Noncompetitive blocking of human GLUT1 hexose transporter by methylxanthines reveals an exofacial regulatory binding site

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GLUT1, a protein that consists of 12-transmembrane helixes, with the NH2- and COOH-termini facing the cytosolic side of the protein and an N-linked glycosylation site on its extracellular face (20). GLUT1 is located in the plasma membrane of most animal cells, where it is considered to be responsible for the basal uptake of glucose, playing a central role in the energetics of the cell (17). In addition, since GLUT1 constitutes near 2–3% of total membrane protein in red blood cells, the kinetic properties of the glucose transport facilitated by GLUT1 have been well characterized (6). In recent years, GLUT1 has gained attention in cancer biology because glucose uptake and consumption are highly enhanced in malignant cells. Unlike normal cells, cancer cells upregulate glycolytic and pentose phosphate pathways even in the presence of oxygen, a phenomenon known as the Warburg effect. It is generally accepted that the Warburg effect not only assures an immediate production of ATP by the rapid degradation of glucose but also increases the availability of biosynthetic intermediates, which results in supporting the highly proliferative nature of cancer cells (12, 18, 32). Consistently, tumor cells overexpress GLUT1 in their plasma membranes, which results in enhanced glucose uptake (29, 51). In addition, cancer cells are very sensitive to glucose deprivation, as a limited supply of glucose not only prevents cell proliferation but also induces apoptosis (11, 12, 29, 30). Hence, restricting glucose uptake by inhibition of GLUT1 is a promising antiproliferative and proapoptotic strategy against cancer cells. However, to be therapeutically useful, it is important to identify compounds whose inhibitory concentration could be attained in serum. Therefore, detailed information regard the mechanism and kinetic consequences of compounds that impair the activity of GLUT1 is needed.

GLUT1 activity is inhibited by a number of molecules that have been used to study the kinetic properties and structure of the transporter (2, 4, 28). Interestingly, these inhibitors are not structurally related to glucose. For instance, barbiturates such as pentobarbital, thiobutabarbital, and barbital inhibit hexose transport by interacting with GLUT1 in cultured cells and in human red cells (13). Cytochalasin B, the best-characterized GLUT1 inhibitor, binds to the transporter and acts as a competitive inhibitor in zero-trans entry assays and as a noncompetitive blocker in zero-trans exit assays, suggesting its interaction with the cytosolic face of the transporter (2, 8). Indeed, a domain comprising residues 388 and 412 seems to be involved in cytochalasin B binding (16, 19). GLUT1 also contains at least three functional regulatory binding sites for natural flavonoids and a subgroup of synthetic tyrosine kinase inhibitors that inhibit glucose transport in human myeloid HL-60 cells, Chinese hamster ovary-1 cells, and human erythrocytes (33, 48, 49). Methylxanthines are a family of compounds with a vast variety of physiological effects, including inhibition of glucose transport. For example, theophylline (1,3-dimethylxanthine) relaxes smooth muscle, stimulates heart activity, activates the central nervous system, and increases diuresis. Caffeine (1,3,7-trimethylxanthine) acts at the level of skeletal muscle, increasing the contractile response. On the other hand, pentoxifylline; caffeine

THE GLUCOSE TRANSPORTERS (GLUTs) are a family of transmembrane proteins that catalyze the facilitative transport of sugars across biological membranes (3, 5, 6, 20, 31). There are 14 known isoforms of GLUTs, which differ in their kinetic parameters, substrate specificity, and level of expression in different cell types (1). The most widely studied of these transporters is GLUT1, a protein that consists of 12-transmembrane helixes, with the NH2- and COOH-termini facing the cytosolic side of the protein and an N-linked glycosylation site on its extracellular face (20). GLUT1 is located in the plasma membrane of most animal cells, where it is considered to be responsible for the basal uptake of glucose, playing a central role in the energetics of the cell (17). In addition, since GLUT1 constitutes near 2–3% of total membrane protein in red blood cells, the kinetic properties of the glucose transport facilitated by GLUT1 have been well characterized (6). In recent years, GLUT1 has gained attention in cancer biology because glucose uptake and consumption are highly enhanced in malignant cells. Unlike normal cells, cancer cells upregulate glycolytic and pentose phosphate pathways even in the presence of oxygen, a phenomenon known as the Warburg effect. It is generally accepted that the Warburg effect not only assures an immediate production of ATP by the rapid degradation of glucose but also increases the availability of biosynthetic intermediates, which results in supporting the highly proliferative nature of cancer cells (12, 18, 32). Consistently, tumor cells overexpress GLUT1 in their plasma membranes, which results in enhanced glucose uptake (29, 51). In addition, cancer cells are very sensitive to glucose deprivation, as a limited supply of glucose not only prevents cell proliferation but also induces apoptosis (11, 12, 29, 30). Hence, restricting glucose uptake by inhibition of GLUT1 is a promising antiproliferative and proapoptotic strategy against cancer cells. However, to be therapeutically useful, it is important to identify compounds whose inhibitory concentration could be attained in serum. Therefore, detailed information regard the mechanism and kinetic consequences of compounds that impair the activity of GLUT1 is needed.

