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Adenosine and adenosine triphosphate modulate the substrate binding affinity of glucose transporter GLUT1 in vitro

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Abstract

Evidence indicates that a large portion of the facilitative glucose transporter isoform GLUT1 in certain animal cells is kept inactive and activated in response to acute metabolic stresses. A reversible interaction of a certain inhibitor molecule with GLUT1 protein has been implicated in this process. In an effort to identify this putative GLUT1 inhibitor molecule, we studied here the effects of adenosine and adenosine triphosphate (ATP) on the binding of D-glucose to GLUT1 by assessing their abilities to displace cytochalasin B (CB), using purified GLUT1 in vesicles. At pH 7.4, adenosine competitively inhibited CB binding to GLUT1 and also reduced the substrate binding affinity by more than an order of magnitude, both with an apparent dissociation constant (K_D) of 3.0 mM. ATP had no effect on CB and D-glucose binding to GLUT1, but reduced adenosine binding affinity to GLUT1 by 2-fold with a K_D of 30 mM. At pH 3.6, however, ATP inhibited the CB binding nearly competitively, and increased the substrate binding affinity by 4–5-fold, both with an apparent K_D of 1.22 mM. These findings clearly demonstrate that adenosine and ATP interact with GLUT1 in vitro and modulate its substrate binding affinity. They also suggest that adenosine and ATP may regulate GLUT1 intrinsic activity in certain cells where adenosine reduces the substrate-binding affinity while ATP increases the substrate-binding affinity by interfering with the adenosine effect and/or by enhancing the substrate-binding affinity at an acidic compartment.

Keywords: GLUT1; Adenosine; Adenosine triphosphate; Glucose; Cytochalasin B; Equilibrium binding

1. Introduction

The uptake and release of glucose by animal cells is catalyzed by a family of intrinsic membrane proteins, known as the facilitated-glucose transporter or GLUTs, and further regulated by metabolites and/or hormones [1–3]. One well-known mode of acute regulation of GLUT function is the stimulation of glucose uptake by insulin in muscle and adipose cells due to the recruitment of GLUT4 and GLUT1 from intracellular storage sites to the plasma membrane [4,5]. A less well-known though equally important mode of acute GLUT regulation is the stimulation of glucose uptake seen in response to hypoxia and other increased metabolic demands in many animal cells, including avian erythrocytes [6,7], cardiac

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myocytes [8], skeletal muscles [9], clone 9 cells [10] and L6 myocytes [11]. All of these cells express GLUT1 and the stimulation occurs without any increase in the plasma membrane GLUT1 contents, indicating that it is due to an increased catalytic activity of individual GLUT protein [12]. Isoproterenol inhibits and adenosine enhances the insulin stimulated glucose transport in rat adipocytes presumably by modulating intrinsic activity of GLUT protein [13]. These two acute modes of GLUT regulation, however, are apparently not mutually exclusive, and increased catalytic activity of individual GLUT proteins may also in part contribute to the insulin-induced stimulation of glucose uptake by muscle and adipocytes [14–17].

How the intrinsic activities of GLUT proteins are modulated is not well understood. It may involve proteins that modulate GLUT covalently or non-covalently. Phosphorylation of GLUT4 has been shown to decrease its catalytic activity [18]. A study with clone 9 cells [19], on the other hand, has suggested the presence of an inhibitory or masking protein for GLUT1 that interacts non-covalently and results in inactivation of the bound GLUT1, while exposure to azide dissociates the masking protein from GLUT1, leading to activation. A similar role for F-actin as a masking protein for GLUT1 in human erythrocytes has been indicated [20,21]. Possible involvement of small molecules other than proteins in intrinsic activity regulation of GLUT proteins has also been suggested. These include apparent stimulation of GLUT intrinsic activities by adenosine triphosphate (ATP) [22,23], adenosine diphosphate [24], and cadmium [25,26].

In the present study, we have examined the interaction of adenosine and ATP with purified GLUT1 protein and their effects on GLUT1 substrate and inhibitor-binding affinities using D-glucose and cytochalasin B (CB), respectively. We show that adenosine competitively inhibits GLUT1 CB binding and also drastically reduces GLUT1 D-glucose-binding affinity. ATP, on the other hand, had little effect on either glucose or CB binding to GLUT1 at pH 7.4, although it caused a significant increase in the substrate-binding affinity at pH 3.5. Possible significance of these in vitro interactions of adenosine and ATP with GLUT1 in the physiological regulation of GLUT1 intrinsic activity is discussed.

2. Materials and methods

2.1. Materials

Cytochalasin B, D-glucose, adenosine and ATP were purchased from Sigma (St. Louis, MO). [^{3}H]Cytochalasin B was purchased from Amersham. BSA was from Boehringer and collagenase from Warthington.

2.2. Purification and characterization of GLUT1 protein

Human erythrocyte glucose transporter, GLUT1 was purified under reducing conditions using recently outdated human whole blood (obtained from the American Red Cross, Buffalo, NY) and reconstituted in vesicles as described earlier [27]. The GLUT1 in vesicles thus prepared is typically 97% pure in terms of protein staining in sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolution, and 75–80% functional in terms of cytochalasin B binding activity with the specific binding activity of 14.5–15 nmol/mg protein. The GLUT1 in these vesicles is approximately 50% cytoplasmic side-in and 50% cytoplasmic side-out orientations (Pinkofsky and Jung, unpublished data).

