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Effects of ATP depletion on the mechanism of hexose transport in intact human erythrocytes

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Depletion of ATP is known to inhibit glucose transport in human erythrocytes, but the kinetic mechanism of this effect is controversial. Selective ATP depletion of human erythrocytes by 10 μg/ml A23187 in the presence of extracellular calcium inhibited 3-*O*-methylglucose influx noncompetitively and efflux competitively. ATP depletion also decreased the ability of either equilibrated 3-*O*-methylglucose or extracellular maltose to inhibit cytochalasin B binding in intact cells, whereas neither total high-affinity cytochalasin B binding nor its K_d was affected. Under the one-site model of hexose transport these data indicate that ATP depletion decreases both the affinity of the inward-facing glucose carrier for substrate and its ability to reorient outwardly in intact cells.

Sugar transport; ATP depletion; Cytochalasin B; One-site carrier model; (Human erythrocyte)

1. INTRODUCTION

Depletion of erythrocyte ATP by treatment with the ionophore A23187 and calcium to activate Ca^{2+}-ATPase lowers rates of hexose uptake, reflecting a decrease in the V_{max} of net glucose influx with no change in the K_m [1]. Other models of ATP depletion in intact cells or resealed ghosts [2–5] have confirmed an inhibitory effect of ATP depletion, but often with conflicting kinetic results. Reasoning that the transport effects of ATP depletion have the most physiologic relevance in intact cells, and that A23187/calcium treatment is the most specific method for depleting ATP [1], I have used these conditions to define the effects of ATP depletion on the transport mechanism under the one-site or alternating conformation model of transport [6].

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2. EXPERIMENTAL

2.1. *Cell, buffer, and ghost preparation*

Freshly drawn human blood was anticoagulated with heparin (16.7 U/ml blood) and prepared for all subsequent studies as described by Jacquez [1] in a balanced salt solution containing 60 mM NaCl, 75 mM KCl, 1 mM $MgCl_2$, 20 μM $CaCl_2$ and 10 mM Tris, pH 7.4.

2.2. *Hexose transport assays*

The net influx of 3-*O*-[*methyl*-^{14}C]methylglucose was measured as described [7] at 0.01, 0.25, 0.5, 1.0, 2.0 and 4.0 mM 3-*O*-methylglucose.

Kinetic measurements of zero-*trans* efflux of 3-*O*-methylglucose were initiated by equilibrating 0.8 ml of 20% erythrocytes for 30 min at 37°C with labeled and unlabeled 3-*O*-methylglucose (0.5, 1, 2, 4 and 8 mM) in the presence or absence of A23187. The cells were pelleted in a microfuge, the supernatant was aspirated, the tube containing the cell pellet was cooled on ice, and duplicate 10 μl aliquots of packed cells were removed to a culture tube also on ice. The efflux assay was started by adding 1 ml of ice-cold buffer and terminated by vigorously adding 3 ml of ice-cold 'stop' solution containing 10 μM cytochalasin B. The cells were pelleted and the radioactivity of 0.5 ml of supernatant counted. The time of efflux was varied to maintain extracellular radioactivity at the termination of the assay to less than 20% of that present after 30 min at 37°C. Correction was made in each assay for label which was extracellular at the beginning of the assay by subtracting the ex-

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tracellular radioactivity measured when stop solution was added to cells before diluent.

Transport rates for both net influx and efflux were normalized to the equilibrium space of 3-*O*-methylglucose for that sample. Michaelis constants were determined using the nonlinear least-squares method of Wilkinson [8].

2.3. *[^{3}H]Cytochalasin B binding*

Cytochalasin B binding to intact erythrocytes was measured as in [9] in the presence of 100 μM cytochalasin E as well as a range of cytochalasin B concentrations (250, 125, 62.5 or 10 nM) containing 62.5 nCi [4-^{3}H]cytochalasin B. The inhibition of 10 nM [^{3}H]cytochalasin B binding by several concentrations of either maltose or 3-*O*-methylglucose (0, 12.5, 25, 50, 100 mM) was determined under the above conditions and expressed as an apparent inhibitory constant (K_i) according to Gorga and Lienhard [6].

2.4. *Other assays*

Total glutathione content was measured by the method of Hissen and Hilf [10]. Efflux of $^{35}SO_4^{2-}$ from preloaded cells was measured as described [7]. Data are expressed as means ± SE and compared using the Student's *t*-test for paired values.

3. RESULTS

Preliminary studies (not shown) confirmed the initial observations of Jacquez [1] that treatment of erythrocytes with 10 μg/ml A23187 inhibited tracer 3-*O*-methylglucose uptake by 50–60% within 30 min, and that this effect was completely reversed by metabolic repletion with 5 mM inosine, 5 mM pyruvate, 5 mM adenine, and 10 mM glucose for 1 h at 37°C followed by washes and the transport assay. The specificity of A23187 treatment for hexose transport was confirmed by measuring its effects on sulfate efflux and GSH content under the same experimental conditions. Neither rates of sulfate efflux nor intracellular GSH content measured at 30°C and at an extracellular pH of 7.4 were affected by pretreatment with 10 μg/ml A23187 (not shown).