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the other hand, the synthetic methylxanthines pentoxifylline [1-(5-oxohexil)-theobromine] and isobutyl-methylxanthine are prescribed for the treatment of cardiovascular disorders (50, 52). Pentoxifylline increases the erythrocyte deformability of damaged erythrocytes and reduces platelet aggregation, fibrinogen levels, adhesion to the endothelium, and activation of leukocytes and the resulting endothelium damage. It also decreases blood viscosity, thus facilitating microcirculatory perfusion through improved blood flow (37–39). Regarding the molecular targets of methylxanthines, it has been described that they are competitive inhibitors of phosphodiesterases, a family of enzymes that catalyze cAMP breakdown, resulting in increased levels of cAMP in the cell (22). However, this effect occurs at concentrations that exceed therapeutic levels. Inhibition of glucose transport has been described in human erythrocytes (7, 15) and in rat adipocytes (44, 45), and the evidence suggests that caffeine and theophylline interact directly with GLUT1 (44, 45) and that synthetic 3-isobutyl-1-methylxanthine binds to the extracellular face of the protein (7). The inhibitory mechanism of methylxanthines over GLUT1 is, however, still unknown. In the present study, we investigated the effects of natural (caffeine and theophylline) and synthetic (pentoxifylline) methylxanthines on glucose transport in human red blood cells. Our results demonstrate that these drugs inhibit glucose transport by interacting directly with GLUT1, and kinetic assays provide evidence that methylxanthines interact with GLUT1 at a specific site accessible through the exofacial surface of the protein. This regulatory binding site is independent but close to the GLUT1 external β-glucose site.

EXPERIMENTAL PROCEDURES

Materials and solutions. 3-O-methyl-glucose (OMG), caffeine, pentoxifylline, theophylline, β-glucose, phloretin, β-sorbitol, cytochalasin B, cytochalasin E, and 2-deoxy-β-glucose (2DG) were purchased from Sigma Chemical. [3H]OMG, [3H]2DG, and [4-3H(N)]cytochalasin B were purchased from American Radiolabeled Chemicals. Human erythrocytes were purified from fresh blood samples obtained from the Blood Bank Service of the Regional Hospital in Valdivia, Chile.

Cytochalasin B binding to protein-striped membranes. Experiments were done with unsealed erythrocyte ghosts from washed red blood cells (43). β-Glucose-displaceable binding of cytochalasin B to functional GLUTs was estimated from the difference between cytochalasin B bound in the presence of 500 mM β-sorbitol and 500 mM β-glucose. The amount of specifically bound cytochalasin B was estimated by the quantity of radioactive ligand associated with the membrane pellet (49). Every data point represents the mean ± SD of at least three independent determinations.

Kinetic assays. For hexose transport assays, cells were incubated at room temperature in incubation buffer (34) containing 0.1–1 mM of [3H]OMG or [3H]2DG and 0.5–50 mM of unlabeled glucose analogs. Uptake assays with 2DG and OMG were performed as previously described (34). For exit assays, cells previously washed with 1× PBS were incubated with a mixture of [3H]OMG and unlabeled OMG for at least 1 h before assay transport. For equilibrium exchange, cells were preincubated with unlabeled OMG and then as described above for the entry assay. Transport reactions were stopped at 15 s for entry assays and at 10 s for exit assays with stopping solution (cold 1× PBS containing 10 μM HgCl2). Cells were pelleted and washed twice with stopping solution, and radioactivity was then determined as previously described (34).

Net sugar efflux experiments (Sen-Widdas assays). Net sugar efflux experiments (Sen-Widdas assays) were performed as previously described (34). Briefly, we monitored through light scattering the exit rates of β-glucose out of erythrocyte human cells using a Perkin-Elmer spectrophotometer (LS-50) with temperature control. The excitation and emission wavelengths were 650 nm with 5-nm slits. Red blood cells were suspended for at least 2 h in solutions containing 100 mM β-glucose. Aliquots of prewarmed packed cells (3 μl) were added to a fluorescence cuvette containing 3 ml of saline solution at the same temperature and mixed thoroughly, and the changes in light scattering were collected starting within 5 s after mixing. Exit times were determined after nonlinear fitting of the data point of light scattering against time to the following three-parameter exponential model: y = y0 + A × [1 − exp(−k × t)], where y0 is the scattered light (y) at time 0, A is the amplitude of the variation in light scattering, k is the rate constant, and t is time. The exit time corresponds to the half-life (t1/2) of β-glucose exit, which was evaluated as follows: t1/2 = ln2/k. Nonlinear and linear regressions were done with SigmaPlot version 11 software.

