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Hexose Transporter GLUT1 Harbors Several Distinct Regulatory Binding Sites for Flavones and Tyrphostins

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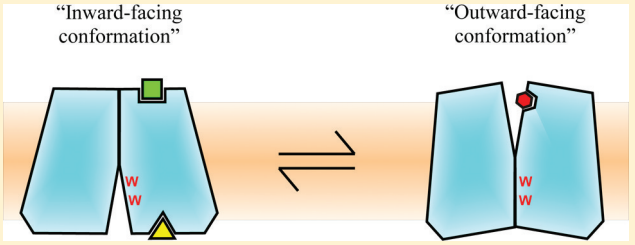
ABSTRACT: The facilitative hexose transporter GLUT1 activity is blocked by tyrosine kinase inhibitors that include natural products such as flavones and isoflavones and synthetic compounds such as tyrphostins, molecules that are structurally unrelated to the transported substrates [Vera, et al. (2001) *Biochemistry*, *40*, 777−790]. Here we analyzed the interaction of GLUT1 with quercetin (a flavone), genistein (an isoflavone), and tyrphostin A47 and B46 to evaluate if they share one common or have several binding sites on the protein. Kinetic assays showed that genistein, quercetin, and tyrphostin B46 behave as competitive inhibitors of equilibrium exchange and zero-trans uptake transport and noncompetitive inhibitors of net sugar exit out of human red cells, suggesting that they interact with the external surface of the GLUT1 molecule. In contrast, tyrphostin A47 was a competitive inhibitor of equilibrium exchange and zero-trans exit transport and a noncompetitive inhibitor of net sugar entry into red cells, suggesting that it interacts with the cytoplasmic surface of the transporter. Genistein protected GLUT1 against iodide-elicited fluorescence quenching and also decreased the affinity of D-glucose for its external binding site, while quercetin and tyrphostins B46 and A47 promoted fluorescence quenching and did not affect the external D-glucose binding site. These findings are explained by a carrier that presents at least three binding sites for tyrosine kinase inhibitors, in which (i) genistein interacts with the transporter in a conformation that binds glucose on the external surface (outward-facing conformation), in a site which overlaps with the external binding site for D-glucose, (ii) quercetin and tyrphostin B46 interact with the GLUT1 conformation which binds glucose by the internal side of the membrane (inward-facing conformation), but to a site accessible from the external surface of the protein, and (iii) the binding site for tyrphostin A47 is accessible from the inner surface of GLUT1 by binding to the inward-facing conformation of the transporter. These data provide groundwork for a molecular understanding of how the tyrosine kinase inhibitors directly affect glucose transport in animal cells.



Uptake of glucose in mammalian cells is facilitated by a family of membrane transporters known as GLUTs. In humans, 14 forms of GLUTs have been identified and cloned, most of them in the past years as a result of the human genome project. The Glut protein family belongs to the major facilitator superfamily (MFS) of membrane transporters,[1] and the Glut family members can be grouped into three different classes based on their sequence similarities.[2] GLUT transporters share common structural characteristics such as 12-transmembrane helices, an N-linked glycosilation site, intracellular NH_2 and COOH termini, and several conserved residues and motifs.[3] These GLUT proteins differ on their substrate specificity, kinetics of transport, tissue distribution, and cellular localization. GLUT1 is the most studied GLUT transporter from a structural and kinetic point of view,[4] and its properties have been studied in great detail using human erythrocytes (where GLUT1 corresponds to 2−3% of membrane protein) or by expression in *Xenopus* oocytes.

GLUT1 activity is inhibited by molecules such as cytochalasin B, phloretin, and the diterpene forskolin which are structurally unrelated with glucose, and these blockers have been used to elucidate the structure and functional properties of this transporter.[5−7] It has been also found that barbiturates such as pentobarbital, thiobutabarbital, and barbital inhibit hexose transport interacting with GLUT1 in cultured cells and in human red cells.[8] The most used and characterized inhibitor of GLUT1 is the fungal alkaloid cytochalasin B. This compound binds to the internal face of the transporter and acts as a competitive inhibitor in zero-trans exit assays and as a noncompetitive blocker in zero-trans entry assays.[7,9] Photolabeling of tryptophanyl residues 388 and 412 with cytochalasin B reduces cytochalasin B binding, and replacement of both residues with leucine inhibits the photolabeling of the

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protein.[10,11] These data suggest that these tryptophan residues are involved in GLUT1 cytochalasin B binding.

Several tyrosine kinase inhibitors have been also used as tools for detection of sites with functional characteristic of nucleotide binding sites in GLUT1.[12,13] These inhibitors correspond to natural flavonoids and a subgroup of synthetic tyrosine kinase inhibitors that potently inhibit glucose transporters function in human myeloid HL-60 cells, Chinese hamster ovary (CHO)1 cells overexpressing the glucose transporter GLUT1, and human erythrocytes. The characteristics and the specificity of the inhibition and the results of studies showing that the tyrosine kinase inhibitors affect the glucose-displaceable binding of cytochalasin B to GLUT1 in human erythrocytes indicate that the inhibitory effect on transport is related to the direct interaction of the inhibitors with GLUT1. Only those tyrosine kinase inhibitors that compete with ATP binding to the tyrosine kinases inhibited transport through GLUT1 in a competitive manner.[12,13] Accordingly, three short sequence segments that are highly homologous to consensus nucleotide-binding sequences present in protein segments that are discontinuous in the primary sequence and form the ATP binding sites in several ATPases and other nucleotide-binding proteins have been identified on GLUT1,[14,15] indicating that these domains may be important for the interaction of the tyrosine kinase inhibitors with the transporter.

The interaction of GLUT1 with compounds functionally defined by their specific interaction with protein tyrosine kinases poses a number of questions regarding both the identity of the amino acid residues forming part of the binding site(s) and the functional characteristics of these sites as they relate to the overall regulation of glucose transporter function. Our data points to the existence of three (motifs I, II, and III) discrete structural motifs in GLUT1 with the functional properties expected for nucleotide binding sites, motifs that may be responsible for the effect of the tyrosine kinase inhibitors on transport. GLUT1 is, however, a bidirectional transporter of the facilitative type, and therefore the net flux of hexoses depends on the substrate chemical gradient across the plasma membrane and does not require energy from ATP hydrolysis. On the other hand, evidence has been gathered indicating that ATP actually binds to GLUT1 protein and regulates glucose entry into cells under several conditions.[16−21]

An interesting possibility is that flavones and tyrphostins inhibit GLUT1 by binding directly to nucleotide sites on the transporter.[13] It is not clear if some of these domains functionally correspond to an allosteric site implied in the modulation of the activity of GLUT1. However, a recent study using photoaffinity labeling and peptide mapping identified a 10 kDa peptide of GLUT1 which was labeled with azide-ATP and that contains the putative nucleotide-site motif III.[20]

GLUT1 shows an intrinsic fluorescence that is quenched by binding of substrates and inhibitors, and this property has been employed to characterize ligand binding to the transporter.[16,22] This type of analysis has revealed the occurrence of important conformational changes associated with the substrate transport cycle in GLUT1. Similarly, ligand-induced quenching of intrinsic fluorescence has been used to probe the binding of flavonoids to the ATP-binding site of P-glycoprotein.[23−26] It is reasonably then to propose that the interaction of flavones and tyrphostins that competitively blocks GLUT1 activity could change GLUT1 intrinsic fluorescence. In this work, we analyzed the quenching of fluorescence induced by these compounds to test the mechanism by which they bind to the transporter. We also examined if the flavones and tyrphostins are able to perturb the exofacial glucose binding site on the transporter using infinite-cis exit transport assays (Sen−Widdas assays).

