Biochimica et Biophysica Acta, 1110 (1992) 105–111

BBAMEM 75742

Phloretin *keto-enol* tautomerism and inhibition of glucose transport in human erythrocytes (including effects of phloretin on anion transport)

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(Received 4 February 1992)

Key words: Erythrocyte; Hexose; Anion transport; Phloretin; Phloroacetophenone

Under various pH conditions phloretin demonstrates *keto-enol* tautomerism with a pK value of 7.26 ± 0.06. As Wilbrandt has shown ((1950) Arch. Exp. Pathol. Pharmacol. 212, 9–29) phloretin added to erythrocytes inhibits glucose efflux, but not glucose influx. At pH 6.5 a K_i value of 0.36 and at pH 9 of 22.7 μM was measured; only the ketonic form of phloretin contributes to the inhibition of glucose efflux. This was also the case for inhibition of galactose efflux and anion exchange. The geometry optimization of a large number of conformations of the ketonic and enolic forms of phloretin demonstrates different shapes of the molecules. Only the ketonic form shows several overlapping structures with β-D-glucopyranose. Considering surplus binding of phloretin under glucose efflux conditions as being equivalent to the number of glucose transporters, a number of about 200000 molecules was determined. By two independent methods 210000 and 171000 molecules per cell were calculated. This result is in close agreement with the number of glucose transporter sites of the erythrocyte.

Introduction

Wilbrandt discovered in 1950 [1] that in human erythrocytes phloretin, the aglucone of phlorizin, is many times more potent in inhibition of glucose transport than the glucoside. The K_i values for phlorizin, phloretin and polyphloretin phosphate, measured by the Sen-Widdas technique [2], were reported to be 150, 1.6 and 2.4 μM, respectively. The inhibition was claimed to be reversible and competitive for the three inhibitors [2,3]. In addition the inhibitors demonstrated asymmetry of inhibition by the fact that only the exit of glucose is significantly inhibited but not the entrance [1,3]. Rosenberg and Wilbrandt [4] focussed their attention on the hydroxyl groups of phloretin, which could be blocked by methylation. The experiments showed that substitution for any one of the hydroxyl hydrogens by a methyl group in the phloretin molecule reduces the inhibitory potency by at least 95%. Methylation at two or three points abolished nearly completely the inhibitory activity with the exception of 2,4-dimethyl phloretin, which was even slightly more active than phlorizin.

LeFevre and Marshall [5] investigated the attachment of phloretin to human erythrocytes in connection with inhibition of glucose transport. They observed by investigation of the pH dependence that only the ketonic form of phloretin binds to the erythrocytes and inhibits glucose transport. However, data about the inhibitory potency of phloretin in relation to pH were not given. It was claimed that the grouping involved in this tautomerism does not itself provide the essentials for the attachment process and that the transport sites are saturated even at a few μM of phloretin, the lowest level which could be detected chemically. ^{3}H-labeled phloretin was shown to be bound reversibly by human erythrocytes and ghost membranes but not to penetrate across them in either direction [6]. By enclosing phloretin into ghosts, monosaccharide influx but not efflux was inhibited. Thus phloretin is active only at the side towards which the transport proceeds.

The glucose transporter of human erythrocytes is a transmembranous glycoprotein of 492 amino acids, identified as the band 4.5 protein, with a molecular mass of approx. 55 kDa [7]. Analysis of the primary stucture suggests the presence of twelve membrane-spanning domains. Several of these may form amphi-

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pathic helices and contain abundant hydroxyl and amide side chains that could participate in glucose binding. The glucose transport inhibitor cytochalasin B was found only in band 4.5 [8] and the total number of cytochalasin B binding sites amounts to about 250000 copies per cell. Since a monoclonal antibody to the human glucose transporter recognized the cytochalasin B binding tryptic fragments [9], it can be assumed that cytochalasin B binding is equivalent to the number of transporters.

In this study the inhibition of glucose and galactose net efflux in human erythrocytes by phloretin was investigated in relation to the pH-dependent *keto-enol* tautomerism. Not only glucose transport but also bicarbonate-chloride exchange were inhibited by the ketonic form of phloretin. Chemical structure and energy calculations of phloretin based on molecular mechanics demonstrated structure differences between the minimized ketonic and enolic forms. The ketonic form of phloretin was compared with the transported β-D-glucopyranose molecule. In addition, binding of phloretin was investigated during glucose influx, efflux and equilibrium conditions.