RESULTS

Methylxanthines inhibit cytochalasin B binding to erythrocyte membranes. Since it has been reported that pentoxifylline has the ability to increase membrane fluidity of human erythrocytes (37–39), methylxanthines may alter the properties of membrane transporters as a result of nonspecific conformational changes arising from the alteration of the membrane instead of direct interactions with the GLUT1 carrier. On the other hand, cytochalasin B binds directly to the GLUT1 hexose transporter, and its dissociation is not dependent of membrane fluidity (21). Thus, we used cytochalasin B displacement assays to verify whether or not methylxanthines interact with the transporter. To determine how efficient the methylxanthines are in displacing cytochalasin B from its GLUT1-binding site, we measured the amount of cytochalasin B (0.1 μM) that remained bound to erythrocyte membranes as a function of increasing concentrations of methylxanthines. Approximately 1 mM pentoxifylline, 3 mM theophylline, or 3.5 mM caffeine displaced the cytochalasin B already bound to the transporter by 50%, and total displacement was observed at 30 mM for every methylxanthine (Fig. 1A). Pentoxifylline, caffeine, and theophylline competitively displaced the cytochalasin B bound to erythrocyte ghosts (Fig. 1, B–D). A secondary plot of the apparent IC50 for cytochalasin B binding as a function of concentration was linear for every methylxanthine and allowed us to derive inhibition constant (Ki) values of 0.64, 1.5, and 3.2 mM for pentoxifylline, caffeine, and theophylline, respectively (Fig. 1E). These data are consistent with the notion that methylxanthines interact directly with the GLUT1 hexose transporter protein.

Inhibition of 2DG transport by methylxanthines in human erythrocytes. GLUT1 is especially abundant in human erythrocytes, and most of the information on the structure and function of GLUT1 has been obtained using the erythrocyte transporter (5). We tested the effect of different concentrations of methylxanthines in 2DG uptake using human erythrocytes and radiolabeled substrate. The three tested methylxanthines inhibited the zero-trans uptake of 2DG in a dose-dependent manner, with 50% inhibition observed at 7.5, 17.1, and 19.8 mM pentoxifylline, theophylline, and caffeine, respectively (Fig. 2A). As a positive control, we used phloretin, a known inhibitor of GLUT1, which blocked the transport of 2DG in our system with an IC50 value of 41.4 μM (Fig. 2B), consistent with results previously described by other authors (2, 23). As expected, cytochalasin E, a cytochalasin unable to inhibit...
glucose transport (35), did not inhibit hexose uptake in red blood cells (Fig. 2B). These results argue against the possibility that the decreased transport in the presence of methylxanthines was due to an unspecific event caused by perturbation of the membrane structure. Thus, the most logical interpretation is that methylxanthines interfere with glucose transport by a direct interaction with the sugar transport protein.

Uncompetitive blocking by methylxanthines of 2DG transport in human erythrocytes. To further characterize how these methylxanthines interact with GLUT1, we decide to study the effect of different fixed concentrations of methylxanthines on the uptake of variable 2DG concentrations using zero-trans entry conditions. Figure 2, C–F, shows the results for pentoxifylline, caffeine, theophylline, and phloretin, respectively. The effects of methylxanthines were consistent with mixed inhibition, since they altered both \( K_m \) and \( V_{max} \) values for substrate transport. Surprisingly, the effect of each xanthine showed a notable uncompetitive component because the lines trended to converge at the \( x \)-axis. This feature contrasts with the clear competitive behavior exhibited by phloretin, where the lines converged to a common point on the \( y \)-axis (Fig. 2F), indicating that the \( V_{max} \) value did not significantly change by the addition of the inhibitor. The absence of a competitive component on the Eadie-Hofstee plot shows that methylxanthines do not interfere with substrate binding to the exofacial glucose site on GLUT1. However, the specific displacement of cytochalasin B bound to the transporter caused by methylxanthines indicates that these inhibitors do bind to the transporter. Therefore, we suggest that GLUT1 harbors a methylxanthine-binding site whose occupation by any of them alters glucose transport. Thus, it is plausible that methylxanthines may preferentially interact with GLUT1 when the transporter has a bound substrate, which could explain the uncompetitive character of the inhibition, as the decrease of glucose transport may be due to the formation of a stable ternary complex that locks the transporter in a nonfunctional conformation.