EXPERIMENTAL PROCEDURES

Materials. D-Glucose, sodium bisulfite, sodium phosphate dibasic anhydrous, sodium chloride, potassium chloride, magnesium chloride, potassium phosphate monobasic, EDTA, HEPES, dimethyl sulfoxide, dithiothreitiol, and TRIS-base were obtained from JT Baker. Cytochalasin B, genistein, quercetin, tyrphostin A47, tyrphostin B46, phloretin, ATP, and octylglucoside were obtained from Sigma Chemical Co., St. Louis, MO. Potassium iodide was purchased from Mallinckrodt. DEAE-cellulose was from Whatman.

Erythrocyte Isolation and Preparation of Membranes. Overall procedures were based on methods previously described.[27] Blood was obtained from units (containing dextrose, adenine, and sodium citrate as anticoagulant) provided by the Blood Bank Unit of the Valdivia Regional Hospital. Erythrocytes were suspended in HEPES saline (150 mM HEPES pH 7.3, 135 mM sodium chloride, 50 mM potassium chloride, 18 mM calcium chloride, 8 mM magnesium chloride) and washed by centrifugation in a clinical centrifuge, and the cell pellet was resuspended in HEPES saline. Before resuspension, the white layer on top of the erythrocytes, containing neutrophils, macrophages, and lymphocytes, was removed by aspiration with a pipet. This process of centrifugation−resuspension was repeated at least three times to obtain a clear supernatant. The washed red cells were maintained in HEPES saline until used. After the final wash, the erythrocytes were lysed by hypotonic stress by adding 5 vol of 5 mM sodium phosphate, pH 7.4, and the mix was maintained on ice for 10 min. The membranes were collected by centrifugation at 10 000 rpm for 25 min at 4 °C. The supernatant was discarded, and the pellet was suspended in 10 mM sodium phosphate, pH 7.4. This centrifugation and suspension process was repeated three times to obtain erythrocyte membranes completely devoid of cytoplasm. These membranes were suspended in 10 mM sodium phosphate, pH 7.4, and subsequently they were treated with a basic solution (25 mL of 10 mM NaOH) for 45 s, washed with phosphate saline, pH 7.4, and finally suspended in 5 mM phosphate buffer for fluorescence perturbation experiments or with 50 mM TRIS-HCl, pH 7.4, for subsequent purification of the transporter.

Purification and Reconstitution of GLUT1 on Proteoliposomes. To a suspension of peripheral protein-free membranes (~2.1 mg of protein/mL), dissolved in 50 mM TRIS-HCl pH 7.4, was added a solution consisting in 46 mM octylglucoside and 2 mM ditiotreitiol. After 20 min of gentle stirring at 4 °C the sample was dispensed in a polycarbonate tube and centrifuged for 20 min in a RC-5 Sorvall centrifuge, SS-34 rotor, at 10 000 rpm without brake. The supernatant was carefully removed with a pipet, avoiding contamination with the sediment, and solid NaCl was added for a final concentration of 25 mM. This extract was applied to a DEAE-cellulose column (Whatman DE-52) which was previously equilibrated with 10 vol of 50 mM TRIS-HCl, pH 7.4, washed with 1 vol of equilibrium buffer, and eluted with 34 mM octylglucoside in 50 mM TRIS-HCl, pH 7.4, 25 mM NaCl, 2 mM dithiothreitol, and then continued elution of the column with the same solution. Fractions of 1 mL were collected, including an aliquot

from the initially applied sample, and proteins were detected by measuring absorbance at 280 mM; eluted fractions showing an absorbance higher than 0.2 were combined. After addition of 25 μL of 4 M NaCl per mL (100 mM final) and 5 μL of 200 mM EDTA per mL (1 mM final), the pooled sample was dialyzed against 1 L of 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA. The dialyzed sample was stored for no longer than 5 days between 0 and 4 °C until use.

Fluorescence Determination. The procedures for studies of intrinsic fluorescence quenching of GLUT1 are based on those described by Pawagi and Deber,[28] with minor modifications. For these studies, samples of membranes or purified and reconstituted carrier with or without addition of inhibitor were tittered directly on a 3 mL cuvette with a freshly prepared 5 M KI stock solution containing 0.1 mM $Na_2S_2O_3$, controlling that the total volume increase did not exceed 10%. The determinations were performed on a Perkin-Elmer LS-50 spectrofluorimeter at 25 °C. The sample was excited at 295 nm to minimize interference by fluorescence of tyrosine residues, with excitation and emission slits of 8.0 and 2.5 nm, respectively. When using erythrocyte membranes, samples were suspended in 5 mM Na_2HPO_4, 0.1 M NaCl, 0.1 mM EDTA, while determinations done with the purified transporter were performed in 50 mM Na_2HPO_4 pH 7.4, 0.1 M NaCl, 1 mM EDTA. For addition of tyrosine kinase inhibitors, stocks solutions were freshly prepared at concentrations such as that when added directly to the cuvettes containing the membranes; the final dimethyl sulfoxide concentration never exceeds 0.2%. Each emission spectrum collected was corrected by the dilution factor of the protein and the inhibitor inner filter effect.

D-Glucose Exit Transport Assays. This method is based on that described by Sen and Widdas.[29] Previously washed erythrocytes were incubated at 30 °C for at least 1 h in 100 mM D-glucose. The exit rate of D-glucose was recorded using a Perkin-Elmer LS-50 spectrofluorimeter with temperature controlled at 30 °C and equipped with a magnetic stirrer. The excitation and emission wavelengths were both 650 nm, with 5 nm slits in both cases. The assay was started by adding 5 μL of cells loaded with D-glucose ($(50-60) \times 10^{-6}$ cells/mL), over 2.5 mL of HEPES saline pH 7.4 with or without inhibitor, in a 3 mL quartz cuvette. Concentrated stock solutions of genistein, quercetin, tyrphostin B46, and tyrphostin A47 in dimethyl sulfoxide were diluted in saline HEPES, pH 7.4, at concentrations calculated to maintain final concentration of dimethyl sulfoxide below 0.5%.

Zero-Trans and Exchange Assays. Uptake assays were performed as previously described.[30,31] Briefly, cells were incubated at room temperature in incubation buffer containing 2-[1,2-^{3}H(N)]-deoxy-D-glucose (specific activity 36.2 Ci/mmol, American Radiolabeled Chemicals Inc.) or 3-*O*-[methyl-^{3}H]-methyl-D-glucose (specific activity 86.7 Ci/mmol, American Radiolabeled Chemicals Inc.) and adequate concentrations of the respective unlabeled compounds for the times indicated in the figures. For exchange assays, cells were equilibrated with the desired concentrations of cold substrate at least 30 min before performs the transport assays.

RESULTS

Perturbation of Intrinsic Fluorescence in Human Erythrocyte Ghosts. GLUT1 fluoresces because of the presence of six tryptophanyl residues on its primary structure. Figure 1A shows that the fluorescence of human erythrocyte ghosts exhibit a maximum at 338 ± 2 nm when they are excited

