibitor of zero-trans transport, but a competitive inhibitor of equilibrium-exchange transport (Figs. 3 and 4). The percent inhibition of transport, at tracer concentrations of substrate, by 10 μM SB203580 was similar to that previously reported (6). However, at higher concentrations of internal 3-*O*-methyl-

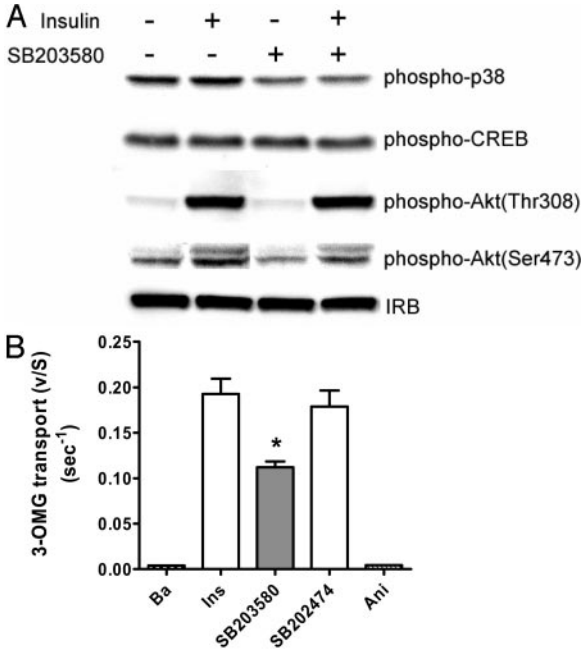

FIG. 2. Inhibition of kinase activity by SB203580. A, Rat adipose cells were stimulated with 20 nM insulin for 10 min after a 20-min pretreatment with (+) and without (−) 10 μM SB203580. Cell lysates were analyzed by Western blotting with antibodies to phospho-p38 MAPK, phospho-CREB, phospho-Akt(Thr^{308}), and phospho-Akt-(Ser^{473}). Blotting of the insulin receptor β-subunit (IRB) was used as a loading control. The blot is representative of three separate experiments. B, The active MAPK inhibitor SB203580 (10 μM), but not the inactive control SB202474 (10 μM), led to reduced transport activity. Anisomycin treatment alone (at 37 μM for 20 min) did not extensively stimulate transport. The data are the mean and SEM from three separate experiments. *, $P < 0.05$, by unpaired *t* test *vs.* uninhibited control.

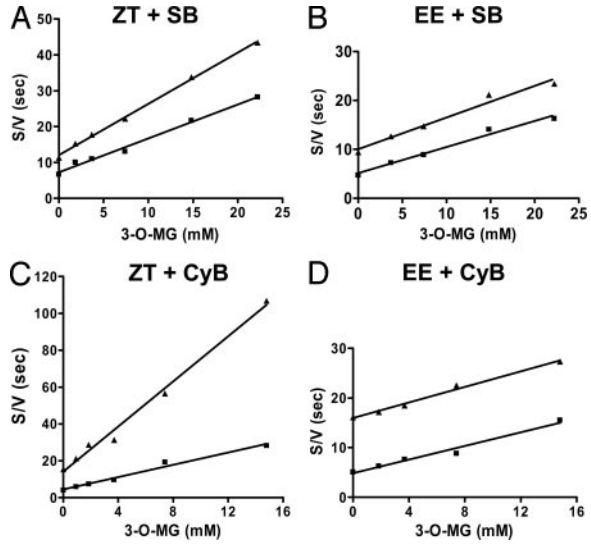

FIG. 3. Zero-trans and equilibrium-exchange inhibition of glucose transport activity. Rat adipose cells were stimulated with 20 nM insulin for 30 min, and then either zero-trans (ZT in A and C) or equilibrium exchange (EE in B and D) uptake of 3-*O*-methyl-D-glucose was determined in the presence (▲) or absence (■) of inhibitor. Uptake was determined in cells that had been pretreated for 15 min before insulin treatment with 10 μM SB203580 (A and B) or with 1 μM cytochalasin B (C and D). Hanes plots of the reciprocal of the rate constant, or S/v, *vs.* the 3-*O*-methyl-D-glucose substrate concentration were used to analyze the type of inhibitor interaction. Varying slopes and convergence on the *abscissa* in the ZT plots are indicative of noncompetitive inhibition. Equal and parallel slopes in the EE plots are indicative of competitive inhibition. Results are from single experiments representative of three or four separate experiments for each condition.

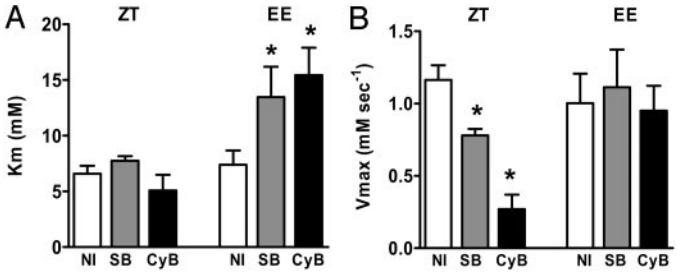

FIG. 4. Analysis of transport kinetic parameters for SB203580 and cytochalasin B inhibition of glucose transport activity. K_m (A) and V_{max} (B) values were compared for zero-trans (ZT) and equilibrium exchange (EE) in noninhibited insulin-treated cells (NI) or cells incubated with 10 μM SB203580 (SB) or with 1 μM cytochalasin B (CyB) before insulin treatment. Results are the mean ± SEM of three or four experiments for each condition and include the single representative experiments in Fig. 3. *, $P < 0.05$, by *t* test (SB paired, CyB unpaired) *vs.* uninhibited control.