Characterization of OMG transport inhibition by methylxanthines. Because methylxanthines bind to the GLUT1 and in uptake experiments they behaved as mixed inhibitors of glucose transport, we reasoned that there are two possibilities explaining the result: 1) methylxanthines interact with GLUT1 at the endofacial binding site for glucose or 2) methylxanthines bind to the transporter at a site independent of either the exofacial or endofacial glucose-binding sites. In the first case, methylxanthines should act as competitive inhibitors of the transport when the endofacial site is accessible for the substrate: equilibrium exchange and zero-trans exit assays. In the second case, methylxanthines should behave as noncompetitive inhibitors of glucose transport in equilibrium exchange and zero-trans entry and exit conditions. Thus, to discriminate between the two options, we used zero-trans entry (no substrate inside), zero-trans exit (no substrate outside), and equilibrium exchange (substrate inside and outside) transport assays to analyze the effects of pentoxifylline, theophylline, and caffeine on OMG transport.

In Fig. 3, we show the inhibition pattern of methylxanthines on OMG transport under equilibrium exchange (top row), zero-trans exit (middle row), and zero-trans entry conditions (bottom row). For equilibrium exchange assays, described as an unambiguous test to differentiate between competitive and noncompetitive inhibitors (9), the apparent \( K_i \) values were 1.8, 2.9, and 4.7 mM for pentoxifylline, caffeine, and theophylline, respectively (Fig. 3A). The slopes of the lines in the Eadie-Hofstee plots (Fig. 3, B–D) represent \( K_m \) values, whereas the intercepts on the \( y \)-axis correspond to the \( V_{max} \) at that particular inhibitor concentration. Notably, these methylxanthines did not affect \( K_m \) values as inhibitor concentration varied, but \( V_{max} \) decreased as the inhibitor concentration increased, a behavior that is consistent with noncompetitive inhibitors. Secondary plots of the reciprocal of the \( V_{max} \) values as a function of methylxanthine concentration were linear (data not shown), as the \( K_i \) values extrapolated from those plots were 2.0, 5.9, and 7.3 mM for pentoxifylline, caffeine, and theophylline, respectively. Thus, these results provide convincing evidence that methylxanthines interact with GLUT1 by binding to a site that is different from the external or internal substrate-binding sites of the carrier.

As shown in Fig. 3 (middle row), the inhibition pattern was also noncompetitive in the zero-trans efflux assays with apparent \( K_i \) values of 1.2, 2.3, and 3.2 mM for pentoxifylline,
caffeine, and theophylline, respectively (Fig. 3E). Eadie-Hofstee plots of inhibition of OMG transport at different concentrations of pentoxifylline (Fig. 3F), caffeine (Fig. 3G), and theophylline (Fig. 3H) showed parallel lines, indicative of the noncompetitive character of the inhibition. Secondary plots of the reciprocal of the $V_{\text{max}}$ data as a function of methylxanthine concentration were also linear (data not shown), as the $K_i$ values extrapolated from those plots were 2.8, 4.5, and 5.3 mM for pentoxifylline, caffeine, and theophylline, respectively. These results, together with the zero-trans exit and equilibrium exchange assays, reinforce the existence of a regulatory binding site different from the internal and external glucose-binding site and whose occupancy by methylxanthines is remarkably favored by previous binding of the substrate to the external but not to the internal glucose-binding site.

Methylxanthines inhibit glucose exit from human erythrocytes. To further confirm that methylxanthines and $\alpha$-glucose do not share a common binding site on the external face of the transporter, the effect of each methylxanthine on glucose efflux was evaluated under infinite-cis conditions. In these assays, human erythrocytes were preloaded with 100 mM glucose, and changes in light scattering were recorded upon dilution of the cells in medium containing increasing concentrations of $\alpha$-glucose (41). Pentoxifylline, caffeine, and theophylline inhibited the exit of $\alpha$-glucose in human erythrocytes in a dose-depen-
dent manner in the millimolar range, with 50% inhibition observed at 1.2, 3.9, and 4.8 mM for pentoxifylline, caffeine, and theophylline, respectively (Fig. 4A). The specificity of the methylxanthine’s effect on glucose transport was confirmed in control experiments using phloretin, cytochalasin B, and cytochalasin E. Accordingly, phloretin and cytochalasin B blocked glucose exit in the micromolar range (apparent $K_i$ values of 0.5 and 1 μM, respectively), and no inhibition was seen with cytochalasin E.