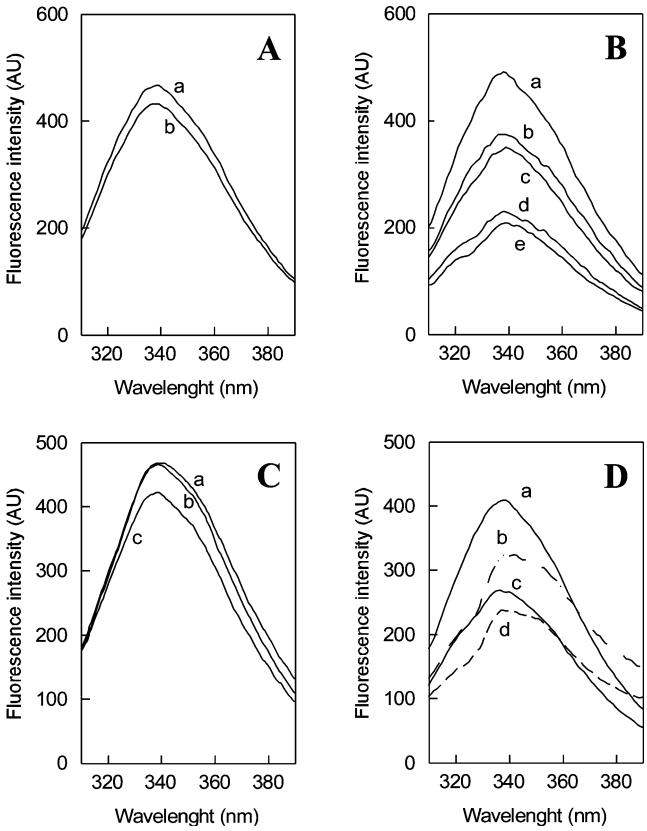

Figure 1. Fluorescence emission spectra of human erythrocyte membranes. The spectra correspond to (A) (a) control, no additions (b) 180 mM D-glucose; (B) (a) control, no additions, (b) 10 μM genistein, (c) 15 μM tyrphostin A47, (d) 15 μM tyrphostin B46, and (e) 10 μM quercetin. (C) Effect of hexoses on the fluorescence emission spectra of purified and reconstituted GLUT1. The spectra correspond to (a) control, no additions, (b) 180 mM L-glucose, and (c) 180 mM D-glucose. (D) Effect of tyrosine kinase inhibitors on the fluorescence emission spectra of purified and reconstituted GLUT1. The spectra correspond to (a) control with no additions, (b) 10 μM tyrphostin A47, (c) 10 μM quercetin, and (d) 10 μM genistein.

at 295 nm. Since protein tryptophan fluorescence is sensitive to the polarity of the local environment, showing a maximum at 350 and 329 nm in water or dioxin, respectively, GLUT1 overall fluorescence emission is probably a mixed function of the fluorescence of tryptophan residues located in a polar environment as well as nonpolar regions, probably localized within the hydrophobic portions of the membrane lipid bilayer. D-Glucose produced a decrease of an 8% in the fluorescence intensity at 338 nm, with no change in the maximum of the spectra. The tyrosine kinase inhibitors caused a greater decrease in fluorescence intensity (Figure 1B); the intensities at 338 nm were 24, 30, 53, and 57% lower after the addition of genistein, tyrphostin A47, tyrphostin B46, and quercetin, respectively.

Perturbation of Fluorescence of GLUT1 Reconstituted in Proteoliposomes. The previous results were obtained using isolated alkaline-treated membranes from human erythrocytes, which still contains a complex repertoire of the red cell integral proteins. It could be argued that a sizable fraction of the fluorescence change elicited by the tyrosine kinase inhibitors could be due to binding to integral proteins instead of GLUT1. To verify the interaction of these compounds with GLUT1, we repeated these experiments using preparations of GLUT1 solubilized from red cell

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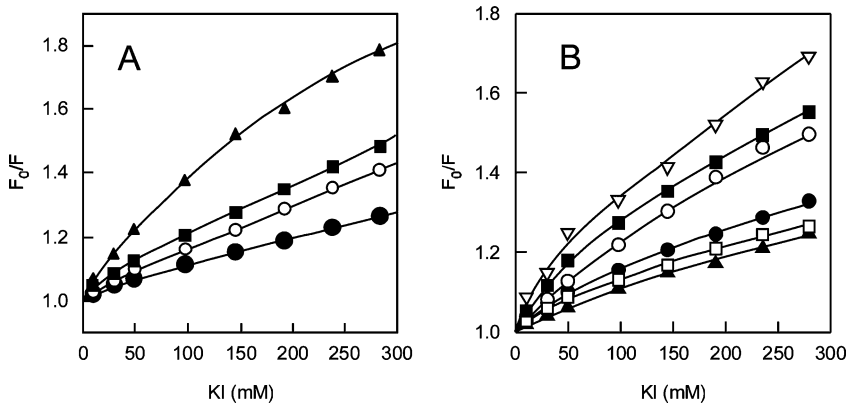

Figure 2. Quenching of GLUT1 fluorescence by iodide. (A) Effect of ATP and glucose on the quenching of GLUT1 in erythrocyte membranes. The symbols correspond to (○) control with no additions, (●) 180 mM D-glucose, (■) 2 mM ATP, and (▲) 4 mM ATP. The lines represent the fit of the data to the Stern–Volmer equation. (B) Stern–Volmer plot of the fluorescence quenching of purified and reconstituted GLUT1. The symbols correspond to (●) control with no additions or in the presence of (▲) 180 mM D-glucose, (□) 6 μM genistein, (○) 4 mM ATP, (■) 10 μM tyrphostin A47, and (▽) 30 μM quercetin.

