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D-Glucose, forskolin and cytochalasin B affinities for the glucose transporter Glut1 Study of pH and reconstitution effects by biomembrane affinity chromatography

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Abstract

The affinities of D-glucose and the transport inhibitors, forskolin and cytochalasin B (CB), for Glut1 were studied by frontal affinity chromatography at pH 5–10 on sterically immobilized proteoliposomes with reconstituted human red cell glucose transporter Glut1. The affinity of D-glucose for Glut1 became slightly weaker as the pH was increased. The inhibitor affinities decreased and became immeasurably weak above pH 9. At pH 7.4, the dissociation constants were 44 m*M* for glucose, 1.8 μ*M* for forskolin and 72 n*M* for CB. The affinities of these solutes for Glut1 in red cell membrane vesicles and particularly for Glut1 in red cells were higher, as shown by chromatographic analyses.

Keywords: Glucose transporters; Biomembrane affinity chromatography; pH effects; Glucose; Forskolin; Cytochalasin B; Glut1; Proteins

1. Introduction

The human red cell D-glucose transporter, Glut1, is a transmembrane protein that mediates glucose diffusion across the cell membrane [1–3]. Useful tools in studies of Glut1 have been the transport inhibitors cytochalasin B (CB) [4,5], which binds at the inward-facing glucose binding site of Glut1 [6], and the diterpene, forskolin [6–9]. These inhibitors compete with D-glucose and with each other for binding to Glut1 [4–6,8,9]. Glucose-displaceable binding of forskolin to Glut1 in human red cell membranes (ghosts) has been analyzed by centrifugation methods [8,9].

In previous work from our research group, pH effects were observed on the transport retention of D-glucose on Glut1 proteoliposomes and the transport activity of reconstituted Glut1 [10], and on retardation of CB on Glut1 proteoliposomes [11]. For analyses of pH effects on the glucose affinity for the inward-facing binding site of Glut1, and on the competing affinities of forskolin and CB, zonal or frontal affinity chromatography on proteoliposomes immobilized in gel beads by freeze–thawing is well suited. We have named this and related methods biomembrane affinity chromatography (BAC). Several applications have been reported [11–15].

In this work, we used BAC in the frontal mode to study the effects of pH on glucose, forskolin and CB affinities for Glut1 and on the number of operative CB binding sites, and revealed effects of reconstitution of Glut1. The affinities at pH 7.4 of the above

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solutes for reconstituted non-purified and purified Glut1, Glut1 in red cell membrane vesicles depleted of peripheral proteins and Glut1 in red cells [14] were determined. The use of BAC analysis for all systems allowed a reliable comparison.

2. Experimental

2.1. Materials

Forskolin (7β-acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one) (98%) was bought from Sigma (St. Louis, MO, USA) and [4-^{3}H]CB was from NEN Life Science Products (Boston, MA, USA). The commercial sources of other materials were as listed in Ref. [15] or as described below.

2.2. Preparation and steric immobilization of membrane vesicles and proteoliposomes

Human red cell concentrate (five–six weeks old), tested for the absence of viral infection, was bought from the Blood Bank of the Uppsala University Hospital (Uppsala, Sweden). Red cell membranes were prepared and stripped of peripheral proteins, whereupon membrane vesicles were formed [16]. A P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for the chromatographic elutions. Glut1 and other membrane components were solubilized with octyl glucoside. Non-solubilized material was removed by ultracentrifugation to obtain non-purified Glut1, from which purified Glut1 was prepared by ion-exchange chromatography at pH 7.4. Non-purified or purified Glut1 was reconstituted with egg yolk phospholipids (70% phosphatidylcholine, 21% phosphatidylethanolamine) to obtain proteoliposomes (denoted NP-Glut1 or P-Glut1, respectively) and the proteoliposomes were concentrated [11]. Membrane vesicles and concentrated NP-Glut1 and P-Glut1 proteoliposomes were immobilized in Superdex 200 prep-grade gel beads [11,15]. The materials were washed five times by centrifugation at 350 *g* with eluent of pH 7.4 and packed at 1.0–1.5 ml/min for 30–60 min in 0.5 cm I.D. glass columns (HR 5/2 or 5/5, Pharmacia Biotech) to obtain a 0.34-ml vesicle gel bed or 0.82–0.94 ml proteoliposome gel beds.

2.3. Preparation and immobilization of non-freeze–thawed small proteoliposomes by use of hydrophobic ligands

Hydrophobic ligands were attached to Superdex 200 prep-grade gel beads essentially as described previously for other gels [10,17,18] to obtain an estimated ligand concentration of 4 μmol/ml of gel bed. Non-freeze–thawed and non-concentrated small P-Glut1 proteoliposomes (10 ml) were prepared by gel filtration as described in Ref. [15]. Derivatized gel was mixed for 20 h with these proteoliposomes and was packed into a 0.82-ml gel bed in a HR 5/5 column. Non-immobilized proteoliposomes were circulated through the gel bed for 20 h at room temperature. A control gel bed was prepared as described in Section 2.2 by freeze–thawing with concentrated proteoliposomes prepared as above from the same batch of purified Glut1 as used for the above gel bed (see Table 1, below).