Figure 4, C–E, shows Sen-Widdas plots of glucose exit in the absence or presence of methylxanthines. The intercepts on the x-axis correspond to negative values of $K_d$ for the external

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**Equilibrium-exchange assays**

**A**

- Transport (% of control)
  - Methyloxanthine (mM)

**B**

- Pentoxifylline
  - v (pmoles/min·10^6 cells)
  - v/S

**C**

- Caffeine
  - v (pmoles/min·10^6 cells)
  - v/S

**D**

- Theophylline
  - v (pmoles/min·10^6 cells)
  - v/S

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**Zero-trans efflux assays**

**E**

- Transport (% of control)
  - Methyloxanthine (mM)

**F**

- Pentoxifylline
  - v (pmoles/min·10^6 cells)
  - v/S

**G**

- Caffeine
  - v (pmoles/min·10^6 cells)
  - v/S

**H**

- Theophylline
  - v (pmoles/min·10^6 cells)
  - v/S

---

**Zero-trans efflux assays**

**I**

- Transport (% of control)
  - Methyloxanthine (mM)

**J**

- Pentoxifylline
  - v (pmoles/min·10^6 cells)
  - v/S

**K**

- Caffeine
  - v (pmoles/min·10^6 cells)
  - v/S

**L**

- Theophylline
  - v (pmoles/min·10^6 cells)
  - v/S
glucose site of GLUT1 (41). In the absence of inhibitor, the $K_d$ value of glucose for the external site varied between 2 and 7 mM, which is consistent with previously described values (2, 34). These $K_d$ values were not affected by the addition of caffeine or theophylline at levels that inhibit glucose transport (Fig. 3A). Exit rates were slower in the presence of these methylxanthines without affecting the affinity of glucose for the external glucose-binding site of GLUT1. A different result was obtained with pentoxifylline, where the intercept on the $x$-axis changed as the pentoxifylline concentration increased (Fig. 4C), indicating that pentoxifylline affects the binding of glucose to the exofacial glucose-binding site. A secondary plot of the apparent $K_d$ values for glucose as a function of pentoxifylline concentration showed that the inhibition was linear and permitted us to extrapolate a $K_i$ value of 4.4 mM for pentoxifylline (data not shown).

We used phloretin and cytochalasin B as controls to test if this assay under infinite-cis conditions is able to discriminate
the action of inhibitors on the affinity of the GLUT1 external $\beta$-glucose site. The kinetic effects of cytochalasin B and phloretin on hexose transport mediated by GLUT1 are well known. Cytochalasin B is a competitive inhibitor of glucose exit out of cells and is noncompetitive on the incorporation of sugar into the cell (2, 8), whereas phloretin is a noncompetitive inhibitor of glucose exit out of cells and competitive on the incorporation of sugar into the cell (23). These behaviors are consistent with the fact that cytochalasin B binds to a site accessible by the endofacial face of the transporter, whereas phloretin competitively binds to the glucose external site (8, 23). As expected, cytochalasin B inhibited $\beta$-glucose exit ($IC_{50} = 0.5 \mu M$) but did not affect the affinity of $\beta$-glucose for its external binding site. Phloretin also blocked sugar exit ($IC_{50} = 1 \mu M$), but, in this case, it significantly altered the affinity of $\beta$-glucose on the external glucose-binding site, where the $K_d$ value for the $\beta$-glucose external site increased from 4 to 15 mM in the presence of 5 $\mu M$ phloretin (data not shown). These results validate our approach to distinguish the face of the transporter where the inhibitor binds and further confirm that the binding sites for cytochalasin B and phloretin are located at opposite faces of GLUT1.

**Evidence for a common methylxanthine site on the transporter.** The results of our Sen-Widdas assays suggest that caffeine and theophylline bind to the transporter at a binding site different from the external $\beta$-glucose-binding site, whereas pentoxifylline may share some binding determinants with the external $\beta$-glucose-binding site. Thus, we wondered whether there is one common binding site for the three methylxanthines or there are at least two sites, one site responsible for caffeine and theophylline binding and another for pentoxifylline. To discern between these possibilities, we performed simple coinhibition assays where we measured the transport rate as a function of inhibitor concentration. We reasoned that if the presence of a second compound was able to change the apparent $IC_{50}$ value for the first inhibitor, both compounds would be competing for the same binding site. In contrast, if the second inhibitor was unable to affect the binding site of the first blocker, the apparent $IC_{50}$ value for the first compound would not be altered.

Table 1 shows the results of an inhibition test for different pairs of inhibitors for glucose exit under infinite-cis conditions, combining the methylxanthines with themselves or with external $\beta$-glucose. The results are presented as ratios of apparent $IC_{50}$ values obtained in the presence or absence of the fixed concentration of the second inhibitor. The level of the second inhibitor was chosen as its respective $IC_{50}$ value. The data revealed that the three methylxanthines compete with each other for binding to GLUT1, suggesting the presence of at least one methylxanthine-binding site on the transporter where all three methylxanthines bind. In contrast and consistent with the Sen-Widdas assays (Fig. 4C), pentoxifylline competed with $\beta$-glucose for binding to the external glucose-binding site, whereas caffeine and theophylline did not. Conversely, when we used $\beta$-glucose as the second inhibitor, it effectively affected pentoxifylline binding to the transporter but did not affect the binding of caffeine or theophylline to GLUT1 protein, which confirms the complexity of the inhibition pattern shown by pentoxifylline.