Methods

Cell preparation. Freshly drawn human erythrocytes with heparin as anticoagulant were washed three times with ice-cold 150 mM NaCl, 20 mM Hepes buffer adjusted to the pH values given in the experiments and made up to a hematocrit of 10 or 20%.

Net glucose efflux experiments. The efflux of glucose was followed in cells after loading the cells initially with 300 mM glucose or galactose for 1 h at 37°C. A right angle light-scattering method was used to measure efflux as described by Fuhrmann [10]. In order to compensate for the 300 mM glucose or galactose in the cell interior in addition to the osmotic pressure of normal Hepes buffer, 150 mM NaCl were added to the exit solution. If not otherwise stated 100 μl of a solution with 10% cells loaded with 300 mM sugar were pipetted into 15 ml exit solution with 1.33% ethanol at 20°C to start the net efflux (sugar outside concentration 2 mM).

$HCO_3^- - Cl^-$ exchange experiments. Heteroanion exchange was measured also by the right angle light-scattering method [10]. 100 μl of three times washed erythrocytes at a hematocrit of 20% were added to 15 ml 150 mM NH_4Cl, 20 mM Hepes buffer adjusted to the pH given in the experiments with 1.33% ethanol at 20°C into the light scattering measurement cuvette. The light-scattering signal obtained immediately after suspension of the cells was kept constant by automatic titration of a 4.5 M NaCl solution into the cuvette. The μl of NaCl solution titrated per unit time by the osmotitration method are directly proportional to the heteroanion exchange [10].

Absorbance spectra. The spectra of phloretin and phloroacetophenone (2,4,6-trihydroxyacetophenone) were recorded on a Kontron UVIKON 810 spectrophotometer. Spectra were scanned from 260 to 360 nm. The absorbance at 285 and 320 nm from pH 6 to pH 10 were analysed by a computer program Kinfit [11] for pK values.

Cell volume and cell number. Cytocrit was determined at $14000 \times g$ for 5 min and the cell number counted in a Sysmex CC 800.

Phloretin binding studies. Phloretin was analysed from the erythrocyte pellet and from the cell-free supernatant. Phloretin binding was calculated per erythrocyte by the number of cells counted and by using Avogadro's number ($6.023 \cdot 10^{23}$ mol^{-1}). If not otherwise stated the pH was 6.5 and immediately after establishing the glucose gradient the erythrocytes were spun down and the pellet as well as the supernatant were analysed for phloretin (all experiments with 1.33% or 2% ethanol for solubilisation of phloretin). The erythrocyte sediments (5 or 20 μl packed cells in 7.5 or 30 ml buffer solution pH 6.5) were hemolysed by ultra sonication for 8 min or addition of 100 μl H_2O. Proteins were pelleted by addition of 100 μl acetonitrile. Samples of 20 μl (protein-free extract from pellet) or 100 μl (cell-free supernatant from 0.4% erythrocytes) were analysed by reversed-phase high-performance liquid chromatography (HPLC). HPLC System Gold Beckman with a progammable Solvent Module 116 and Detector Module 166 was used. Separation was obtained with an SERVA Octadecyl = Si100, 5 μm column with 20 or 50 mM KH_2PO_4 and 30% acetonitrile pH 8.5 as solvent, flow rate 0.8 ml/min and phloretin detection at 320 nm.

Computational chemistry. For molecular modeling and energy minimization the program MACROMODEL, version 1.1 was used [12]. Calculations have been performed without lone pair electrons at oxygen.

Materials. Hepes was obtained from Serva (Heidelberg, Germany). Phloretin was obtained from Fluka, Switzerland. Phloroacetophenone (2,4,6-trihydroxyacetophenone) was obtained from Aldrich, Germany. All other reagents were analytical grade.