2.4. Immobilization of red cells

Fresh human blood from healthy adult donors, collected in EDTA tubes and tested for the absence of viral infection, was bought from the Blood Bank of the University Hospital. The plasma and the buffy coat were removed and the red cells were washed and immobilized in particles of a gel synthesized from derivatized acrylamide monomers, as described in Ref. [14].

2.5. Quantitative frontal affinity chromatography

The BAC system and the conditions used were as described in Refs. [12,15]. The eluents contained 150 m*M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* dithioerythritol and 10 m*M* citric acid–NaOH (pH 4–6.4), 10 m*M* Tris–HCl (pH 7.4–8.7), 10 m*M* Tris–NaOH (pH 9.1) or 10 m*M* glycine–NaOH (pH 9.4 and 10). For analyses with red cells, the eluents and conditions were as described in Ref. [14]. Forskolin (0–10 μ*M*) or D-glucose (0–100 m*M*) was present as a competitor in runs of 15–40 ml samples with 1 n*M* [^{3}H]CB. For runs in the absence of competitor, samples containing 1 n*M* [^{3}H]CB were supplemented with CB to a concentration of 1–80 n*M*. The temperature was 23°C. The elution profiles were

Table 1
Dissociation constants at pH 7.4 for solute interactions with human red cell Glut1 in immobilized red cells, red cell membrane vesicles depleted of peripheral proteins or proteoliposomes (NP-Glut1 or P-Glut1)

Solute	K_d			
	Cells	Membrane vesicles	Reconstituted NP-Glut1	Reconstituted P-Glut1
D-Glucose (m*M*)	7[a]	19	43	45[b]; 55[c]
Forskolin (μ*M*)	0.7[d]	1.7	1.7	1.8
CB (n*M*)	33	52	72	72

Estimated relative error limit: ±10%.
[a] Value from Ref. [14].
[b] Average of the value of 35 m*M* obtained in the present work and five values giving an average value 48 m*M*, reported in Ref. [12].
[c] Value obtained with small P-Glut1 proteoliposomes immobilized on hydrophobic ligands. The same P-Glut1 batch was used as for the freeze–thawed P-Glut1 proteoliposomes that gave the value of 35 m*M* cited in footnote b.
[d] With forskolin as the sample and glucose as the competitor, a value of 1.8 μ*M* was obtained previously [14].

similar to those illustrated in Ref. [15]. The release of Glut1 from a P-Glut1 gel bed over a period of three months of runs was 25%, according to amino acid analyses as described in Refs. [10,15]. Most of the loss occurred over the first few days after immobilization [12,15]. The BAC analyses were done when the column had stabilized.

Dissociation constants and the amount of operative CB binding sites (CB binding activity) were calculated as described in Ref. [15] by use of the theory adapted from Ref. [19]. In the present work, [A] in Eqs. (1) and (2) of Ref. [15] represents the concentration of D-glucose or forskolin. The later theory by Winzor and Jackson [20] was not used (see Ref. [12]).

3. Results

3.1. Effects of pH on D-glucose, forskolin and CB interaction with Glut1

Egg phospholipid proteoliposomes with reconstituted Glut1 were sterically immobilized in gel beads and used as a stationary phase for studies of the affinities of D-glucose and the transport inhibitors forskolin and CB for Glut1 by BAC in the frontal mode.

The affinity of glucose for Glut1 decreased with increasing pH, particularly between pH 5 and 5.5. The dissociation constant, K_d(glc), was 31 m*M* at pH 5 and 48 m*M* at pH 8.7 (Fig. 1). At pH values of 9.1, 9.4 and 10, the affinity of CB for the transporter was very weak, as shown by small retardations, and no K_d value could be determined. The inactivation at these pH values was reversible; the activity of Glut1 at pH 5 and 7.4 could be regained. At pH 4, irreversible inactivation occurred, consistent with earlier results [10,11].

The dissociation constant for forskolin interaction with NP-Glut1 was essentially constant over the pH range 5–7, but increased strongly from pH 8 to 8.7 (Fig. 2A). For P-Glut1 (Fig. 2B), the increase at high pH values was smaller. The affinity of CB for NP-Glut1 and P-Glut1 decreased in a manner similar to that of forskolin with increasingly high pH values (Fig. 2 C,D).