**DISCUSSION**

The main findings of the present study can be summarized as follows: 1) the methylxanthines pentoxifylline, caffeine, and theophylline behave as effective blockers of the transport of OMG, 2DG, and $\beta$-glucose, all substrates that cross cell plasma membranes through the facilitative hexose transporter GLUT1; 2) the inhibitory effect on glucose transport is due to the direct interaction of methylxanthines with GLUT1 and is not related to perturbation of another property, such as membrane fluidity; and 3) there is a common binding site for methylxanthines at the external face of GLUT1, which is different but located close to the glucose-binding site.

The direct effect of methylxanthines on GLUT1 is supported by the observation that cells did not require a preincubation period, as we (present study) and others (15) have shown that inhibition of glucose transport was observed immediately after the addition of methylxanthines to living cells in a dose-dependent manner. Indeed, the results were not affected by preincubation of the cells with methylxanthines (data not shown). Likewise, a direct interaction of the synthetic IBMX with GLUTs has been reported in adipocytes (22). Nevertheless, it is recognized that pentoxifylline has the ability to affect membrane fluidity in human erythrocytes (38), and it may be argued that the inhibition of glucose transport is due to a nonspecific phenomenon product of the alteration of membrane properties. To rule out this possibility, we tested the effect of methylxanthines on the $\beta$-glucose-displaceable binding of cytochalasin B to GLUT1. This alkaloid competes for substrate binding at the endofacial face of the transporter; therefore, this approach is used as evidence of a direct interaction of other compounds with GLUT1 protein (4). Every methylxanthine was able to displace cytochalasin B from GLUT1, indicating that the interaction of methylxanthines with the carrier is direct and not the result of a nonspecific event at the membrane level. The observation that bound cytochalasin B was almost completely displaced at high methylxanthine levels together with

**Table 1. Coinhibition analysis of the interaction of methylxanthines with glucose transporter 1**

<table>
<thead>
<tr>
<th>Fixed Inhibitor</th>
<th>Pentoxifylline</th>
<th>Caffeine</th>
<th>Theophylline</th>
<th>$\beta$-Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline (1 mM)</td>
<td>2.1 ± 0.1 (3)</td>
<td>2.1 ± 0.1 (4)</td>
<td>2.0 ± 0.2 (6)</td>
<td></td>
</tr>
<tr>
<td>Caffeine (5 mM)</td>
<td>2.0 ± 0.5 (3)</td>
<td>2.0 ± 0.1 (3)</td>
<td>1.1 ± 0.1 (5)</td>
<td></td>
</tr>
<tr>
<td>Theophylline (5 mM)</td>
<td>1.9 ± 0.2 (3)</td>
<td>2.1 ± 0.1 (3)</td>
<td>1.1 ± 0.2 (5)</td>
<td></td>
</tr>
<tr>
<td>$\beta$-Glucose (5 mM)</td>
<td>2.1 ± 0.1 (5)</td>
<td>1.0 ± 0.1 (4)</td>
<td>1.3 ± 0.3 (4)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of R values; numbers in parentheses correspond to numbers of independent determinations. The effect of increasing concentrations of a lead inhibitor was measured in the absence or presence of a fixed concentration of a second inhibitor. The concentration of inhibitor causing 50% inhibition ($IC_{50}$) in exit assays of $\beta$-glucose under infinite-cis conditions was determined by nonlinear fitting. The parameter $R$ is defined as the ratio between the $IC_{50}$ for the first inhibitor measured in the presence of a fixed concentration of the second inhibitor over the $IC_{50}$ value estimated in the absence of the other effector.
the linear dependence of the secondary Scatchard plots suggests that the displacement was competitive in nature. Competitive blocking of binding may imply that the xanthenes compete with cytochalasin B for the same protein site. Although there are obvious chemical and structural differences between the methylxanthines and cytochalasin B, the literature reports that the endofacial cytochalasin B site (2, 8) can promiscuously interact with a variety of different chemical entities, including steroids and benzoic acid derivatives (10, 26, 27). Alternatively, xanthenes may actually bind to an independent site whose occupancy renders the carrier refractory to attach cytochalasin B, an option supported by our kinetic data indicating that GLUT1 harbors a xanthen-binding site accessible from its external surface. This would be similar to the displacement of bound cytochalasin B by phloretin or genistein, molecules that are known to interact with the external face of the carrier and behave as negative allosteric ligands of cytochalasin B binding (2, 23, 24, 33).