2.3. Equilibrium CB binding assay in the presence of glucose, adenosine and/or ATP

Equilibrium binding of CB in the absence and presence of specified concentrations of D-glucose, adenosine, or ATP were measured as described [26,28] using purified GLUT1 in vesicles. D-Glucose, adenosine and ATP were added to purified GLUT1 in vesicle suspension and the suspension was subjected to three cycles of freeze–thawing (each cycle consisted of 2 min in liquid nitrogen followed by 10 min at room temperature). This procedure was shown to effectively introduce the ligands into intra-vesicular space and result in intra-vesicular concentrations indistinguishable from those of the suspension (not shown). Binding assays were done using either a fixed (10^{-7} M) or six different CB concentrations (10^{-8}–10^{-5} M) and a fixed tracer amount of [^{3}H]cytochalasin B, and at room temperature and at either pH 7.4 or 3.6 as specified. Radioactivities were

measured in an LKB 1209 Rackbeta Liquid Scintillation spectrometer. Unless otherwise mentioned, 25 μg of purified GLUT1 protein was used in 1 ml of the binding assay mixture. CB bound to GLUT1 was expressed in percent of the total amount of CB in the binding assay mixture.

2.4. Data analysis by multiple association model

Analysis of CB binding in the presence of ATP or adenosine, and glucose, was carried out as described [26] with slight modifications. Briefly, a simple multiple association is assumed in which a complex can form between GLUT1 (denoted by the symbol T) and CB, glucose, ATP, or adenosine. Thus,

$$[TC] = [T] \times [C] / K_C$$

$$[TG] = [T] \times [G] / K_G$$

$$[TX] = [T] \times [X] / K_X$$

Here G and C denote glucose and CB, respectively, and X denotes either Adenosine or ATP, depending upon which system is under study. The K's are the respective dissociation constants for the binding reactions, while brackets indicate concentrations. These relationships can be conveniently re-expressed in terms of concentrations normalized by the corresponding dissociation constant; for example

$$[TC] = [T] \times \underline{C} \text{ where } \underline{C} \equiv [C] / K_C$$

Similar notations are used for the normalized concentrations of glucose and X, such that $\underline{G} \equiv [G] / K_G$ and $\underline{X} \equiv [X] / K_X$.

In addition, it is assumed that ternary complexes, involving different ligands, can form in accord with the following scheme:

$$[TCX] = [TX] \times [C] / K_{CX} = [TX] \times [C] \times \alpha_{CX} / K_C$$

Thus, the coefficient α_{CX} expresses the effect that binding of X has upon the subsequent association with CB: a value of α_{CX} being zero ($\alpha_{CX} = 0$), for example, means formation of the ternary complex is precluded, and X and CB are strictly competitive. $\alpha_{CX} = 1$, on the other hand, indicates the presence of X has no effect upon the binding affinity of CB. From thermodynamic considerations, the association of GLUT1 with CB must have the identical effect upon the binding characteristics of X.

With the extension of these definitions to the other complex forms, we then obtain the set of relations:

$$[TC] = [T] \times \underline{C}$$

$$[TG] = [T] \times \underline{G}$$

$$[TX] = [T] \times \underline{X}$$

$$[TXC] = [T] \times \underline{X} \times \underline{C} \times \alpha_{CX}$$

$$[TXG] = [T] \times \underline{X} \times \underline{G} \times \alpha_{GX}$$

$$[TCG] = [T] \times \underline{C} \times \underline{G} \times \alpha_{CG}$$

The total number of GLUT1 moieties, T_T, is given by the sum of all complex forms and free T. Then

$$T_T = [T] + [TC] + [TG] + [TX] + [TXC] + [TXG] + [TCG] = [T](1 + \underline{C} + \underline{G} + \underline{X} + \underline{X} \times \underline{C} \times \alpha_{CX} + \underline{X} \times \underline{G} \times \alpha_{GX} + \underline{C} \times \underline{G} \times \alpha_{CG}) \quad (1)$$

The amount of CB bound, C_{Bnd}, can be expressed by the sum of all forms containing CB:

$$C_{Bnd} = [T](\underline{C} + \underline{X} \times \underline{C} \times \alpha_{CX} + \underline{C} \times \underline{G} \times \alpha_{CG}) \quad (2)$$

Taking the ratio between these equations, with a little rearrangement, yields

$$\frac{T_T}{C_{Bnd}} = 1 + \frac{1 + \underline{G} + \underline{X} + \underline{X} \times \underline{G} \times \alpha_{GX}}{\underline{C}(1 + \underline{X} \times \alpha_{CX} + \underline{G} \times \alpha_{CG})} \quad (3)$$

This equation can be further rearranged into

$$[C](T_T / C_{Bnd} - 1) = K_C \frac{1 + \underline{X} \times \underline{G}(1 + \underline{X} \times \alpha_{GX})}{1 + \underline{X} \times \alpha_{CX} + \underline{G} \times \alpha_{CG}} \equiv K_C' \quad (4)$$

The expression on the left-hand side, composed of experimentally measured quantities, provides a convenient form in which the binding data can be expressed, taking the form of an apparent dissociation constant for the binding of CB to GLUT1. The manner in which this apparent coefficient, K_C', varies with glucose and X (ATP or adenosine) provides a means of estimating the values of the α's, as well as the dissociation constants for glucose and X.