In kinetic transport studies, A23187 treatment halved the V_{max} of net 3-*O*-methylglucose influx, without an appreciable effect on the K_m (table 1). On the other hand, under similar conditions of cell number, buffer composition, and temperature of the transport assay (3–5°C), A23187 approximately doubled the K_m of net efflux, without affecting the V_{max} (table 1).

Again under the same conditions, A23187 treatment had no significant effect on K_d or total specific binding of cytochalasin B to the glucose carrier in intact cells (table 1). However, A23187 treatment doubled the apparent K_i of equilibrated 3-*O*-methylglucose for the carrier, assessed by the ability of the sugars to inhibit cytochalasin B binding (table 1). The ability of extracellular maltose to inhibit cytochalasin B binding was even more strongly decreased by A23187 treatment (table 1).

Table 1

Effects of ATP depletion on transport kinetics and cytochalasin B binding

Measurement	Control	*p*	A23187 treatment	*N*
Net influx				3
K_m	1.3 ± 0.1	NS	1.1 ± 0.1	
V_{max}	3.7 ± 0.3	<0.02	2.0 ± 0.2	
Net efflux				9
K_m	4.4 ± 1.0	<0.02	10.5 ± 1.6	
V_{max}	15.7 ± 3.6	NS	17.1 ± 3.4	
Cytochalasin B binding				
K_d	137 ± 11	NS	138 ± 12	4
B_0	5.2 ± 0.4	NS	5.5 ± 0.3	4
3-*O*-Methylglucose – K_i	15 ± 4	<0.01	32 ± 3	7
Maltose – K_i	24 ± 3	<0.01	72 ± 8	5

Erythrocytes at 20% hematocrit were incubated for 30 min at 37°C with or without 10 μg/ml A23187 followed by the appropriate assay as described in section 2. *N*, number of experiments; *p*, statistical significance. Parameter units: K_m (mM); V_{max} ($mol^{-1} \cdot l^{-1} \cdot s^{-1}$); K_d (nM); B_0 (pmol/ml packed cells); K_i (mM)

4. DISCUSSION

Treatment of freshly prepared human erythrocyte with the calcium ionophore A23187 in the presence of extracellular calcium depletes cellular energy stores, primarily in the form of ATP, resulting in decreased rates of hexose transport [1]. As shown initially by Jacquez [1], and confirmed herein, the effects of A23187 on hexose entry are completely restored by metabolic repletion. The effect of A23187 treatment also appears to be selective for hexose transport, since under identical incubation conditions neither sulfate efflux nor GSH content was affected in the present studies. Bursaux et al. [11] similarly found no effect of several different methods of ATP

depletion on erythrocyte sulfate efflux at an extracellular pH of 7.2 or greater.

The initial findings of Jacquez [1] that A23187 treatment lowers the maximal rate of glucose entry without affecting affinity for the sugar were confirmed in the present work using a non-metabolizable glucose analog, 3-*O*-methylglucose (table 1). It was also found that the inhibition of 3-*O*-methylglucose exit by A23187 treatment was due to a fall in the K_m without an effect on the V_{max} (table 1). As noted by Krupka and Devés [12], under the one-site or alternating conformation model of transport [6,12], such decreased affinity at the inward-facing substrate-binding site (i.e. competitive inhibition) will necessarily produce the noncompetitive inhibition of entry observed.

Whereas cytochalasin B is a competitive inhibitor of hexose exit [13], neither its affinity for the inward-facing carrier nor its total binding was affected by A23187 treatment (table 1). On the other hand, the ability of 3-*O*-methylglucose equilibrated with both sides of the membrane to inhibit cytochalasin B binding was diminished by A23187 treatment (table 1). This is consistent with the observation by Jacquez [1] that A23187 treatment prevented the expected inhibition of cytochalasin B photolabeling by even high concentrations of D-glucose in resealed ghosts, while cytochalasin B photolabeling itself was little affected. Possible explanations for the different effects of A23187 treatment on substrate and cytochalasin B binding are that the binding sites for substrate and cytochalasin B on the inward-facing carrier are not identical [13], or that a conformational change in the carrier induced by A23187 treatment selectively blocks substrate binding.

Maltose does not enter erythrocytes [7,14] and its displacement of internally bound cytochalasin B provides strong support for the one-site model of transport. The decreased ability of extracellular maltose to inhibit cytochalasin B binding (table 1) further suggests that the carrier in A23187-treated cells has a decreased capacity to reorient outwardly and bind substrate. The combined transport and cytochalasin B-binding data are compatible with the notion that ATP depletion affects both the ability of the inward-facing carrier to bind substrate, and to reorient to its outward-facing form.

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