Our kinetic experiments revealed that pentoxifylline, caffeine, and theophylline are effective inhibitors of hexose transport at millimolar concentrations, affecting the transport of substrate in zero-trans entry and exit assays and under equilibrium exchange conditions. Pentoxifylline was the more potent inhibitor compared with caffeine and theophylline. The zero-trans entry IC50 values determined for each methylxanthine were approximately two times higher when we use 2DG instead of OMG as the substrate. These values correspond to apparent \(K_v\) values measured at fixed substrate concentrations, which are dependent on the level of saturation of the transporter when the inhibitors follow a noncompetitive or an uncompetitive mechanism (40). Thus, under our experimental conditions (2 mM OMG and 0.5 mM 2DG as the substrate), the transporter is expected to be more saturated when OMG is used (\(K_m = 8.5\) mM) than when 2DG is the substrate (\(K_m = 6.5\) mM). The net result is that methylxanthines (noncompetitive/uncompetitive inhibitors) will exhibit an apparent higher affinity for the transporter when OMG is the substrate.

The kinetic treatment of reversible inhibition of transport systems was described by Deves and Krupka (9). They provided simple kinetic tests to determine the mechanism of inhibition of transport, and we performed some of those tests to discriminate whether methylxanthines interact with the substrate-binding site, the cytochalasin B-binding site, or an unrelated site in GLUT1 protein. Under equilibrium exchange and zero-trans efflux conditions, the kinetic behavior of the inhibition of OMG transport by the three methylxanthines was noncompetitive: \(V_{\text{max}}\) values decreased and \(K_m\) values were not altered as inhibitor levels increased. Under zero-trans influx assays, using OMG or 2DG as substrates, the pattern of inhibition was mixed, although a notable uncompetitive component was observed. In other words, \(V_{\text{max}}\) and \(K_m\) values decreased in a manner such that regression lines tended to intersect on a common point on the x-axis in the Eadie-Hofstee plots. Thus, the mixed inhibition pattern provided convincing evidence that methylxanthines block GLUT1 by binding to a specific noncatalytic site on the protein surface that differs from the exofacial or endofacial \(\alpha\)-glucose-binding sites, impairing the transporter to change conformations after substrate (glucose) binding. Our data also indicated that substrate affinity for GLUT1 was reduced only when the transporter was oriented toward the extracellular face, suggesting that the binding site for methylxanthines is located at the extracellular face of the transporter. The fact that \(K_v\) values obtained for each methylxanthine were similar in the different experimental settings is also consistent with a common mechanism and binding site. Pentoxifylline appeared to be more potent than caffeine and theophylline as an inhibitor under every tested condition.

Our kinetic analysis provides evidence that the methylxanthine site lies on the external surface of the transporter, so we may suppose that the methylxanthines bind to the outward-facing conformation of the carrier. Compared with the IC50 values under equilibrium exchange experiments, the apparent affinities of methylxanthines for GLUT1 seem to be slightly higher under zero-trans exit assays and lower in zero-trans entry assays. These differences in the apparent affinities for OMG transport could be reconciled with the expected changes in the steady-state distribution of the inward and outward transporter conformations under the different experimental settings. Assuming the classical carrier model, GLUT1 catalyzes glucose transport by a kinetic scheme comprising at least four steps: 1) rapid binding of glucose to an external sugar site on the transporter, 2) translocation of the sugar-transporter complex, 3) release of sugar from the internal (endofacial) site, and, finally, 4) a relaxation step of free carrier that regenerates the substrate exofacial binding site. In human erythrocytes, it is known that the relaxation corresponds to the rate-limiting step; thus zero-trans exit assays should displace the equilibrium toward the outward-facing conformation and favor methylxanthine binding. The converse should occur for zero-trans entry assays. This equilibrium displacement could explain the slight decrease under exit conditions and the increase under entry conditions for the IC50 values for each methylxanthine compared with the IC50 values under exchange conditions.