Table 1
Equilibrium binding of CB to GLUT1, measured and expressed as the percentage of CB bound to GLUT1 (% Bnd), as described in Section 2

A. Effects of adenosine on CB binding at pH 7.4

[CB]	0.01		0.10		0.30		1.0		3.0		10.0	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[Adenosine]												
0	69.7	0.209	66.6	0.207	59.7	0.204	36.6	0.197	14.7	0.221	5.1	−0.63
0.1	66.0	0.237	63.5	0.228	56.1	0.229	33.8	0.240	15.7	−0.050	5.1	−0.95
1	61.2	0.292	58.1	0.290	51.3	0.291	30.9	0.337	15.1	0.039	5.2	−1.03
3	54.3	0.387	50.7	0.398	46.3	0.373	28.9	0.420	13.6	0.335	5.5	−1.47
10	34.8	0.863	33.2	0.859	29.4	0.895	16.2	1.546	11.6	0.869	4.4	0.43
20	23.3	1.51	22.0	1.55	21.7	1.43	16.6	1.47	9.6	1.62	4.7	−0.26

B. Effects of adenosine and D glucose on CB binding at pH 7.4

[Glucose]	0		3		10		30		100		300	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[Adenosine]												
0	69.5	0.206	67.8	0.224	64.4	0.263	57.3	0.360	41.8	0.694	25.1	1.537
1	63.4	0.275	62.4	0.287	59.3	0.330	52.9	0.434	37.8	0.826	25.0	1.546
3	58.3	0.417	53.3	0.427	48.6	0.519	46.3	0.573	34.9	0.940	22.9	1.737
5	48.4	0.535	48.9	0.524	45.2	0.612	41.8	0.708	29.0	1.276	17.0	2.602
7.5	41.1	0.729	41.4	0.720	40.3	0.755	36.7	0.885	28.0	1.342	19.0	2.264
10	36.7	0.870	35.7	0.909	35.8	0.906	32.4	1.061	26.0	1.460	18.2	2.342
10[a]	39.3	0.789	38.3	0.824	37.6	0.850	33.9	1.006	27.7	1.363	18.4	2.358
20	23.3	1.533	22.0	1.656	21.7	1.690	16.6	2.373	9.6	4.529	4.7	9.774
20[a]	23.6	1.704	22.8	1.785	23.4	1.724	21.2	1.966	17.7	2.475	12.6	3.728

C. Effects of adenosine on CB binding at pH 3.6

[CB]	0.01		0.10		0.30		1.0		3.0		10.0	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[Adenosine]												
0	60.0	0.208	56.3	0.203	46.8	0.202	24.9	0.208	9.9	0.206	3.9	−1.67
5	51.0	0.301	46.4	0.314	39.5	0.305	22.7	0.311	9.8	0.227	3.7	−1.38
7.5	45.3	0.369	43.3	0.350	36.2	0.355	21.0	0.378	9.2	0.342	3.4	−0.88
10	38.6	0.423	35.8	0.420	30.1	0.419	17.6	0.439	7.9	0.397	3.2	−1.51
20	27.5	0.705	26.2	0.687	22.5	0.700	14.0	0.802	7.4	0.610	3.1	−1.33

D. Effects of adenosine and D glucose on CB binding at pH 3.6

[Glucose]	0		3		10		30		100		300	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[Adenosine]												
0	62.5	0.205	61.2	0.246	56.6	0.302	55.0	0.323	28.5	1.057	23.9	1.357
5	56.8	0.299	52.3	0.363	47.3	0.449	38.0	0.672	20.5	1.666	11.0	3.552
7.5	52.9	0.354	47.8	0.439	41.6	0.573	31.7	0.901	16.7	2.161	9.8	4.066
10	49.6	0.407	46.3	0.468	41.7	0.571	31.0	0.933	20.6	1.655	8.6	4.697
20	36.4	0.723	33.9	0.811	31.4	0.915	22.2	1.499	13.3	2.847	6.0	6.912

E. Effects of ATP on CB binding at pH 3.6

[CB]	0.01		0.10		0.30		1.0		3.0		10.0	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[ATP]												
0	60.1	0.207	56.4	0.203	46.7	0.204	24.9	0.207	9.8	0.224	3.6	−1.17
5	23.5	0.870	24.0	0.778	20.8	0.790	13.6	0.858	7.0	0.771	3.0	−1.11
7.5	23.2	0.855	20.3	0.939	18.3	0.916	12.0	1.028	6.8	0.789	2.9	−1.09
10	19.0	1.10	16.7	1.21	15.3	1.18	10.9	1.23	6.3	1.09	3.0	−1.40
20	16.1	1.30	14.1	1.44	13.3	1.38	10.1	1.32	5.5	1.47	2.8	−1.04

Table 1 (*continued*)

F. Effects of ATP and D glucose on CB binding at pH 3.6

[Glucose]	0		3		10		30		100		300	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[ATP]												
0	58.0	0.204	55.0	0.233	47.8	0.320	36.9	0.518	20.9	1.21	12.2	2.35
5	29.4	0.748	20.8	1.22	12.4	2.32	6.5	4.78	2.6	12.4	1.5	21.9
7.5	28.2	0.796	19.1	1.36	11.6	2.51	5.3	5.98	2.4	13.6	1.4	23.7
10	20.1	1.28	12.8	2.23	7.4	4.19	3.6	9.01	1.9	17.9	1.1	30.2
20	7.4	4.17	3.5	9.20	1.9	17.2	0.7	47.4	0.6	55.3	0.4	80.5

G. Effects of ATP and adenosine on CB binding at pH 7.4

	Run 1		Run 2		Run 3	
	% Bnd	K_C'	% Bnd	K_C'	% Bnd	K_C'
[ATP]						
0	28.1	1.52	29.0	1.52	28.8	1.54
10	30.4	1.35	31.7	1.34	30.8	1.39
20	32.5	1.22	35.8	1.10	33.3	1.24
30	32.0	1.25	36.0	1.09	33.7	1.21
40	33.8	1.15	35.7	1.11	34.3	1.18
50	33.5	1.17	37.0	1.04	35.2	1.13

[Adenosine] = 20 mM

Measurements were done in the absence or presence of D-glucose (B, D, F and G), adenosine (A, B, C, D and G) and/or ATP (E, F and G), whose concentrations are shown in mM. In measurements A, C and E, six different concentrations of CB, specified in μM, were used. In measurements B, D, F and G, a single CB concentration of 0.10 μM was used. Also shown is the corresponding *apparent* dissociation constant for CB binding (K_C') as defined in Eq. 4 of the text.