Results

(1) Chemistry of the keto-enol forms of phloretin

We calculated the lowest energy conformations of the ketonic and enolic forms of phloretin using the MM2 force field parameters [13] and the program Macromodel [12]. The most stable conformations of the two isomeric forms are shown in Figs. 1A and 1B. They have been obtained by optimization of 1296 different starting geometries for the ketonic and enolic forms, which have been produced by rotating around the central four C-C bonds in increments of 60°. As

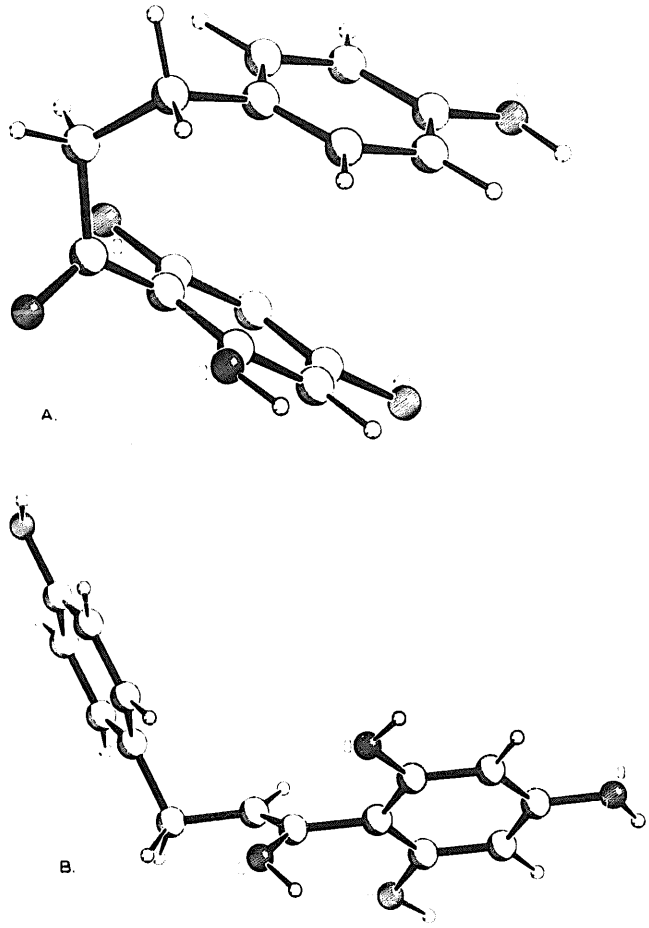

Fig. 1. Minimized forms of *keto*-phloretin (A) and of enol-phloretin (B).

can be seen from Figs. 1A and 1B both molecules depict completely different shapes of their minimal energy forms. The calculated minimal energy for the ketonic phloretin molecule (Fig. 1A) is 7.7 kJ/mol (1.84 kcal/mol) and for the enolic form (Fig. 1B) 32.7 kJ/mole (7.81 kcal/mol). Not only the shape and minimal energies are significantly different but also the surface charge distributions of the ketonic and enolic form of phloretin (not shown). Also the dipole moment of both forms are different with 4.7 against 12.5 Debye.

By comparing the positions of the 4-hydroxyl- and the keto-group of phloretin with the hydroxyl groups of β-D-glucopyranose several overlapping structures can be obtained (not shown), which demonstrate the close structural relationship between *keto*-phloretin and glucose.

As has been known for a long time [5] the *keto-enol* tautomerism can be demonstrated by the effects of hydrogen ions on the ultraviolet absorption spectrum of phloretin (Fig. 2). In order to calculate the pK value of this reaction we analysed the *keto-enol* peaks at 285 and 320 nm in relation to the pH values under our experimental conditions. By nonlinear regression analysis [11] a pK value of 7.26 ± 0.06 was estimated.