Figs. 1 and 2 together indicate that from pH 5 to 5.5, the forskolin and CB affinities for Glut1 were constant, whereas the glucose affinity decreased. In contrast, the inhibitor affinities were more sensitive to high pH than was the glucose affinity.

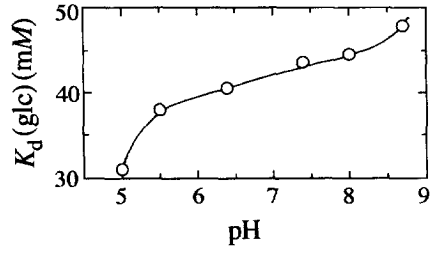

Fig. 1. K_d values versus pH for D-glucose interaction with Glut1 in NP-Glut1 proteoliposomes. Single determinations with estimated error limits ±2 m*M*.

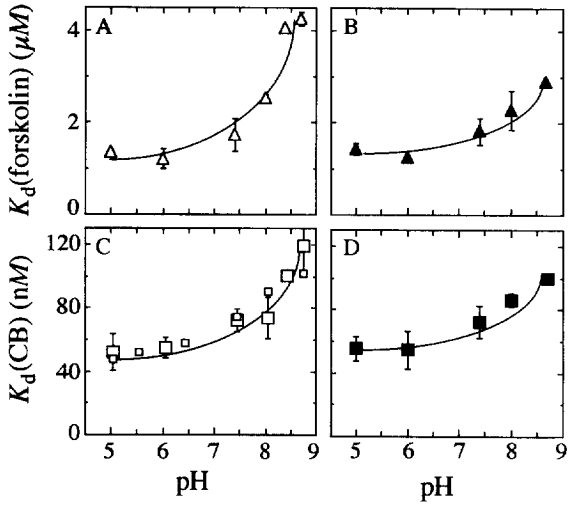

Fig. 2. K_d values versus pH for (A, B) forskolin and (C, D) CB interaction with Glut1 in (A, C) NP-Glut1 (open symbols) and (B, D) P-Glut1 proteoliposomes (closed symbols). CB was applied as the sample and forskolin as the competitor in the eluent (except for values represented by small squares, which were obtained with D-glucose as the competitor; experiments illustrated in Fig. 1). Average values from determinations on two–three columns, except that a single determination was done at pH 8.4 in (A) and (C). In some cases, the error limits fall within the symbols.

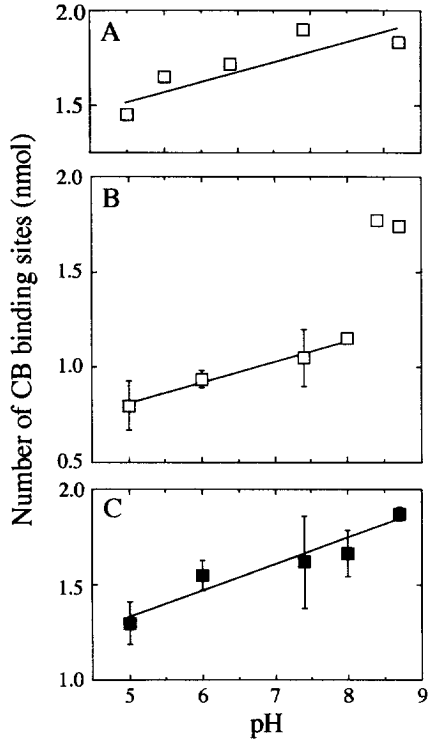

Fig. 3. The number of operative CB binding sites determined on NP-Glut1 with (A) D-glucose and (B) forskolin as the competitor, and (C) on P-Glut1 with forskolin as the competitor. Three NP-Glut1 columns were used, a–c. The values shown in (A) were determined on column c and those in (B) on columns a–c (a, b at pH 5–7.4; b, c at pH 8 and 8.7, c at pH 8.4). In (A, B), single determinations were done at pH 8 and 8.4, otherwise two–four determinations were performed. In (B), the values from columns a and c were normalized to agree at pH 7.4 and 8, respectively, with the values for column b. For this purpose, the values from column c were multiplied by 0.70. The glucose-displaceable CB sites in (A) can be compared with the forskolin-displaceable CB sites in (B) after multiplication by this factor. In (C), values from two P-Glut1 columns were normalized to agree at pH 7.4. Two or more determinations were done for each point.

The number of operative NP-Glut1 CB binding sites increased slightly from pH 5 to pH 8.7 in analyses with D-glucose as the competitor (Fig. 3A). A large reversible increase in the number of CB binding sites was seen from pH 8 to 8.4 with forskolin as the competitor (Fig. 3B). The reason for this has not yet been studied.

The molar ratio between the amounts of CB binding sites at pH 7.4 and P-Glut1 monomers was about 0.34, in agreement with previous values [12,15].