[a]Duplicate runs.

3. Results

3.1. Effects of adenosine and ATP on equilibrium CB binding to GLUT1 in vitro

The association of adenosine and ATP with the GLUT1, and the effect these moieties have upon glucose binding to the transporter, are studied by measuring the effects of these ligands on equilibrium CB binding to GLUT1, at pH of 7.4 and 3.6. Results were expressed as the percent of CB bound to GLUT1 and shown in Table 1. Data include the CB binding measured in the presence of varying concentrations of adenosine or ATP with varying concentrations of CB (Table 1A,C,E). Data also include the CB binding as affected by an increasing concentration of D-glucose in the presence of varying concentrations of either adenosine or ATP, measured at a single CB concentration (10^{-7} M) (Table 1B,D,F). The last set of the data (Table 1G) illustrates the CB binding in the presence of varying ATP concentrations and a fixed concentration (20 mM) of adenosine measured at a fixed CB concentration of 10^{-7} M. Also included in this table are calculated apparent CB dissociation constants, K_C', as defined in Eq. 4 above. It is clear in this table that adenosine at both pH 7.4 and 3.6, and ATP at pH 3.6 affect both CB binding to GLUT1, and the displacement of CB by D-glucose significantly. The case of ATP at pH 7.4 represents a special circumstance, in that no experimentally significant effect of ATP upon either CB binding, or glucose displacement of CB, was observed at this physiological pH (data not shown in Table 1). However, since ATP is known to quench intrinsic tryptophan fluorescence of purified GLUT1 at pH 7.4 [22], this would indicate that while ATP binds to GLUT1 at this pH, it has no effect upon either the CB- or glucose-binding processes or their interaction ($\alpha = 1$ in each case). On the other hand, an interaction between adenosine and ATP binding at pH 7.4 is clearly observed (Table 1G). This behavior provides the basis for estimating the dissociation constant for ATP at pH 7.4 (vide infra).

Data obtained earlier [20,26,29] indicate that CB and glucose are competitive in their association with GLUT1. That finding is reproduced in the present

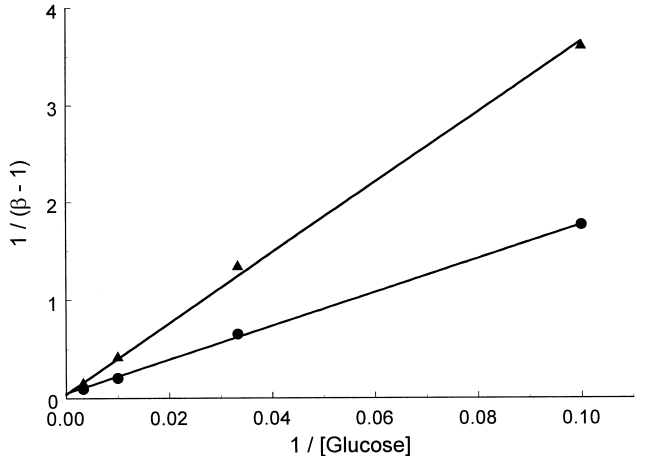

Fig. 1. Binding of D-glucose to GLUT1 at pH 7.4 (▲) and 3.6 (●), as computed from the data of CB binding to GLUT1 measured in the absence and presence of D-glucose at varying concentrations. The computation assumes the simple association of the ligands as shown in Eq. 5. Lines are drawn based on the least square linear regression analyses (AXUM, Version 5.0) and represent $y = 0.080 \pm 0.05 + (35.5 \pm 0.90)x$ (▲), and $y = 0.045 \pm 0.02 + (17.25 \pm 0.32)x$ (●). Each point represents the average of three independent measurements. Note that the y-axis intercept is $\alpha_{CG}/(1-\alpha_{CG})$, while the slope gives $K_G/(1-\alpha_{CG})$, as shown in Eq. 5.

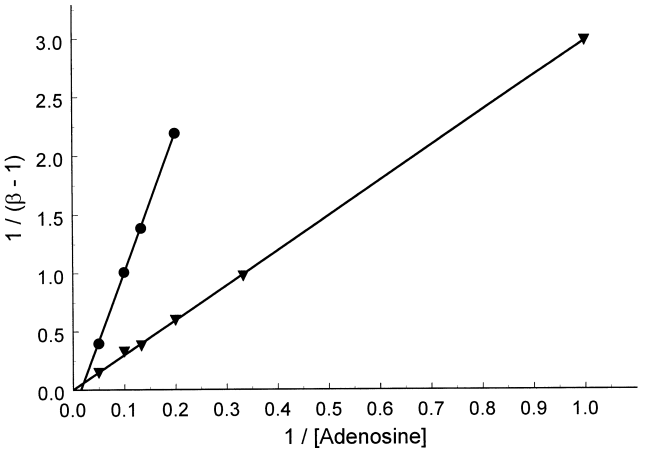

Fig. 2. Binding of adenosine to GLUT1 at pH 7.4 and 3.6, computed from the data of CB binding to GLUT1 measured in the absence and presence of varying concentrations of adenosine. The function $1/(\beta-1)$ was computed and plotted against reciprocal adenine concentration, according to Eq. 8 as detailed in the text, where the plot gives $\alpha_{CA}/(1-\alpha_{CA})$ at the y-axis intercept, and the slope of the plot is proportional to K_A. Each data point represents the average of three independent measurements at pH 7.4 (▼) and 3.6 (●). Lines are the least square linear regression analyses (AXUM, Version 5.0) and represent $y = -0.002 \pm 0.010 + (2.98 \pm 0.023)x$ (▼), and $y = -0.196 \pm 0.014 + (11.94 \pm 0.11)x$ (●).