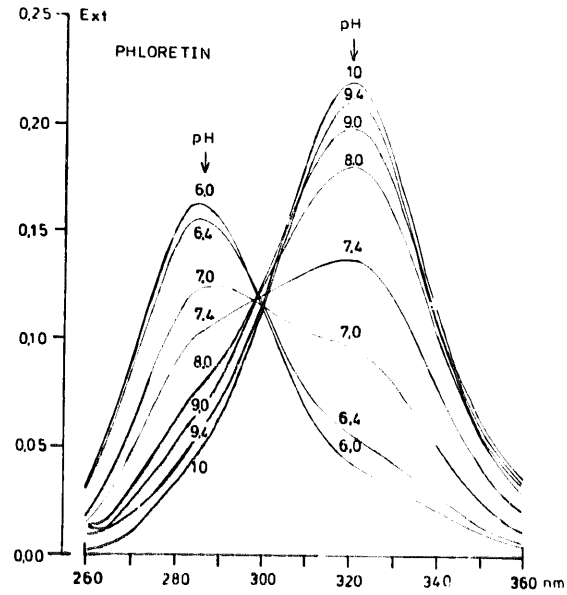

Fig. 2. Absorbance spectra of phloretin between 260 and 360 nm at pH values from 6 to 10.

(2) Inhibition of glucose and galactose net efflux and $HCO_3^- - Cl^-$ exchange transport by phloretin in relation to pH

We confirm here the fact that phloretin added externally inhibits glucose efflux and not influx as originally shown by Wilbrandt [1,3]. Therefore, only the glucose efflux, as described in Methods, was measured at several phloretin concentrations in between pH 6.5 and 9. Fig. 3 demonstrates the linear relationship of inhibition in a normalised Dixon plot. The interception with the abscissa ($-x$) corresponds to the apparent K_i value, which is the concentration required for 50% inhibition. In Table I the apparent K_i values are listed

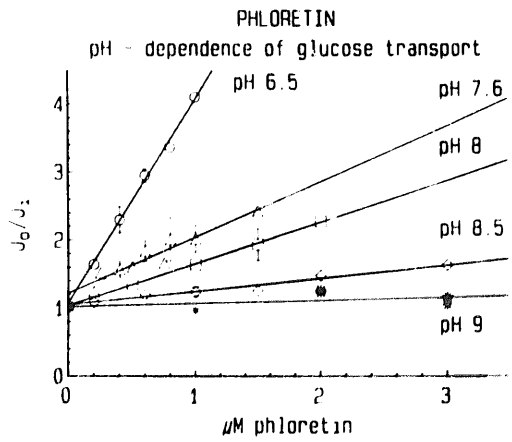

Fig. 3. pH-dependence of glucose efflux in normalized Dixon-plots with phloretin as inhibitor. J_o represents the glucose efflux without phloretin and J_i the glucose efflux with phloretin. Points with statistics mean values of four experiments ± S.D.

TABLE I

Inhibition of glucose efflux by phloretin, Dixon-plot

pH	K_i (μM)	Affinity ($1/K_i$)	Correlation coefficient
6.5	0.44	2.25	0.987
6.5	0.34	2.97	0.978
6.8	0.31	3.21	0.987
6.8	0.40	2.52	0.994
6.8	0.31	3.23	0.999
7.0	0.34	2.94	0.996
7.2	0.42	2.36	0.997
7.2	0.41	2.44	0.995
7.6	0.80	1.26	0.993
7.6	1.67	0.60	0.962
7.6	1.11	0.90	0.984
7.6	0.57	1.76	0.993
8.0	1.58	0.63	0.981
8.0	1.85	0.54	0.954
8.5	4.65	0.22	0.982
8.5	4.94	0.20	0.974
9.0	22.67	0.04	0.573

for different pH values with the highest affinities in the low pH range. Table II includes apparent K_i values of phloretin in galactose efflux experiments and K_i values in $HCO_3^- - Cl^-$ exchange experiments at different pH values. Again phloretin inhibits strongly at the low pH values investigated.

By plotting the apparent affinity of phloretin to the glucose transporter ($1/K_i$) against pH (Fig. 4A) a close relationship to the pH-dependent *keto-enol* tautomerism (Fig. 4B) becomes obvious. The corresponding value to the pK value of 7.26 ± 0.06 measured spectroscopically in Fig. 4B is matched by a value of 7.33 ± 0.1 in Fig. 4A. Both curves are similar in shape, demonstrating that the ketonic-form of phloretin is the