D-glucose (as occurred in the equilibrium exchange experiment), the percent inhibition of transport fell markedly.

Discussion

The described studies demonstrate that rat adipose cells differ from cultured 3T3-L1 and L6 cells in the extent to which insulin can activate p38 MAPK. This kinase is not significantly activated under conditions in which insulin activates glucose transport and the key signaling molecule Akt by 10-fold or greater. In this respect, rat adipose cells are similar to human adipose cells, because studies on these cells have also failed to show insulin-stimulated activation of p38 MAPK (22). Small increases in the activation of other MAPKs, including p44/42 MAPK and SAPK/JNK MAPK, were observed in rat adipose cells. However, the observed small increases were transitory and returned to basal levels over 30 min. We also observed small reductions in the phosphorylation of Akt at serine 473 after treatment of rat adipose cells with the MAPK inhibitor SB203580. Approximately 30% reductions in Akt activity using 10 μM reagent are known to occur, both using recombinant enzyme (18) and in studying Akt activity in 3T3-L1 cells (23). It is unlikely that these levels of reduction in Akt signaling are sufficient to be relevant to inhibition of transport activity. This is because only relatively small increases in Akt activity are necessary for GLUT4 translocation (24), and the translocation of epitope-tagged GLUT4 is unimpaired by SB203580 treatment (12).

Collectively, the signaling studies suggest that an insulin activation of glucose transport activity by p38 MAPK after the recruitment of GLUT4 to the plasma membrane is unlikely. Therefore, we have tested whether the observed inhibition of transport activity by the MAPK inhibitor SB203580 could be due to a direct interaction with the transporter, rather than being mediated by inhibition of a kinase activity. We have determined that SB203580 is a competitive inhibitor of glucose transport activity and that, based on a kinetic test, the site of interaction is at the endofacial surface of the transporter. Occupation of the endofacial substrate site reduces V_{max} under zero-trans conditions, because return of the substrate site to the outside is blocked, and there is no substrate inside to displace the inhibitor. Under equilibrium-exchange conditions, internal substrate competitively displaces inhibitor, and the transport K_m is increased. An endofacial site of action is consistent with other aspects of SB203580 inhibition of transport activity. The requirement for a preincubation step is most likely due to a slow penetration of the compound across the plasma membrane bilayer for interaction with the inside of the transport system. Once inside the cell, it is probably difficult to remove the reagent because it is hydrophobic, and a portion probably remains in association with the membrane lipid surrounding the GLUT4 molecule.

Cytochalasin B is known to have a similar endofacial mode of interaction with GLUT1 (21, 25), and we have shown here that cytochalasin B is an endofacial inhibitor of GLUT4. Many hydrophobic compounds, including steroids, isoflavones, and anesthetics, also interact with GLUT1 in this way and at its endofacial surface (26). Pharmacophor studies of SB203580 and related inhibitors suggest that they occupy the ATP-binding pocket of p38 MAPK (27, 28). It has been suggested that GLUT1 has an ATP-binding pocket (29), and that occupation of this site by ATP antagonizes and reverses the inhibition of transport by the pharmacological ligands listed above (26). Endofacial domains of GLUT4 may also resemble an ATP-binding pocket, and it may be these domains that become occupied by SB203580. Interaction with these allosteric domains would have to be in competitive equilibrium with the substrate binding site, because substrate displaces the SB203580 inhibitor. Alternatively, and more simply, SB203580 may have a spacing of hydrogen donating and accepting groups that allows direct interaction at the endofacial substrate-binding site.

Although the present data provide evidence against a role for p38 MAPK in regulating the intrinsic activity of GLUT4, other recent studies suggest that translocation can be separated from transporter activation. Studies in 3T3-L1 cells have suggested that the concentration of wortmannin required for inhibition of insulin-stimulated glucose transport activation is significantly lower than that required to block translocation of GLUT4 and insulin-responsive amino peptidase to the cell surface (20, 30). These data have led to the suggestion [reviewed by Kandror (3)] that two phosphatidylinositol 3-kinase-dependent steps with differing wortmannin sensitivities comprise the overall process of GLUT4 translocation and activation. More recent studies have compared the GLUT4 translocation with the corresponding transport increase induced by introduction of inositol phospholipids together with a capping glucosamine-polymer neomycin (5) or by adding neomycin alone (31). Large discrepancies are found between these two parameters. It has therefore been concluded that phosphatidylinositol 4,5-triphosphate generation or insertion into the plasma membrane is sufficient to cause GLUT4 translocation, but is insufficient to lead to activation of these transporters (5). Similar conclusions have been reached on the basis of capping of phosphoinositides by PBP10, a phosphoinositide-binding domain from gelsolin (4). It would therefore be of interest in the future to kinetically analyze potential GLUT4 intrinsic catalytic activation and inhibition by the phosphoinositides and their capping compounds. As we have shown here, the most appropriate and revealing kinetic test would

be the equilibrium exchange experiment, because this would be sensitive to interactions at both the exofacial and endofacial surfaces of the transporter.

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