study (Fig. 1) when data were analyzed based on the simple multiple association model described above in Eqs. 1–4. Defining the ratio β as

$$\beta \equiv K_C' / K_C$$

in the absence of adenosine or ATP ($\underline{X} = 0$), Eq. 4 can be rearranged into the form

$$1/(\beta-1) = \alpha_{CG}/(1-\alpha_{CG}) + (1/[G]) \times K_G/(1-\alpha_{CG}) \quad (5)$$

This function is linear in $1/[G]$, and α_{CG} can be estimated from the intercept of the plot at the y-axis. With the value of α_{CG} in hand, K_G can be estimated from the slope of the line. Fig. 1 shows these plots at both pH 7.4 and 3.6. In each case, the intercept at the y-axis is zero within the experimental limits of this data, indicating $\alpha_{CG} = 0$. This is in accord with results obtained previously: CB and glucose are strictly competitive at pH 7.4, and this is also true at pH 3.6. From the slope of the plots, K_G is estimated to be 35.5 mM at pH 7.4, and 17.3 mM at pH 3.6. The value at pH 7.4 closely reproduces previously reported values of 43 mM [29] and 33 mM [26]. It is also very similar to the dissociation con-

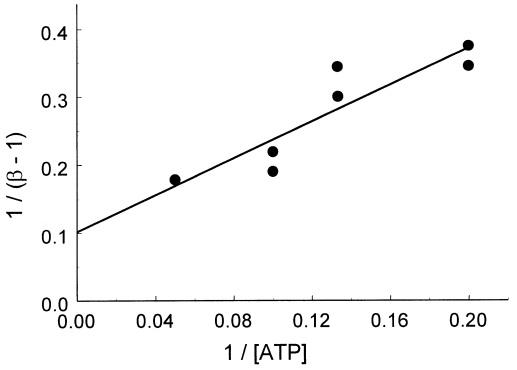

Fig. 3. Binding of ATP to GLUT1 at pH 3.6, as computed from data of CB binding to GLUT1 measured in the absence or presence of varying concentrations of ATP. Values for $1/(\beta-1)$ were computed from data and plotted against 1/[ATP] according to Eq. 8. Each point represents the average of two independent measurements. The line is drawn based on the least square linear regression analysis of the data and represents $y = 0.103 \pm 0.041 + (1.34 \pm 0.29)x$. Similar to the interpretation of Figs. 1 and 2, the slope and y-axis intercept of this plot provide an estimate of K_Z and α_{CZ}, as discussed in the text.

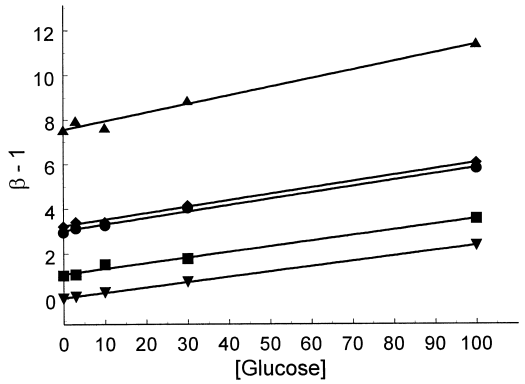

Fig. 4. Interaction of adenosine with D-glucose in binding to GLUT1 at pH 7.4, computed from the data of the CB binding to GLUT1 measured in the absence and presence of varying D-glucose concentration, with adenosine concentration as a parameter. Data are plotted according to Eq. 9 shown in the text. The slopes of these lines provide the basis for estimating the interaction parameter α_{GA} characterizing the effect adenosine binding exerts upon concurrent glucose binding. Adenosine concentrations used are 0 (▼), 3 (■), 10 (♦, ●), and 20 mM (▲). Lines are the least square linear regression analyses of the data, and represent; $y = 0.023 \pm 0.012 + (0.024 \pm 0.001)x$ for 0 mM adenosine; $y = 1.080 \pm 0.072 + (0.025 \pm 0.002)x$ for 3 mM adenosine; $y = 3.021 \pm 0.063 + (0.028 \pm 0.001)x$ for 10 mM adenosine; $y = 7.56 \pm 0.14 + (0.038 \pm 0.003)x$ for 20 mM adenosine.

stant of 35.7 mM calculated based on the dose-dependent increase in the 315 nm emission peak of GLUT1 intrinsic fluorescence by D-glucose [27].

With $\alpha_{CG} = 0$, then basic Eq. 4 can be further simplified to

$$K_C' = [C](T_T/C_{Bnd} - 1) = K_C \frac{1 + \underline{X} + \underline{G} \times (1 + \underline{X} \times \alpha_{GX})}{1 + \underline{X} \times \alpha_{CX}} \quad (6)$$

3.2. Estimation of the binding parameters for adenosine or ATP, K_X and α_{CX}

With reference to Eq. 6, in the absence of glucose, we have for K_C'

$$K_C' = K_C \times (1 + \underline{X}) / (1 + \underline{X} \times \alpha_{CX}) \quad (7)$$

This equation can be rearranged into a form analogous to Eq. 5, yielding

$$1/(\beta - 1) = \alpha_{CX}/(1 - \alpha_{CX}) + (1/[X]) \times K_X/(1 - \alpha_{CX}) \quad (8)$$

Plotting the quantity on the left side of Eq. 8, as determined from experimental measurement, against the reciprocal of ligand concentration, 1/[X], should yield a linear relationship from which both α_{CX} and K_X can be evaluated. Note that in the specific consideration of adenosine or ATP, X is replaced by A for adenosine, and by Z for ATP.