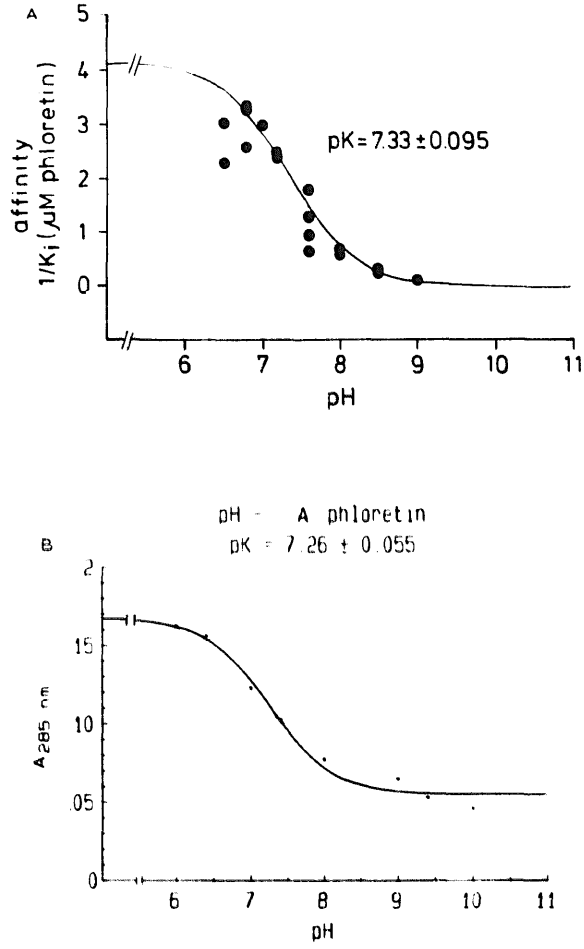

Fig. 4. Plot of apparent affinities of phloretin to the glucose transporter ($1/K_i$ = inhibitor concentration required for 50% inhibition) against pH. Experimental values are given in Table I. The curve is fitted by Kinfit, a computer program that performs nonlinear regression analysis, for pK determination (Fig. 4A). In Fig. 4B the absorbance of phloretin at 285 nm is plotted versus pH. The curve is calculated by Kinfit.

TABLE II

Inhibition of galactose efflux by phloretin, Dixon-plot

pH	K_i (μM)	affinity ($1/K_i$)	Correlation coefficient
6.5	0.20	5.00	0.997
7.0	0.27	3.74	0.997
7.0	0.26	3.78	0.989
7.6	0.40	2.47	0.998
7.6	0.47	2.11	0.996
8.0	0.96	1.04	0.993
8.0	0.86	1.16	0.991
8.5	2.33	0.43	0.842

Inhibition of anion exchange by phloretin, Dixon-plot

pH	K_i (μM)	Affinity ($1/K_i$)	Correlation coefficient
6.6	1.9	0.526	0.976
7.5	40.1	0.025	0.736
7.6	24.3	0.041	0.934
8.5	85.3	0.012	0.641

active compound. The same behaviour can be demonstrated by the affinity of phloretin for galactose transport against pH (not shown). The corresponding value here to the pH is 7.4 ± 0.1. The same tendency can also be assumed for the $HCO_3^- - Cl^-$ exchange, i.e., that only the ketonic form of phloretin inhibits the transport process.

(3) Comparison of phloretin inhibition with that of phloroacetophenone in glucose efflux experiments

A *keto-enol* tautomerism is also present in phloroacetophenone, which is part of the structure of phloretin. The *keto-enol* tautomerism can be seen by the ultraviolet absorption spectrum (Fig. 5). In relation to pH, which is quite similar to that of phloretin (Fig. 2). The pK value is close to phloretin with 7.25. However, inhibition of glucose efflux is less by a factor of about 400.