Several previous reports have indicated that the functional activity of GLUT is affected by methylxanthines, although the mechanism was not clearly determined. The synthetic IBMX behaves as a mixed inhibitor of net glucose efflux out of red blood cells and ghosts and also of net influx into the cells. The major effect of this methylxanthine in exit assays was a decrease in the \(V_{\text{max}}\) for net efflux and a small increase in \(K_m\). However, in entry assays, a significant reduction of substrate affinity was observed. Thus, the evidence suggests that IBMX binds the GLUT1 carrier from the external solution only (7). On the other hand, Ho et al. (15) showed that caffeine affects glucose uptake (zero-trans entry conditions) in normal human erythrocytes through significant reductions of both \(K_v\) and \(V_{\text{max}}\) values. It is likely that when glucose influx is measured IBMX and caffeine induce different GLUT1 conformational changes and, thus, result in different effects over glucose affinities. Unfortunately, we were unable to characterize the inhibition pattern of IBMX in our experimental system because human red blood cells undergo significant hemolysis at the concentrations of methylxanthine required to inhibit transport. Lower levels of IBMX are required to affect glucose transport activity in adipocytes (14, 22); however, the major GLUT isosform in these cells, in both basal and insulin-stimulated states, is GLUT4, which accounts for >90% of all GLUTs (42, 46). Nevertheless, isolated vesicles from rat adipocyte plasma membranes, Kashiwagi et al. (22) showed that 1 mM IBMX suppressed glucose transport by both decreasing \(V_{\text{max}}\) and increasing \(K_m\). Likewise, Steinfelder and Petho-Schramm (45) showed that the presence of caffeine and theophylline produced an immediate
inhibition of glucose transport in insulin-stimulated rat adipocytes and that this blocking effect could be dissociated from known adenosine receptor antagonist properties mediated by these reagents. These studies disclosed that methylxanthines show no isoform selectivity, as the previously published works suggest that they affect the activity of GLUT1 and GLUT4 with similar affinities. Nevertheless, the kinetic signature of methylxanthines is a decrease in $V_{\text{max}}$ on glucose transport, which is consistent with our observations on the effect of methylxanthines in GLUT1 in our experimental system.

Sen and Widdas developed an assay to determine the affinity of sugars for the external binding site of the transporter based on measuring changes in exit rates of the substrate when the same substrate is varied on the extracellular compartment. The infinite-cis assay data are usually represented in a Sen-Widdas plot, and the apparent half-saturation constant, a measure of affinity, for external glucose may be derived from the intercept on the x-axis on such a graph (41). Our results revealed that caffeine and theophylline do not alter the affinity for the transporter external $\alpha$-glucose-binding site, supporting a non-competitive mechanism for methylxanthine inhibition. In contrast, pentoxifylline increased the apparent $\alpha$-glucose half-saturation constant of the external binding site under infinite-cis conditions. The latter might be indicative of an independent binding site for pentoxifylline; however, our kinetic data support the existence of a common binding site for the three methylxanthines on the external face of the carrier. In consequence, the competition with external glucose could arise from the side chain of pentoxifylline [1-(5-oxohexil)], which is absent in caffeine and theophylline.

The structural bases for the binding of methylxanthines to a common site are difficult to determine in the absence of a crystallographic three-dimensional model of any facilitative glucose transporter. Salas-Burgos et al. (36) proposed a theoretical three-dimensional model of human GLUT1 based on comparative homology (1SKU). The structure exhibits a watery passageway that corresponds to the substrate translocation channel, which is defined by residues that are known to be crucial for transport and putative sugar recognition domains at both ends of the channel. Molecular docking was performed on this model to identify putative binding sites for the substrate glucose and several recognized GLUT1 inhibitors: phloretin, forskolin, and cytochalasin B. Glucose, forskolin, and phloretin docked at sites located in close mutual proximity on the exofacial vestibule of the transporter, whereas forskolin, phloretin, and cytochalasin B docked on the endofacial surface of the model (36). Interestingly, molecular dynamic simulations (400 ps) of this model revealed slight changes in the volume and accessibility of two adjacent cavities on the exofacial surface of the carrier (36). Therefore, site-directed mutagenesis experiments on the transporter may reveal whether or not these cavities exist and if they act as binding sites for methylxanthines.

It is recognized that GLUT1 is responsible for basal $\alpha$-glucose uptake in most mammal cells, and, consequently, its expression and functional activity govern the energy availability for $\alpha$-glucose-dependent organs such as the brain (33). GLUT1 is also overexpressed in most cancer cells as a result of the metabolic switch known as the Warburg effect (12). Hence, blockade of glucose uptake arises as a promising antiangiogenic and proapoptotic strategy against some cancer cells. The mechanism of action of several compounds described as GLUT1 blockers has been analyzed in detail (2, 8, 33, 34). Unfortunately, most of those compounds are competitive inhibitors of glucose transport, (25, 34, 47, 48), and, therefore, their therapeutic advantage is unclear because glucose may accumulate and overcome the inhibitory effects. In that scenario, the noncompetitive nature of glucose transport by methylxanthines provides a potential alternative for cancer therapy where substrate accumulation, in principle, does not limit the effectiveness of the treatment. Further studies will be required to fully understand the molecular mechanism of action of methylxanthines on glucose transport as well as explore their potential usefulness in therapeutics.