Fig. 2 shows these plots for adenosine at pH 7.4 and 3.6, while the case of ATP at pH 3.6 is shown in Fig. 3. For adenosine (Fig. 2), again the y-intercepts of the plots are zero within the experimental limit of this data (see the figure legend), and indicate that $\alpha_{CA} = 0$ at both high and low pH; thus adenosine and CB are competitive in their binding to GLUT1 at both pH values. From the slopes of the plots, the dissociation constants, K_A are estimated to be approximately 3.0 and 12.0 mM, at pH 7.4 and 3.6, respectively. ATP at pH 3.6 (Fig. 3), on the other hand, displays binding which is not completely competitive with CB ($\alpha_{CZ} = 0.10$), and a dissociation constant, K_Z of 1.22 mM.

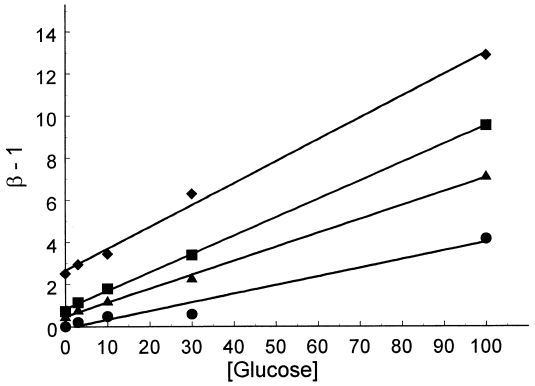

Fig. 5. Interaction of adenosine with D-glucose in binding to GLUT1 at pH 3.6. Experimental procedures and data analyses are essentially identical to those of Fig. 4, except that the lower pH was used here. Slopes of the plots provide the basis for estimating the interaction parameter α_{GA}. See Eq. 9. Adenosine concentrations used are 0 (●), 5 (▲), 7.5 (■), and 20 mM (♦). Lines were drawn according to least square linear regression analyses of the data, and are $y = 0.092 \pm 0.205 + (0.041 \pm 0.004)x$, $y = 0.473 \pm 0.070 + (0.066 \pm 0.001)x$, $y = 0.826 \pm 0.052 + (0.087 \pm 0.001)x$, and $y = 2.66 \pm 0.20 + (0.103 \pm 0.004)x$ for the data of 0, 5, 7.5 and 20 mM adenosine, respectively.

3.3. Evaluation of the interaction parameters α_{GX} between adenosine or ATP and D-glucose binding

Of particular interest is the effect that ligand binding, either adenosine or ATP, has upon the concurrent association of D-glucose, the physiological substrate, with the transporter. This interaction is expressed through the parameter α_{GX}. In order to evaluate this parameter, we refer to Eq. 6, and note that it can be recast in the form

$$\beta-1 = (1-\alpha_{CX})\times\underline{X}/(1+\alpha_{CX}\times\underline{X}) + \underline{G}\times(1+\alpha_{GX}\times\underline{X})/(1+\alpha_{CX}\times\underline{X}) \quad (9)$$

in which, as before, $\beta \equiv K_C'/K_C$. At any particular fixed concentration of X, all terms on the right hand side of this expression, with the exception of α_{GX}, have been evaluated. A plot of $\beta-1$ as a function of [G] should be linear then, with the slope providing the basis to evaluate α_{GX}. Families of such plots, generated by the differing ligand concentration [X], are shown for the systems of adenosine at pH 7.4 (Fig. 4), adenosine at pH 3.6 (Fig. 5), and ATP at pH 3.6 (Fig. 6). As one might expect from the derived nature of this data, the curves reflect a sizeable experimental variation. In the case of adenosine at pH 3.6 (Fig. 5), for example, the values of α_{GA} calculated from the three adenosine concentrations shown, were 0.33, 0.79 and 0.46, with an average of 0.53 ± 0.24 At pH 7.4 (Fig. 4), the computed α_{GA} exhibit nearly the same percentage variation but with an absolute value an order of magnitude lower (0.041, 0.050, and 0.087 for 3, 10, and 20 mM adenosine, respectively), with an average of 0.059 ± 0.024. Perhaps of most interest, however, is the rather large value of α_{GZ} exhibited by ATP at pH 3.6: 4.37 ± 0.61. Thus, in contrast to the case at pH 7.4 where no effect of ATP upon glucose binding at all can be observed, at the lower pH the association of ATP with GLUT1 enhances the binding of glucose by a factor greater than 4. That is, glucose and ATP exhibit strong positive cooperativity in binding to GLUT1 at pH 3.6.