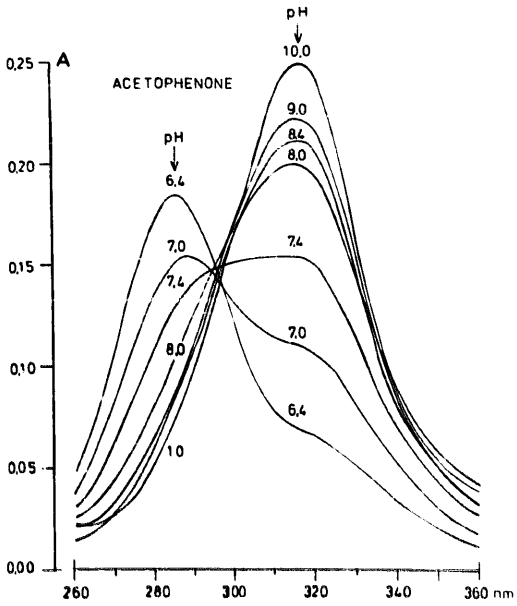

Fig. 5. Absorbance spectra of acetophenone between 260 and 360 nm at pH values from 6.4 and 10.

Table III lists the apparent K_i values derived from the linear Dixon plot with phloroacetophenone as inhibitor of glucose efflux. If one compares the affinities of phloroacetophenone and phloretin against pH, then it can be seen that also two similar curves are obtained (Fig. 6). But it should be noticed, that the K_i value of phloroacetophenone is given in mM and not as for phloretin in μM. It appears that phloroacetophenone is also as phloretin active in the ketonic-form in inhibition of glucose efflux but with a very low affinity.

(4) Binding of phloretin to human erythrocytes

By spectroscopic methods used between pH 7.3 to 7.4 LeFevre and Marshall calculated approx. $2 \cdot 10^6$ molecules of phloretin bound per erythrocyte at a phloretin concentration of 1 μM [5]. In order to improve the detection of phloretin at low concentrations we used separation and detection of phloretin by reversed phase chromatography and an extremely sensitive HPLC photometer. After incubation of a diluted erythrocyte suspension (0.067%) with 0.2 to 4.0 μM

TABLE III

Inhibition of glucose efflux by phloroacetophenone, Dixon-plot

pH	K_i (μM)	Affinity ($1/K_i$)	Correlation coefficient
6.5	152	0.0066	0.999
7.0	160	0.0062	1.000
8.0	410	0.0024	0.999
8.5	566	0.0018	0.979
9.0	963	0.0010	0.993

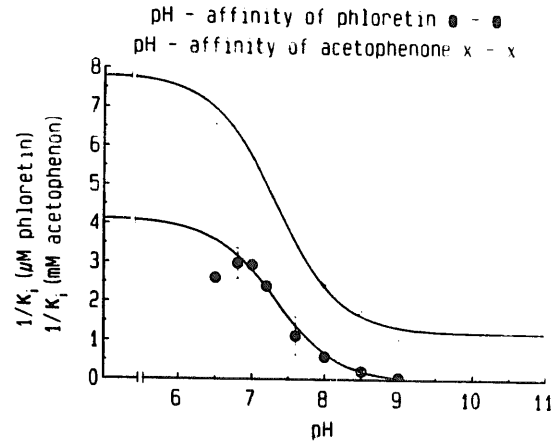

Fig. 6. Dependence of glucose transport affinity of phloretin (●——●) and acetophenone (× ——— ×) versus pH. Curves are calculated by Kinfit. Experimental values are given in Tables I and III.

phloretin the binding to the cells was measured at pH 6.5 and pH 7.6 (Fig. 7). We found for example with 1 μM phloretin at pH 6.5 a number of $4.45 \cdot 10^6$ and at pH 7.6 a number of $2.17 \cdot 10^6$ molecules of phloretin bound per erythrocyte. Thus, the result is in good agreement with the approximation given by LeFevre and Marshall [5].

The binding curve of phloretin is nearly linear for both pH values investigated, which can be demonstrated by the high correlation coefficients (r) of the linear regression analysis. At pH 6.5 an r value of 0.998 and at pH 7.6 of 0.996 was obtained. At pH 6.5 the *keto*-form is calculated to be 85.5% and at pH 7.6 this form is only 31.9%. If binding is related to the ketonic form, a reduction in binding of a factor 2.68 is expected. By comparing the slopes at pH 6.5 and 7.6 in