3.2. Effects of reconstitution on D-glucose, forskolin and CB interaction with Glut1: Comparison of red cells, membrane vesicles and proteoliposomes

At pH 7.4, the K_d values for D-glucose, forskolin and CB interactions with Glut1 were determined by BAC on red cells, membrane vesicles and Glut1 proteoliposomes. The results are shown in Table 1. The glucose affinity was high for Glut1 in red cells, lower with membrane vesicles and lowest with the proteoliposomes. Glucose showed the same affinity for NP-Glut1 and P-Glut1 in proteoliposomes that had become larger upon steric freeze–thawing immobilization [average K_d(glc) 44 mM], but had somewhat weaker affinity for Glut1 in small P-Glut1 proteoliposomes immobilized using hydrophobic ligands directly after gel filtration reconstitution.

Forskolin and CB showed low K_d values on the immobilized red cell column, which indicates that,

like glucose, these inhibitors bind most strongly to Glut1 in the native membrane. Forskolin had about the same K_d value for interaction with Glut1 in the proteoliposomes as for Glut1 in the membrane vesicles. The affinity of CB for Glut1 in the vesicles was intermediate, again making the affinity to reconstituted Glut1 the weakest (Table 1).

4. Discussion

We obtained lower K_d values for all the solutes, glucose, forskolin and CB, on immobilized red cells than on proteoliposomes and membrane vesicles, at pH 7.4 (Table 1). We propose that the lipid environment, exposure of Glut1 to detergent upon solubilization, the orientation of the transporter (essentially random in the proteoliposomes) and the membrane curvature are key factors affecting the solute–Glut1 affinities. The reconstitution methodology must be improved to produce a reconstituted system with protein activities that are identical to those in the native cell membranes.

The K_d values of D-glucose–Glut1 interaction increased only slightly from pH 5.5 to pH 8.0 (Fig. 1), in agreement with the fact that glucose efflux and influx parameters in red cells are essentially constant in the pH range of 5.5–8.5 [21]. However, glucose permeability and influx maxima have been observed at pH 7.2 [22] and 7.5 [23], respectively.

Glucose-displaceable binding of forskolin to Glut1 in human red cell membranes (ghosts) was found to have a K_d value of 2.6 μM at pH 8 (I=0.015) [9] and was 1.8 μM in red cells at pH 7.4 [14]. However, a value of 0.7 μM was obtained for binding of forskolin, determined by forskolin-displacement of CB in red cells at pH 7.4 (Table 1).

The dissociation constants of forskolin and CB increased above pH 7.4 (Fig. 2). CB has been proposed to bind to Glut1 through three hydrogen bonds and to be hydrophobically anchored by the C13–C19 region [4]. Glut1 becomes negatively charged above the isoelectric point, 8.0 [24], to acquire a net charge of approximately −5 at pH 9 [10,25], where the affinity of CB for Glut1 became too weak to be determined. It seems that titration of one or more amino acid side chains with pK_a value(s) of around nine affects the conformation of Glut1 and/or binding of CB to Glut1.

The increase in the number of CB binding sites of NP-Glut1 (Fig. 3B) but not of P-Glut1 (Fig. 3A), found above pH 8 with forskolin as the inhibitor, may reflect either a change in the association state of Glut1 in NP-Glut1, or presentation of a number of weak-affinity forskolin-displaceable binding sites of some protein(s) other than Glut1 among the NP-Glut1 membrane proteins. The fact that the affinities of forskolin and CB for NP-Glut1 at pH 8.7 were relatively low (Fig. 2) may be due to similar reasons.

Glucose transport retention chromatography (retardation of D-glucose relative to L-glucose by uptake only of D-glucose into proteoliposomes) [10,18] has been used to show that protonation of amino acid side chains of $pK_a \approx 4.4$ inactivated Glut1 irreversibly, probably by way of conformational changes. This corresponds to the great increase in Glut1 electrostatic charge as the pH is decreased below pH 5 [25]. At pH 5, the affinity of glucose for Glut1 was stronger than at pH 5.5. Interestingly, the glucose transport (as judged by transport retention) was reversibly decreased at pH 5 compared to the level in the pH range 5.5–10 [10]. The glucose affinity for Glut1 and, presumably, also the Glut1 conformation, were essentially retained in the pH range 5.5–8, over which the net electric charge of Glut1 is small and nearly constant [10,25].

5. Conclusions

Quantitative frontal affinity chromatography with immobilized membrane structures revealed that the affinities of glucose and the inhibitors, forskolin and CB, for Glut1 decreased with increasing pH. Analysis of immobilized cells showed that the solute affinities for Glut1 in a native environment were stronger than for Glut1 in membrane vesicles or reconstituted systems. All the immobilized materials were stable enough to give reasonable accuracy.

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