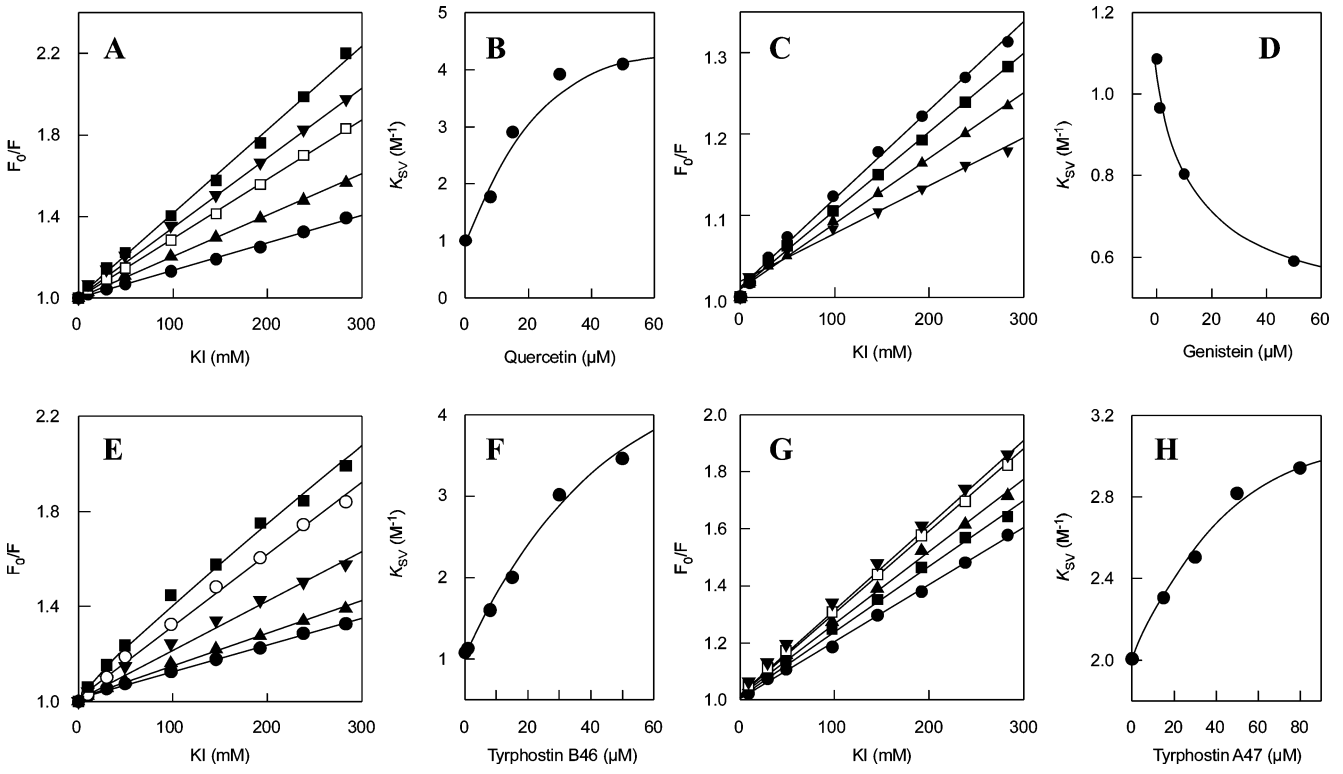

Figure 3. Effect of tyrosine kinase inhibitors on the quenching of intrinsic fluorescence of GLUT1 by iodide. (A) Stern–Volmer graph for the effect of quercetin on the quenching caused by iodide. The symbols correspond to (●) 0, (▲) 8, (□) 15, (▼) 30, and (■) 50 μM quercetin. (B) Analysis of slopes of Stern–Volmer plot based on the concentrations of the flavones. (C) Stern–Volmer graph for the effect of genistein on the quenching by iodide. The symbols correspond to (●) 0, (■) 1, (▲) 10, and (▼) 50 μM genistein. (D) Analysis of slopes of Stern–Volmer plot for genistein data. (E) Stern–Volmer graph for the effect of tyrphostin B46 on the quenching produced by iodide. The symbols correspond to (●) 0, (▲) 8, (▼) 15, (○) 30, and (■) 50 μM tyrphostin B46. (F) Analysis of slopes of Stern–Volmer plot for tyrphostin B46 data. (G) Stern–Volmer graph for the effect of tyrphostin A47 on the quenching produced by iodide. The symbols correspond to (●) 0, (■) 15, (▲) 30, (□) 50, and (▼) 80 μM tyrphostin A47. (H) Analysis of slopes of Stern–Volmer plot for tyrphostin A47 data.

membranes with octylglucoside, purified by exchange-chromatography and reconstituted in proteoliposomes. These preparations were highly enriched, since close to 80% of total protein corresponds to functional GLUT1 (data not shown). After excitation at 295 nm, the proteoliposome-reconstituted GLUT1 exhibited a fluorescence peak at 338 ± 2 nm. The addition of D-glucose provoked a small decrease in fluorescence intensity (Figure 1C) that amounted to a 5% decrease at 340 nm. L-Glucose, a hexose that does not bind to GLUT1, had no effect on the fluorescence spectra. These results agree with those described by Pawagi and Deber.[28] To test the binding of the tyrosine kinase inhibitors to GLUT1, we selected one representative molecule of four different families of tyrosine kinase inhibitors: quercetin, a classical tyrosine kinase inhibitor, was selected as representative of flavones; genistein was selected as an isoflavone; tyrphostin B46 was from the group

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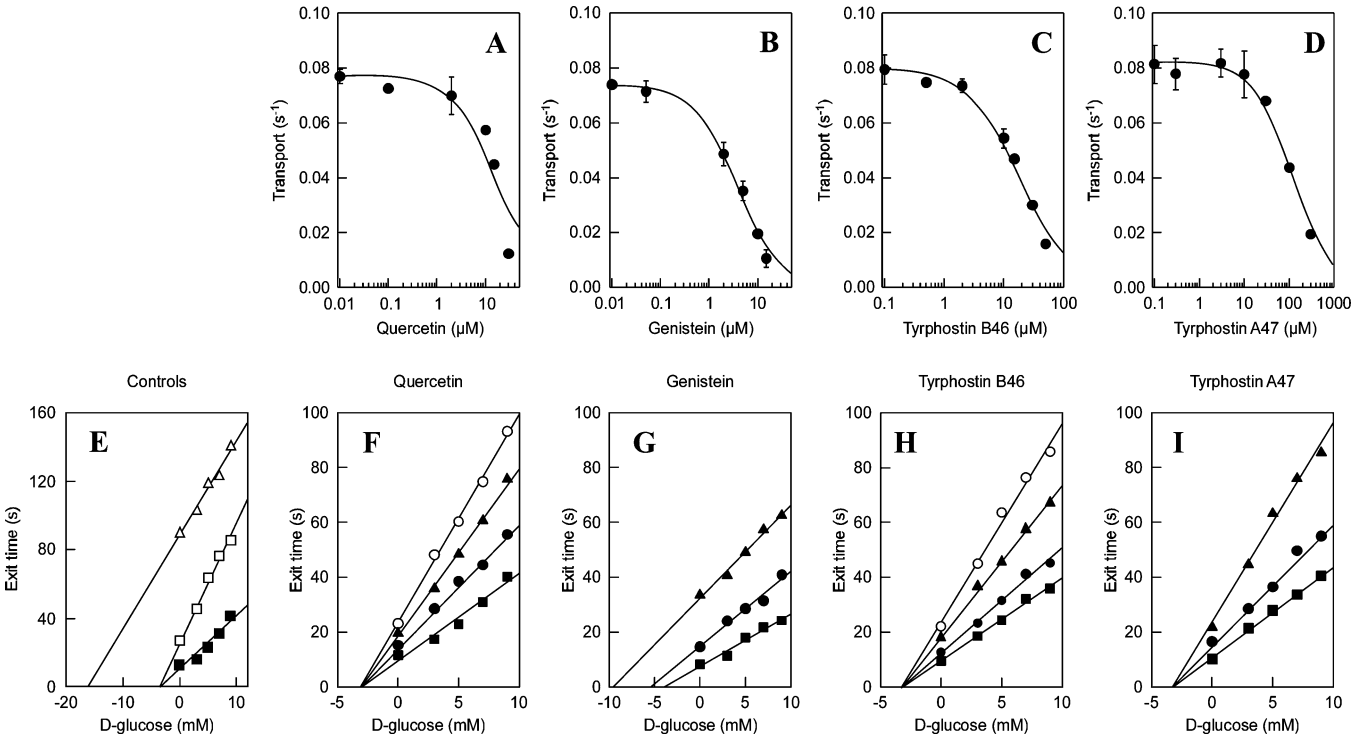

Figure 4. Inhibition of D-glucose efflux in human erythrocytes. The assays were done by measuring light scattering as described in Experimental Procedures. Panels (A–D), Inhibition by tyrosine kinase inhibitors of glucose exit. (A), Quercetin; (B), Genistein; (C), Tyrphostin B46; (D), Tyrphostins A47. The apparent inhibition constants calculated were 4, 16.5, 20 and 115 μM for genistein, quercetin, tyrphostin B46 and tyrphostin A47, respectively. Panels (E–I), Effect of tyrosine kinase inhibitors on the affinity of D-glucose by the hexose external site. (E), Experiments controls: (●) control without inhibitors, (■) cytochalasin B 0.5 μM, (▲) 5 μM phloretin. (F) Effect of quercetin. The symbols correspond to experiments performed in the presence of (■) 0, (●) 10, and (▲) 20 mM quercetin. (G) Effect of genistein. The symbols correspond to experiments carried out in presence of (■) 0, (●) 4, and (○) 10 μM genistein. (H) Effect of tyrphostin B46. The symbols correspond to experiments in the presence of (■) 0, (●) 30, and (▲) 50 μM tyrphostin B46. (I) Effect of tyrphostin A47. The symbols correspond to experiments in the presence of (■) 0, (●) 100, and (▲) 150 μM tyrphostin A47.

of synthetic tyrosine kinase inhibitor with specificity by ATP binding sites;[32,33] and tyrphostin A47 is a tyrosine kinase blocker with specificity for the tyrosine binding site on tyrosine kinases.[34] All the tyrosine kinase inhibitors produced a notable decrease in the fluorescence intensity at 340 nm (Figure 1D), with values ranging from 21% for quercetin to 34% for tyrphostin A47 and a maximal value of 42% for genistein. These results provide persuasive evidence that these tyrosine kinase inhibitors interact directly with the GLUT1 transporter.