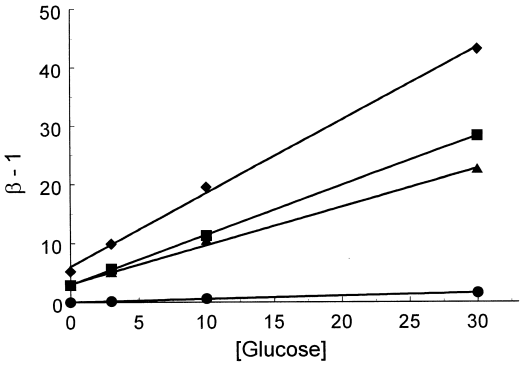

Fig. 6. Interaction of ATP with D-glucose in binding to GLUT1 at pH 3.6. Experimental conditions and data analyses are identical to those of Fig. 5, where adenosine was replaced with ATP. ($\beta-1$) plotted as a function of D-glucose concentration, with ATP concentration as a parameter. The slopes of the plots provide a basis for estimating the interaction parameter α_{GZ}, in accord with Eq. 9. ATP concentrations used are 0 (●), 5 (▲), 7.5 (■), and 20 mM (♦). Lines were drawn by the least square linear regression analyses, and are $y = 0.010 \pm 0.025 + (0.051 \pm 0.002)x$, $y = 3.09 \pm 0.43 + (0.653 \pm 0.027)x$, $y = 2.97 \pm 0.10 + (0.844 \pm 0.006)x$, and $y = 6.03 \pm 0.63 + (1.25 \pm 0.04)x$ for 0, 5, 7.5 and 10 mM ATP, respectively.

Table 2
Binding parameters characterizing interactions of CB, glucose, adenosine, and ATP with GLUT1

Ligands	Parameters	pH 7.4	pH 3.6
CB	K_C	0.20 ± 0.01 μM	0.20 ± 0.01 μM
Glucose	K_G	35.5 ± 0.9 mM	17.3 ± 0.3 mM
Adenosine	K_A	2.98 ± 0.02 mM	11.9 ± 0.1 mM
	α_{CA}	0.0	0.0
	α_{GA}	0.06 ± 0.03	0.53 ± 0.24
ATP	K_Z	30.0 ± 13.0 mM	1.22 ± 0.29 mM
	α_{CZ}	1.0	0.09 ± 0.03
	α_{GZ}	1.0	4.4 ± 0.6
	α_{AZ}	0.47 ± 0.15	N.D.

Results are shown as mean ± S.E.M. from three or more sets of measurements. For details, see text.

3.4. Assessment of the ATP binding parameters K_Z and α_{AZ} at pH 7.4

As mentioned above, the presence of ATP at pH 7.4 produces no detectable effect upon CB binding to GLUT1, or the displacement of CB by D-glucose. The absence of any effect could signify either no association of ATP with GLUT1 at this pH, or alternatively, a significant degree of association but with $\alpha_{CZ} = \alpha_{GZ} = 1.0$. Independent evidence from fluorescence measurements [22] and differential scanning calorimetry (Epand et al., unpublished data) indicates that ATP does indeed associate with GLUT1 at this pH; hence the latter alternative is assumed. In order to assess the properties of this binding, an interaction between ATP and adenosine binding was utilized. Since adenosine competes with CB ($\alpha_{CA} = 0$), any alteration of adenosine binding through interaction with ATP should be signaled by a change in CB association.

Remembering that $\alpha_{CZ} = 1$ and $\alpha_{CA} = 0$, the apparent dissociation constant for CB can be expressed as

$$\beta = 1 + \underline{A} \times (1 + \alpha_{AZ} \times \underline{Z}) / (1 + \underline{Z}) \quad (10)$$

where again β denotes K_C' / K_C. It is assumed that since adenosine and CB are strictly competitive, formation of the quadruple complex, TCAZ, does not occur. The derivation of Eq. 10 proceeds in a manner analogous to Eq. 4. With a little rearrangement, Eq. 10 becomes

$$\underline{A} / (\beta - 1) = 1 + (1 - \alpha_{AZ}) \times \underline{Z} / (1 + \alpha_{AZ} \times \underline{Z}) \quad (11)$$

If the expression on the left of this equation, $\underline{A}/(\beta - 1)$, is defined as R, one further step of rearrangement yields the form

$$1 / (R - 1) = \alpha_{AZ} / (1 - \alpha_{AZ}) + (1 / [Z]) \times K_Z / (1 - \alpha_{AZ}) \quad (12)$$

Thus, plotting $1/(R-1)$ against the reciprocal of ligand concentration should yield a linear relationship, with the intercept at the y-axis being a function of only α_{AZ}, while the slope of the plot provides an means of estimating K_Z. The plot of the average of three sets of data is shown in Fig. 7, with the standard deviations indicated. The best line fit to this data corresponds to $\alpha_{AZ} = 0.47 \pm 0.15$, and $K_Z = 30 \pm 13$ mM.

Table 2 presents the collected binding parameters as estimated by the analysis of this study.

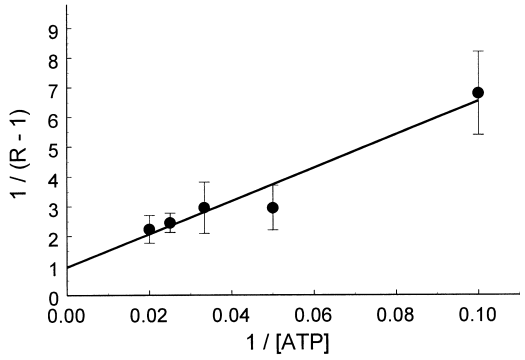

Fig. 7. Interaction of ATP with adenosine binding to GLUT1 at pH 7.4. The function 1/(R−1), defined by Eq. 12 in the text, is plotted against the reciprocal of ATP concentration. Adenosine concentration used was 20 mM. Each point is an average of three determinations, with standard deviations as shown by the error bars. The y-axis intercept is $\alpha_{AZ}/(1-\alpha_{AZ})$, while the slope is given by $K_Z/(1-\alpha_{AZ})$. As detailed in the text and expressed in Eq. 12, the suppression of adenosine binding by ATP was used to compute the interaction parameter, α_{AZ}, and the ATP-GLUT1 dissociation constant, K_Z.