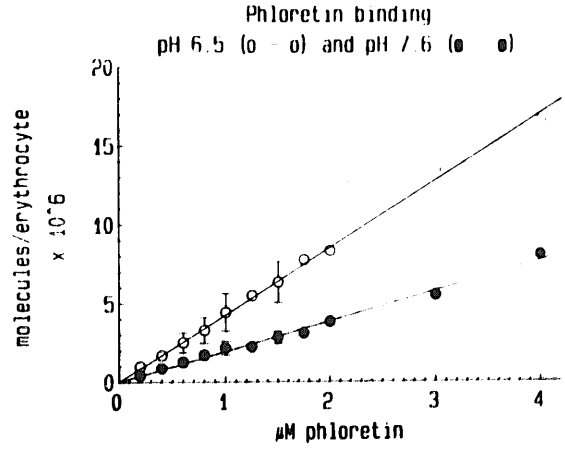

Fig. 7. Molecules phloretin bound *per* erythrocytes versus phloretin concentration at ph 6.5 (○——○) and pH 7.6 (●——●). Mean of three experiments ± S.D.

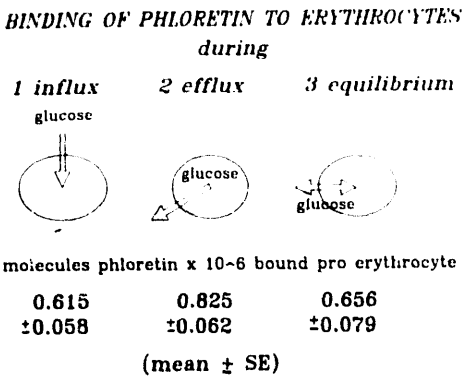

Fig. 8. Binding of phloretin under glucose influx, efflux and equilibrium condition.

Fig. 7, which represents the binding per μM of phloretin, a ratio of 2.21 was calculated from the respective experiments. This result supports the assumption of LeFevre and Marshall [5] that only the ketonic form of phloretin binds to the erythrocytes.

Influence of a glucose gradient on binding. In a number of experiments binding of phloretin to erythrocytes during glucose influx and efflux conditions and glucose equilibrium was investigated. In order to be in the sensitive region of the glucose transporter a low phloretin concentration of 0.2 μM at pH 6.5 was chosen, which is close to the K_i value. In Fig. 8 the molecules of phloretin bound per cell are shown during glucose influx, glucose efflux and glucose equilibrium conditions. In 30 paired experiments the difference in binding between efflux and influx conditions amounts to 210000 molecules per cell. This difference is 'extremely significant' by the paired two-tailed *t*-test resulting in a *P*-value of 0.0002.

Since under glucose influx conditions the outside glucose concentration is relatively high with 300 mM, the above difference might be explained by a glucose-phloretin competition at the transporter site. Therefore, phloretin binding was also measured after glucose efflux from the cells (glucose equilibrium conditions). Because of the small number of cells used in the experiments the outside glucose concentration is in the glucose equilibrium condition and during glucose efflux comperable with approx. 2 mM. In 16 parallel experiments binding under glucose equilibrium resulted in $(0.656 \pm 0.079$ (S.E.)) $\cdot 10^6$ against $(0.992 \pm 0.088$ (S.E.) $\cdot 10^6$ molecules phloretin per cell. Again the difference of 336000 molecules is 'very significant' with the two-tailed *P*-value of 0.0083 in the paired *t*-test. There is, however, no statistical difference between binding under glucose influx and glucose equilibrium conditions. Thus the difference can not be explained by glucose competition.

In addition we measured phloretin in the supernatant and calculated the binding to the erythrocytes. Again a significant difference of 171000 molecules phloretin bound to the erythrocytes between glucose efflux and equilibrium experiments and no statistically significant differences between glucose influx and equilibrium conditions has been measured.

Discussion

The inhibitory action of phloretin has not been tied so far to any particular transport process or chemical reaction. In the literature several energy-independent transport processes in erythrocytes have been reported to be inhibited by phloretin (reviewed in refs. 14–16), that are transports of hexoses, chloride, organic anions, glycerol, urea and methylurea.

In thin lipid membranes phloretin at relatively high concentrations dramatically increases cation conductance and decreases anion conductance in membranes treated with ion carriers [14]. From the fact that the enol form of phloretin is inactive it was concluded that the uncharged ketonic form of phloretin adsorbs near the lipid/water interface probably by the orientation of its own large dipole moment and introduces a dipole moment of opposite polarity to the existing dipoles of the phospholipid head groups. Thus the positive potential in the membrane interior is reduced and the lipid membrane becomes more permeable to cations and less permeable to anions.