Quenching of GLUT1 Fluorescence in Human Erythrocyte Membranes. Since intrinsic fluorescence determinations are acutely dependent on the internal filter effect elicited by a quencher, we decided to use this property to characterize ligand binding by fluorescence quenching, a method that has been reliably used to study the relative accessibility of tryptophan residues in membrane proteins. We examined the nature of the interaction of the ligands with the GLUT1 transporter present in human erythrocytes membranes stripped of peripheral proteins employing potassium iodide as a hydrophilic probe. This type of analysis has permitted to establish the occurrence of important conformational changes associated with the transport cycle in GLUT1.[16,22,27,35–40]

Iodide ions quench membrane fluorescence in a linearly dependent concentration manner. The degree of quenching is analyzed by the Stern–Volmer equation: $F_0/F = 1 + K_{sv} \times [A]$, where F_0 and F correspond to the fluorescence intensities in absence or the presence of iodide, respectively, [A] is the concentration of iodide, and K_{sv} corresponds to the Stern–Volmer quenching constant. In our hands, the K_{sv} value is 1.4 M^{-1}, which is close to that described by others authors.[28] D-Glucose protected GLUT1 tryptophan fluorescence from the quenching induced by iodide, as reflected by a lower slope of the Stern–Volmer line in its presence (Figure 2). In this case, the K_{sv} value decreased to 0.89 M^{-1}. In contrast, the addition of ATP promoted quenching of the fluorescence; at 2 mM ATP the K_{sv} value increased to 1.6 M^{-1}, while that at 4 mM a more acute effect was observed, but in this last case with a clear downward concave curve. This response may reflect the heterogeneous location of the tryptophan residues on GLUT1, with different tryptophan residues showing differential exposures to the solvent and therefore are differentially affected by iodide. These observations indicate that our preparations of membranes are appropriate to develop a detailed study of the interaction of tyrosine kinase inhibitors with the GLUT1 transporter. It is also clear that the transporter shows at least two conformations: one in the presence of D-glucose in which tryptophan residues are somehow protected from iodide quenching and another in the presence of ATP in which those residues are more exposed.

Effect of Tyrosine Kinase Inhibitors on GLUT1 Fluorescence Quenching by Iodide. With quercetin, the slope of the Stern–Volmer quenching curves increased as the concentration of the flavones is increased (Figure 3A). Therefore, it is clear that quercetin promoted iodide quenching of GLUT1 fluorescence as ATP does. A secondary plot of K_{sv} against flavone concentration revealed that the quenching

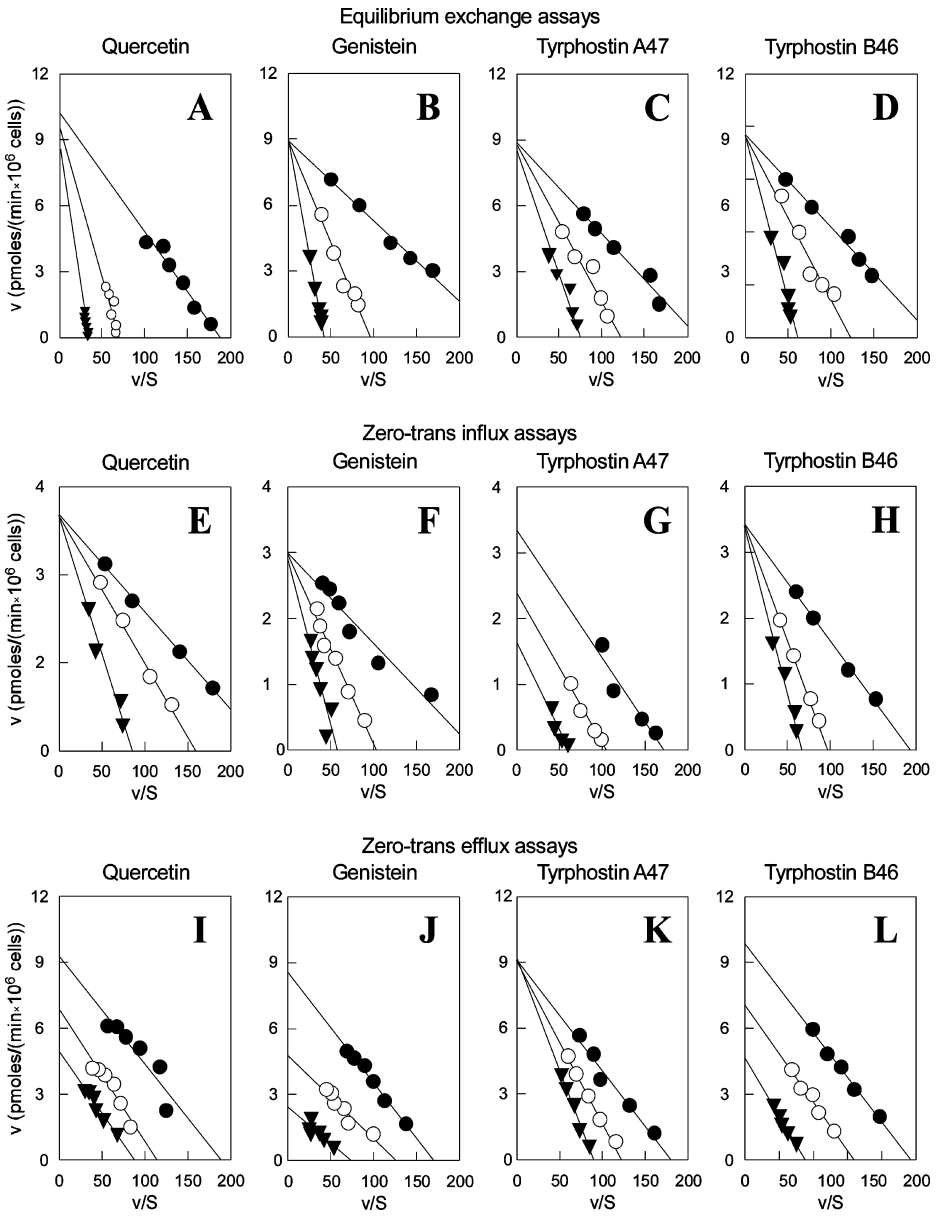

Figure 5. Kinetic characterization of the inhibition of hexose transport by tyrosine kinase inhibitors in human erythrocytes. Eadie−Hofstee plot of the effect of quercetin, genistein, tyrphostin A47, and tyrphostin B46 on the substrate concentration dependence for equilibrium exchange (upper row), zero-trans uptake (middle row), and zero-trans efflux (lower row) of methylglucose in human erythrocytes. On these plots intersects on *x*-axis corresponds to V_{max} while slopes represents the K_M values for methylglucose transport. Therefore, varying slopes and convergence on the abscissa in the plots are indicative of competitive inhibition, while equal and parallel slopes in the plots are indicative of noncompetitive inhibition. (A) Effect of the flavonol quercetin on the substrate concentration dependence for methylglucose exchange in the absence (●) or in the presence of 20 (○) or 40 μM (▼) quercetin. (B) Effect of the isoflavone genistein on the substrate concentration dependence for methylglucose exchange in the absence (●) or in the presence of 30 (○) or 60 μM (▼) genistein. (C) Effect of the tyrphostin A47 on the substrate concentration dependence for methylglucose exchange in the absence (●) or in the presence of 100 (○) or 150 μM (▼) tyrphostin A47. (D) Effect of the tyrphostin B46 on the substrate concentration dependence for methylglucose exchange in the absence (●) or in the presence of 40 (○) or 80 μM (▼) tyrphostin B46. (E) Effect of the flavonol quercetin on the substrate concentration dependence for methylglucose influx in the absence (●) or in the presence of 20 (○) or 40 μM (▼) quercetin. (F) Effect of the isoflavone genistein on the substrate concentration dependence for methylglucose influx in the absence (●) or in the presence of 30 (○) or 60 μM (▼) genistein. (G) Effect of the tyrphostin A47 on the substrate concentration dependence for methylglucose influx in the absence (●) or in the presence of 30 (○) or 60 μM (▼) tyrphostin A47. (H) Effect of the tyrphostin B46 on the substrate concentration dependence for methylglucose influx in the absence (●) or in the presence of 40 (○) or 80 μM (▼) tyrphostin B46. (I) Effect of the flavonol quercetin on the substrate concentration dependence for methylglucose efflux in the absence (●) or in the presence of 4 (○) or 8 μM (▼) quercetin. (J) Effect of the isoflavone genistein on the substrate concentration dependence for methylglucose efflux in the absence (●) or in the presence of 5 (○) or 10 μM (▼) genistein. (K) Effect of the tyrphostin A47 on the substrate concentration dependence for methylglucose efflux in the absence (●) or in the presence of 100 (○) or 150 μM (▼) tyrphostin A47. (L) Effect of the tyrphostin B46 on the substrate concentration dependence for methylglucose efflux in the absence (●) or in the presence of 15 (○) or 30 μM (▼) tyrphostin B46. Results are from single experiments representative of three or four separate experiments for each condition.