4. Discussion

The results of the present study offer compelling evidence that both adenosine and ATP directly interact with purified GLUT1, raising the interesting possibility that adenosine, and possibly ATP, can play a physiological role in the regulation of glucose transport. Using purified and functionally reconstituted GLUT1 in vesicles, we have shown here that adenosine inhibits not only the CB binding activity of GLUT1, but also the binding of its physiological substrate, D-glucose to GLUT1. A straightforward interpretation of this finding then is that adenosine binds to GLUT1 in vitro forming a reversible complex with a simple one-to-one stoichiometry, and that once adenosine binds, GLUT1 is unable to bind CB and also drastically compromised (by 16-fold) in its binding affinity to D-glucose. This is the first demonstration with the purified transporter that adenosine inhibits GLUT1 and does so by a direct interaction. It should be noted that the orientation of GLUT1 in vesicles is not an issue here, as the freeze–thawing protocol resulted in equal ligand concentration inside

and outside of the vesicles, imparting equal access of ligand to GLUT1 regardless of its sidedness.

In the findings of the present study, adenosine is competitive with CB in binding to GLUT1 at both pH 7.4 and 3.6. At the higher pH, adenosine association reduces the affinity of glucose for GLUT1, by a factor of about 16. At the lower pH of 3.6, adenosine binds less strongly, by a factor of about 4, with GLUT1, and its association reduces glucose affinity to about one half its value in the absence of adenosine.

ATP, on the other hand, behaves quite differently. At pH 3.6, ATP binds strongly with GLUT1 (with an apparent dissociation constant of 1.22 mM), and in contrast to the case of adenosine, this association enhances the glucose-binding affinity of GLUT1 by more than 4-fold. Thus, should such low pH conditions prevail in certain subdomain of the plasma membrane, the activity of GLUT1 in that domain would be enhanced by ATP, while being suppressed to a moderate degree by adenosine.

At the more physiological pH of 7.4, adenosine is expected to be more effective as an inhibitor of GLUT1 function, because of its lower dissociation constant ($K_A = 2.98$ mM), and its stronger effect in reducing glucose affinity ($\alpha_{GA} = 0.06$). ATP, on the other hand, would exhibit no direct modulation of GLUT1 function. However, ATP can play a stimulatory role indirectly, by inhibiting the association of adenosine with GLUT1. This latter phenomenon has been exploited to estimate the binding affinity of ATP at this pH, as no displacement of CB by ATP alone is observed.

The observation of concurrent association of both ATP and adenosine at pH 7.4 would indicate that the two molecules bind to the transporter at substantially different sites. This interpretation is consistent with the finding that adenosine, CB, and glucose exhibit mutually competitive, or nearly so, binding, while ATP has no apparent interaction with CB or glucose binding (again at pH 7.4). Over the range of ligand concentrations utilized in this study, the CB displacement data conformed well to the concentration dependence predicted by the simple reversible association and one-to-one stoichiometry assumed in the model by which the data has been analyzed. This conformance over a reasonably wide concentration span lends support to the validity of the model assumed for the basis of analysis. Nonetheless, one cannot claim uniqueness for the model in explaining the experimental data, nor exclude the possibility of more complex behavior that may become apparent at a different concentration range. There is some fragmentary evidence, for example, that ATP interacts with GLUT1 in a more complex manner at low concentrations around 1 mM (M. Lachaal, R.A. Spangler, C.Y. Jung, unpublished data). Nonetheless, in view of the difficulties of the experimental procedure, the scatter in data is reassuringly small. As one might expect, experimental error compounds in the more derived results, such as the calculation of interaction parameters, or the estimation of ATP binding by means of its suppression of CB displacement by adenosine.

The possibility that adenosine may interact with GLUT1 in intact cells was first indicated by the D-glucose-sensitive, CB-sensitive, and L-glucose-insensitive, [^{3}H]8-azidoadenosine photolabeling of a protein in human erythrocytes [30]. Significantly, adenosine indeed was shown to inhibit both glucose uptake and D-glucose-sensitive CB binding in human erythrocytes [31,32]. Our in vitro findings, together with these in vivo findings, would suggest that adenosine is a physiological inhibitor of GLUT1 in cells. It should be noted, however, that the overall adenosine concentration in cells is normally low (0.5 mM or lower) [33]. With the possible exception of conditions found in a specialized subcellular compartment, adenosine levels would never reach the range that we ($K_A = 3$ mM), and others [31,32] (K_I value of 5–9 mM) used for its interaction with GLUT1. Adenosine is also known to enhance glucose transport in insulin-stimulated rat adipocytes without changing plasma membrane GLUT4 contents, suggesting molecular activation of GLUT4 by adenosine. However, this effect was largely due to the effect of adenosine as a purinergic receptor agonist rather than direct interaction with GLUT4 and likely modulating its accessibility to substrates [34]. ATP was also shown to modulate the transport kinetics and intrinsic activity of GLUT1 by modulating the quaternary structure of GLUT1 in human erythrocytes or purified under non-reducing conditions [35,36]. This effect was observed at the ATP concentration range of 1 mM and lower and reduction-sensitive, thus may be unrelated to the ATP effects that we observed in

the present study at much higher ATP concentrations (K_Z of 30 mM at pH 7.4) and using GLUT1 purified under reduced conditions. Further studies are needed to determine physiological significance if any of the in vitro interactions of adenosine and/or ATP with GLUT1 described here.

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