Since membranes of erythrocytes as other cells contain extensive regions of lipid bilayers, phloretin should produce effects on them similar to those seen with artificial bilayers. This has been confirmed in general for erythrocytes [14], however, the concentrations of phloretin necessary were in the order of 250 μM in contrast to a few μM in artificial lipid bilayer. In accordance with the above hypothesis, the inhibitory potency of phloretin as well as a series of analogs on chloride permeability was highly correlated with their dipole moment and lipophilicity [14,16]. The apparent K_i value of phloretin in ox erythrocytes on chloride permeability was 2 μM and for phloroacetophenone 12.6 μM [16].

For the effects of phloretin on small nonelectrolyte permeability such as urea a biphasic effect was seen [15]. At 20 to 60 μM phloretin in the ketonic form an increase in permeability was noted, whereas at 100 μM the effect of phloretin shifted toward inhibition of urea permeability.

As shown by the low K_i values of 0.36 and 0.20 μM phloretin at pH 6.5 in our glucose and galactose efflux experiments, a high affinity of phloretin to the glucose transporter is demonstrated. In addition there is the fact that only efflux of glucose or galactose is inhibited by extracellular added phloretin. Binding of phloretin

at 0.2 μM and pH 6.5 is less than $1 \cdot 10^6$ molecules per cell (Fig. 7). This number is at least more than twice the number of glucose transporter per erythrocyte [8] and about the same number as anion carriers, namely $1.2 \cdot 10^6$ copies per erythrocyte [17]. However, by assuming a linear relationship between binding and inhibition of phloretin at 0.2 μM, only about 25% of the phloretin might be bound to the proteins of the glucose and the anion transporter. This rough estimate serves to show that even at this low phloretin concentration the lipid layer is probably also involved in phloretin binding.

Binding and inhibition of phloretin in glucose, galactose and anion transport follows closely the *keto-enol* tautomerism of phloretin in such a way, that only the ketonic form of phloretin binds to the erythrocyte and inhibits transport. The most favored conformations of the two tautomers exhibit different shapes, and only the ketonic form shows structural similarities to the glucose molecule. The result in inhibition and binding is in close agreement with the qualitative description of LeFevre and Marshall [5], but not with the result of Jennings and Solomon [18], who described a fast penetration of phloretin into the cell and binding mainly to hemoglobin in addition to high-affinity and low-affinity binding to the membrane. We could not detect an penetration of phloretin. The addition of excessive hemolysates in glucose exit experiments did not change the inhibitory potency of phloretin (results not shown). Thus a penetration of phloretin and an interference of hemoglobin with the glucose transporter site could not be detected. Also phloretin inside the cell should inhibit glucose influx [6], but this has not been observed.

The high affinity of phloretin to the glucose transporter is also shown by binding experiments during glucose efflux, glucose influx and in glucose equilibrium conditions. At a low concentration of 0.2 μM phloretin at pH 6.5, which is close to half saturation, there is a significant difference in binding of phloretin to erythrocytes under glucose efflux in comparision to glucose influx and equilibrium conditions. The difference in binding of about 200000 sites is in the order which is expected from the number of copies of glucose transporter per cell.

It can be assumed that only the glucose oriented transporter molecules conditioned by a glucose gradient from inside to outside binds phloretin externally at a transport specific site, which is also the configuration sensitive to inhibition. At equilibrium and in glucose influx conditions, even with an excess of glucose, binding of phloretin is significantly less and there are no differences in phloretin binding under equilibrium and influx. These results are in accordance with an asymmetry of inhibition by phloretin [1,3] and a glucose transporter protein which demonstrates allosteric properties in transport. Thus, phloretin is a highly specific inhibitor at a conformationally altered glucose transporter protein.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemie. It is a great pleasure to thank Professor K.J. Netter and E.S. Vessel for their most valuable comments and suggestions. Furthermore we are grateful to Mrs. H. Radler for her technical assistance.

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