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promoting effect is saturable (Figure 3B). These data and those for tryphostin B46 and A47 (see below) were adjusted to an equation of hyperbolic increment, $K_{sv} = K_0 + K_{max} \times L/(K_d + L)$, where K_0 and K_{max} correspond to the K_{sv} value in absence or presence of saturating concentrations of ligand, respectively, L represents ligand concentration, and K_d is the dissociation constant for the ligand, determined as the concentration of ligand at which a half-maximal quenching is observed. In the case of quercetin, a K_d value of 37 μM was determined. In contrast with the observations with quercetin, genistein protected the tryptophan residues from the quenching of its fluorescence by iodide, as indicated by a decreased slope of the Stern–Volmer lines with increasing genistein concentrations (Figure 3C). The quenching-protecting effect of genistein was also saturable, as verified after plotting of the K_{sv} values against the isoflavone concentration in a secondary plot (Figure 3D). The data were adjusted to an equation of hyperbolic decrement, $K_{sv} = K_0 + K_{max} \times K_d/(K_d + L)$, where K_0 and K_{max} correspond to the K_{sv} value in the absence or in the presence of saturating concentrations of ligand, respectively, L represents ligand concentration, and K_d is the dissociation constant for the ligand, determined as the concentration of ligand at which a half-maximal quenching is observed. The K_d value determined for genistein was 11 μM. In relation with tyrphostins, the addition of tyrphostin B46 or A47 elicited an increase in the slope of the curves of the Stern–Volmer plot (Figure 3E,G). In both cases, the increase was saturable (Figure 3F,H), with estimated K_d values of 88 μM for tyrphostin B46 and of 87 μM for tyrphostin A47.

Our data show clearly that quercetin, tyrphostin B46, and tyrphostin A47 increased the exposure of the tryptophan residues of GLUT1 to iodide quenching, as was observed for ATP. These effects were concentration-dependent and saturable, and the K_d values determined for ligand binding were similar to the K_i values determined for each compound in hexose transport inhibition entry assays in human red cells.[12,13] It is tempting to speculate that these inhibitors share a binding site in GLUT1 that has characteristics of a nucleotide-binding site. In clear contrast, a different result was observed with genistein, since in the presence of the isoflavone, the GLUT1 tryptophan residues become less accessible to iodide quenching. The protective effect was also saturable, and the K_d value determined was in close agreement with the K_i value obtained for this inhibitor in entry-assays in red cells.[12,13] The protective effect of genistein resembles that provoked by the addition of D-glucose, while quercetin and the tyrphostins promote iodide quenching as ATP does. This fact militates against the possibility that genistein share a common binding site with the other tyrosine kinase inhibitors on the transporter.

Sen–Widdas Assays for the Tyrosine Kinase Inhibitors. To evaluate if genistein binding to GLUT1 is distinguishable from that of the other tyrosine kinase inhibitors, efflux assays of D-glucose under infinite-cis conditions were implemented. These assays permit us to evaluate D-glucose affinity for the exofacial glucose site (accessible externally).[29] The temporal course of glucose exit until the external and internal hexose concentrations reach equilibrium follows a monoexponential curve, defined by the following equation: $I(t) = A(1 - e^{-kt})$, where $I(t)$ is the intensity of dispersed light at different times, A is the maximal amplitude of the light intensity signal, and k is the constant rate. Inhibition by the tyrosine kinase inhibitors was evaluated by measuring the values of k in absence or presence of different levels of the blockers. The value for k in absence of inhibitors (30 °C, pH 7.4) was 0.07–0.08 s^{-1}.

Micromolar concentrations of the tyrosine kinase inhibitors blocked D-glucose exit from human red cells (Figure 4). To analyze these curves, data were adjusted to the equation of hyperbolic inhibition: $k_I = k_0 \times K_I/(K_I + I)$, where k_0 corresponds to the rate constant in absence of inhibitor, K_I is the dissociation constant for the inhibitor, and I is the tyrosine kinase inhibitor concentration. The K_I values fluctuated between 4 μM for genistein and 115 μM for tyrphostin A47. These K_I values were in close agreement with those obtained also in human red cells but under zero-trans entry assays.[13]

Sen and Widdas[29] demonstrated that if rate constants obtained under infinite-cis conditions in the presence of different external D-glucose concentrations are plotted as exit time against the external glucose concentration (Sen-Widdas plot), the intercept on the horizontal axis corresponds to the negative value of the D-glucose dissociation constant for the external glucose site on the transporter. In our hands, the K_d value for the external glucose site is 4 mM, in close agreement to the value described previously.[29] Figure 4E shows that cytochalasin B, a classical GLUT1 inhibitor, inhibited glucose efflux (K_I = 0.5 μM) but was unable to alter glucose affinity for the external site. Phloretin also blocked glucose exit (K_I = 0.5 μM), but simultaneously altered the K_d value for glucose binding to the external site; the K_d value increased from 4 to 15 mM in the presence of 5 μM phloretin. These results agree with the proposal that cytochalasin B binds to the internal (endofacial) face of the transporter, while phloretin binds to GLUT1 through its external glucose site. These results validate the use of this experimental approach for testing the effect of the tyrosine kinase inhibitors on the integrity of the external glucose binding site of the transporter.

The more relevant result with the tyrosine kinase inhibitor was that genistein altered the D-glucose affinity for the external glucose site (Figure 4G), in a manner qualitatively similar to the effect of phloretin. The other tyrosine kinase inhibitors failed to affect the external glucose site (Figures 4F,H,I), since K_d values for glucose were not affected by the presence of the inhibitors, a behavior that resemble that of cytochalasin B. These data provide conclusive evidence that the flavone quercetin and the tyrphostins bind to the GLUT1 transporter in a site different from the external glucose site, while genistein binding actually alters D-glucose binding to the external glucose site.

Characterization of the Glucose Transporter Inhibition by Tyrosine Kinase Inhibitors. The absence of a typical competitive inhibitor effect in Sen–Widdas infinite-cis exit experiments implies that quercetin and the tyrphostin A47 and B46 do not react with the external D-glucose site on the transporter protein. This could mean that these blockers bind to an external site different from the external glucose site, or instead they could bind to the transporter through its internal surface. This implies that the competitive nature of the interaction would only be revealed in equilibrium exchange experiments where the competing substrate is present at the putative endofacial inhibitor site. To discriminate between these options, we developed zero-trans influx (no substrate inside), zero-trans efflux (no substrate outside), and equilibrium exchange (substrate inside and outside) transport protocols using methylglucose as substrate.

Figure 5 presents the results of such analysis, represented in the form of Eadie–Hofstee plots. The upper row of Figure 5 shows that under exchange conditions the saturation curves for

methylglucose transport in the presence of different concentrations of the tyrosine kinase inhibitors generate lines with higher slopes at increasing inhibitor levels, lines that extrapolate to a common point on the abscissa of the Eadie−Hofstee plots. High substrate concentrations relieve transport inhibition, since the blockers are unable to affect the V_{max} but they increase the K_M values for methylglucose transport (Figure 5, panels A−D). These results demonstrate that the blockers act like competitive inhibitors of methylglucose exchange, therefore indicating that they bind to the transporter in a manner that competes with glucose sites either on the external or internal sites. The middle row of Figure 5 shows the data obtained under zero-trans influx assays. As with the exchange assays, the equal V_{max} and varying K_M values in the presence of different concentration of quercetin, genistein, and tyrphostin B46 on the Eadie−Hofstee plots (Figure 5, panels E, F, and H) indicate that these compounds inhibit methylglucose entry to the red cells in a competitive manner. In contrast, we observed that tyrphostin A47 actually decreased the V_{max} without altering the K_M for methylglucose transport (Figure 5, panel G). This behavior corresponds to that expected for a noncompetitive inhibitor, indicating that tyrphostin A47 is unable to compete for methylglucose for binding to the substrate external site. The lower row of Figure 5 shows the data obtained with zero-trans assays for methylglucose efflux out of the red cells. With quercetin, genistein, and tyrphostin B46, the curves on the Eadie−Hofstee plots shows variable and decreasing intersects in the *x*-axis but similar slopes, indicating that although the K_M values are preserved, the V_{max} for methylglucose transport decreased with increasing levels of the blockers (Figure 5, panels I, J, and L). These results indicate that under zero-trans efflux the reagents are noncompetitive blockers of the erythrocyte transporter. In clear contrast, the effect of tyrphostin A47 was clearly competitive, since the lines of the Eadie−Hofstee plot showed variable slopes with a common intersection on the *x*-axis; therefore, K_M values are higher at increasing tyrphostin A47 levels, but V_{max} is unaffected. Collectively, the kinetic analysis provides persuasive evidence indicating that quercetin, genistein, and tyrphostin B46 bind to the transporter in a site accessible from its external surface. For tyrphostin A47 instead, the kinetic data provide convincing evidence that its binding site is located on the cytoplasmic face of the transporter.

DISCUSSION

GLUT1 is a typical facilitative transporter that allows the swapping of substrates across the plasma membrane down a concentration gradient, requiring no energy input. The molecular mechanism by which GLUT1 moves its substrate across the membrane remains unclear, mainly because of the lack of a structural model of the protein. This deficiency is explained by the inability to obtain crystals of this membrane protein, which is why several indirect analytical methods have been developed to elucidate its structure. Studies of proteolysis and labeling of glycosylation epitopes confirmed the 12-helix model for this transporter.[41−49] Likewise, the use of site directed mutants coupled with photolabeling have confirmed the existence of exo- and endofacial binding sites for glucose on GLUT1 which are indispensable for substrate binding and transport.[50−53] The existence of endo- and exofacial substrate binding sites is consistent with the functional evidence indicating that GLUT1 is a bidirectional transporter which transports hexose into and out of the cell.

It is known that GLUT1 binds molecules that have no structural relationship with the substrate, such as phloretin and cytochalasin B. In human erythrocytes, cytochalasin B is known to competitively inhibit only at the inside site. Basketter and Widdas[7] and Deves and Krupka[9] 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 binding site for cytochalasin B has been identified in the cytoplasmic region transmembrane between segments 10 and 11, suggesting that this region of GLUT1 is in or near of the internal binding of glucose.[10] Phloretin is a competitive and noncompetitive inhibitor of sugar influx and efflux, respectively, in human red cells,[54] indicating that it reacts with the external orientation of the GLUT1 molecule.

There is evidence that ATP binds GLUT1[16,18−21,27] and a detailed analysis of the primary structure of GLUT1 revealed the presence of three short amino acid sequences (motifs), separated from each other in the primary structure, which show sequence homology with domains present in typical ATP binding sites of ATPases and other proteins dependent on ATP binding and hydrolysis for their function. This led us to suggest that there is a relationship between the presence of putative nucleotide-binding motifs in GLUT1 and the ability of certain flavones and tyrphostins to block the functional activity of the transporter. We show here that GLUT1 has binding sites for flavones and tyrphostins accessible by the endofacial and exofacial side of the transporter. By kinetic assays under zero-trans entry conditions tyrphostin B46, genistein and quercetin were found to be competitive inhibitors, while in zero-trans exit assays these reagents acted as noncompetitive inhibitors. These results indicate that these compounds would be accessible from the exofacial surface of the carrier, and we postulate that they could bind to a site formed by residues containing the putative nucleotide binding motif I. In contrast, tyrphostin A47 showed a noncompetitive behavior in zero-trans entry assays but competitive in zero-trans exit assays, entailing that it interact with a site accessible by the endofacial face of GLUT1. In the latter case it might be thought that its binding site could be formed by residues including the putative nucleotide binding motifs II and III.

We expected that the interaction with GLUT1 of flavones and tyrphostins that inhibit glucose transport would cause significant changes in the quenching of the sugar transporter intrinsic fluorescence. This is why we examined the binding of tyrosine kinases inhibitors such as the flavone quercetin, the isoflavone genistein and the tyrphostins A47 and B46, by analyzing their effect on GLUT1 intrinsic fluorescence in human erythrocyte membranes and in isolated transporter reconstituted in proteoliposomes. The tyrosine kinase inhibitors significantly reduced the intrinsic fluorescence of the red cell sugar transporter, both in membrane preparations and in purified and reconstituted transporter. This decrease can be explained by suggesting that tyrosine kinases inhibitors stabilize the transporter in a conformation in which there is an increased exposure of some tryptophan residues. In membrane preparations the potency of compounds in decreasing order was quercetin > tyrphostins B46 > tyrphostin A47 > genistein, while in purified reconstituted transporter this order was slightly different genistein > quercetin > tyrphostin A47 (tyrphostin B46 was not tested with the reconstituted transporter). The differences could be attributed to unspecific binding to other proteins in the membrane preparation. The

data indicate that the tyrosine kinase inhibitors perturb GLUT1 intrinsic fluorescence, and it is possible that a direct interaction between the inhibitor and one or several tryptophan residues may exist. Testing this possibility will requires further studies.

We further analyzed the interaction of the flavones and tyrphostins with GLUT1 by following the fluorescence quenching of the purified sugar transporter by iodide, a hydrophilic quencher. Quercetin, tyrphostin B46, and tyrphostin A47 increased quenching in isolated membranes and in proteoliposomes containing purified and reconstituted transporter, in a manner similar to the effect of ATP. These results are consistent with the possibility that the compounds stabilize a conformation of the transporter that would expose one or more tryptophan residues. These residues in the transporter without ligands would be in an environment inaccessible to the quencher and only after exposure to the ligands can be quenched by the iodide molecule.

Infinite-cis exit assays showed that quercetin and tyrphostin B46 and A47 did not affect the affinity for D-glucose in the transporter external site, suggesting that their binding sites comprises residues not involved in the binding of D-glucose to the exofacial surface. On the other hand, zero-trans entry assays provided evidence indicating that quercetin and tyrphostin B46 are competitive inhibitors, indicating the presence of an exofacial binding site. It should be stressed, however, that even though our data suggest a competitive mechanism with external D-glucose, we should not necessarily infer that these inhibitors bind exactly to the substrate site; thus, binding of the inhibitors to the transporter may trigger a conformational change that affects the structure of the catalytic site obliterating external D-glucose binding. Our results for quercetin and tyrphostin B46 clearly favor the latter option. Regarding tyrphostins A47, our data suggest that it binds to GLUT1 at the endofacial face, as described for cytochalasin B. Accordingly, it is not expected that this inhibitor would be able to alter the D-glucose affinity for the hexose external binding site. We expect that the mechanism by which tyrphostins A47 interacts with the transporter is most probably different from that of cytochalasin B, since tyrphostin A47 promotes fluorescence quenching, while cytochalasin B has no effect or show a slight protective effect (data not shown).

One very interesting observation was that the effect on the fluorescence quenching induced by the isoflavone genistein was distinguishable from that of the other inhibitors; different from ATP and similarly to D-glucose, genistein induced protection against iodide quenching. Moreover, the Sen–Widdas assays showed that genistein decreased the affinity of the external site for D-glucose, while phloterin also affects glucose binding to the external site. The sidedness of phloretin binding to the glucose carrier has been previously determined by comparing the type of inhibition produced in zero-trans entry and zero-trans exit experiments. The inhibition of glucose entry by phloretin was competitive, and the inhibition of exit noncompetitive, suggesting that phloretin interacts with the outward-facing form of the glucose carrier, but not with the inward-facing form.[54] The isoflavone genistein shows a structural similarity with the chalcone phloretin, and we also established that genistein competitively inhibited the entry of hexose in human red cells, but noncompetitively blocked glucose efflux.[12,13] Therefore, it is conceivable that these compounds may share a binding site on GLUT1. The simplest interpretation of competition with extracellular glucose is that genistein (and by extension phloretin) competes with D-glucose for binding to a site accessible from the external surface of the transporter. However, a ligand could, in principle, competitively displace extracellular glucose from the outward-facing conformation but do so by binding at the endofacial surface of GLUT1. Therefore, in the absence of direct physical evidence showing interaction of genistein with the outer surface of the transporter, our proposal of an external genistein inhibitory site should remain as tentative.

A strong interaction between ATP and genistein, estradiol, and cytochalasin B at the endofacial site of the carrier has been described in experiments done with fresh drawn red cells.[55] Since we used aged (probably ATP-depleted) red cells it could be envisioned that genistein interaction with the inward-facing conformation could occur in the presence of ATP. However, control experiments done with fresh erythrocytes bearing normal ATP levels confirm the altered affinity of external D-glucose inhibition of infinite-cis efflux and also the noncompetitive inhibition pattern into the zero-trans assays (not shown). Afzal et al.[55] showed that K_i values for genistein, estradiol, and cytochalasin B inhibition of glucose exit from fresh whole red cells and ghosts with no ATP were similar. However, raising ATP in ghosts to 2–4 mM increased these K_i values, suggesting that ATP have no effect or actually competitively antagonizes genistein, estradiol, and cytochalasin B inhibition of glucose exit. These observations do not favor an enabling effect of ATP on genistein interaction with the inward form of the transporter. However, that ATP affect in some extent the interaction of genistein and quercetin with GLUT1 is a possibility that merit analysis. More conclusive data may be obtained in further studies using erythrocyte ghost prepared with varying ATP levels.

It is thought that the mechanism by which GLUT1 catalyzes glucose transport implies alternating conformational changes which expose sequentially a hexose binding site on the protein facing the outer or the inner surface of the plasma membrane.[56–58] The mechanism of net transport of sugar into the cell (influx) is usually explained by a kinetic scheme comprising at least four steps: (1) a rapid binding 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 stage of relaxation of free carrier that regenerates the exofacial binding site for the substrate. Conversely, the complete transport cycle in exit assays of substrate (efflux) involves (1) the binding of substrate to the internal sugar site, (2) translocation of the sugar–transporter complex, (3) release of sugar from the external (exofacial) site, and (4) a relaxation of the free carrier that regenerates the endofacial binding site. In this regard, it has been suggested that a region that includes part of transmembrane helices 10 and 11 and contains tryptophan residues 388 and 412 participates in cytochalasin B binding.[11,28] Given the conformational dynamics associated with the transport cycle, it can be proposed that this region is exposed in the conformation that binds D-glucose on the cytoplasmic side, while it is hidden in the conformation that binds D-glucose at the extracellular face of the transporter.

In summary, we consider more likely that GLUT1 harbors three binding sites for the tyrosine kinase inhibitors, defined by the ability of the blockers to bind to different conformations of the transporter (Figure 6): (1) genistein would bind to the carrier in the conformation that displays the site of glucose into the outer surface ("outward-facing conformation") at a site that overlaps with the external binding site for D-glucose, (2) the

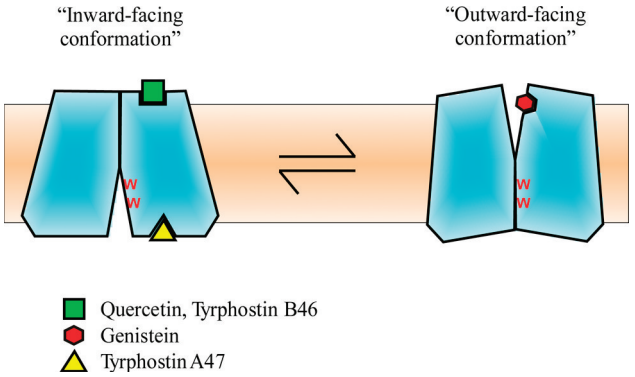

Figure 6. Putative binding sites for tyrosine kinase inhibitors on GLUT1. The GLUT1 transporter undergoes a conformational change between two conformations, the exo form ("outward-facing") and endo ("inward-facing") conformation during the catalytic cycle of transport. The red "W" represents the arrangement of a population of tryptophan residues whose exposure is affected by this conformational change, showing less exposure in the outward-facing conformation. Genistein would bind to the outward-facing conformation of the transporter in an accessible location for its external side, where it competes for binding of D-glucose to the external site; therefore, it protects the tryptophan fluorescence quenching caused by iodide. Quercetin and tyrphostin B46 would link to a site also accessible by the external face of the transporter, but in the inward-facing conformation of the transporter, thus promoting the fluorescence quenching by iodide. Tyrphostin A47 would join a site accessible on the inner face of the transporter, distinguishable from the endofacial cytochalasin B site, stabilizing the inward-facing conformation.

flavone quercetin and tyrphostin B46 would bind instead to the conformation of the transporter that shows its binding site toward the inner side of the membrane ("inward-facing conformation"), but at a site accessible from the outer surface of the protein, and (3) the binding site for tyrphostin A47 on the transporter would be accessible from the inner surface of the plasma membrane, to a conformation of the transporter with the binding site exposed to the interior of the cell ("inward-facing conformation"). On this model one can propose that the tryptophanyl moieties, whose fluorescence is shielded by iodide, are accessible in the inward-facing conformation and are protected in the outward-facing conformation of the carrier. On the basis of the foregoing, it can be argued that the conformation that shows a sugar site toward the outer surface ("outward-facing conformation") is stabilized by D-glucose and genistein, whereas quercetin, tyrphostin B46, and ATP stabilize the inward-facing conformation. This view is also compatible with the results of quenching of intrinsic fluorescence which indicated that the transporter displays two conformations: one in the presence of glucose, in which certain indol moieties are protected from quenching, and another in the presence of ATP, in which some tryptophanyl residues are heavily exposed. Direct evidence to prove this assertion should wait for further studies, e.g., by assaying accessibility on several Trp point mutants of Glut1.

The fact that different tyrosine kinase inhibitors target various discrete allosteric sites on the GLUT1 transporter is of particular interest because of the role played by the transporter in human physiology. Located on the plasma membrane of most animal cells, GLUT1 is responsible for basal D-glucose uptake, and consequently its expression and functional activity govern the energy availability of D-glucose-dependent organs such as the brain.[59] GLUT1 is also overexpressed in most cancer cells, obeying to the metabolic switching on the malignity process to an anaerobic profile for D-glucose utilization, involving an exacerbated glycolytic flux.[60] Hence, blocking of glucose uptake has been invoked as a promising antiproliferative and pro-apoptotic strategy against cancer cells. It is obvious that tyrosine kinase inhibitors are unsuitable for that purpose since they affect cellular tyrosine kinases. However, the fact that GLUT1 could be affected by tyrosine kinase inhibitors at different levels stresses the need to design new derivatives able to specifically block glucose uptake through GLUT1. In fact, the presence of different allosteric regulatory sites on GLUT1 opens up the possibility that combinations of different derivatives could be highly efficient in blocking glucose entry into cells compared with using any isolated single compound. Of course, we would have to neutralize the expected adverse effect on glucose transport through the blood–brain barrier by a controlled use of a cetogenic diet, as is actually recommended for GLUT1 deficiency syndrome patients.[61,62]

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ABBREVIATION

GLUT1, human erythrocyte glucose transport protein.

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