ELSEVIER

Biochimica et Biophysica Acta 1369 (1998) 295–303

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The effect of ATP-depletion on the inhibition of glucose exits from human red cells

Martha Kaloyianni [1], Graham F. Baker *

Department of Biological Sciences, Royal Holloway, University of London, Egham Hill, Surrey TW20 0EX, UK

Received 16 June 1997; revised 16 September 1997; accepted 24 September 1997

Abstract

The effect of ATP-depletion or its consequence, by metabolic inhibition, on the inhibition of glucose transport by various inhibitors was studied in human red cells. In cells depleted of ATP, glucose exit times were longer than in normal cells and the times increased with the duration of depletion. The K_m for external glucose was higher in ATP-depleted cells than in normal undepleted cells (3.0 mM c.f. 2.5 mM at 30°C). In contrast, the apparent K_i for cytochalasin B decreased from 0.85 μM in the normal cells to 0.5 μM after ATP-depletion. Half-maximal rates of glucose exit in the absence, and in the presence of 2 μM cytochalasin B were found at ATP concentrations of 0.43 and 0.68 μM, respectively. Although glucose exits from ATP-depleted cells exposed to the irreversible inhibitor of glucose transport, 1-fluoro-2,4-dinitrobenzene (FDNB) were slower than in normal cells, the relative degrees of inhibition were not significantly different. However, normal and ATP-depleted cells responded differently to treatment with 1,2-cyclohexanedione, a modifier of arginine residues which inhibits glucose exit. While normal cells were markedly inhibited, depleted cells were much less affected and the inhibitory effect of cytochalasin B seen in normal cells was reduced. These findings demonstrate that the glucose transport system of human red cells is affected by intracellular ATP and that ATP alters the affinity of the transporter for certain inhibitors. The implications of these findings are discussed. © 1998 Elsevier Science B.V.

Keywords: Red cell; Glucose transport; ATP-depletion; (Human)

1. Introduction

The glucose transport system in human erythrocytes is usually regarded as the epitome of a passive transporter, requiring no input of metabolic energy in order to function. However, there have been several reports in the literature, of the modulation of glucose transport in red blood cells by ATP [1–3].

Jaquez [1] reported that the depletion of energy stores in human red cells decreased their maximum capacity for glucose transport to a value of one-third or less of that found in red cells from freshly drawn blood and Weiser et al. [2] and Jensen and Brahm [3], using outdated bank blood, also found a reduction in the permeability for glucose, albeit less marked. Carruthers and Melchior [4] found that the transporter behaved asymmetrically in the presence of a cytosolic factor which Hebert and Carruthers [5] demonstrated could be ATP.

In a series of detailed experiments, Carruthers et al. [5–7] examined the effect of ATP on the glucose

* Corresponding author: Fax: +44 1784 470756; E-mail: g.baker@rhbnc.ac.uk

[1] Present address: Department of Zoology, Science School, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece.

PII S0005-2736(97)00234-4

transporter and showed that the changes it produced were due to an activation of transporters rather than a recruitment and that ATP was bound but not hydrolysed in the process. Further, it was shown that the effect is only produced by ATP (and some synthetic analogues) and not by ADP and AMP, although these nucleotides are also able to bind to the transporter.

However, there were conflicting reports in the literature as to the kinetic effects of ATP. Thus, although both Jacquez [1] and Carruthers and Helgerson [7] found that ATP increased the rate of sugar entry into red cells and ghosts, respectively, Jaquez interpreted the effect as due to an increase in V_{max} while Carruthers and Helgerson suggested that it was due to a decrease in K_m and V_{max}. Furthermore, Carruthers [6] reported that in resealed ghosts, containing ATP, the K_m for the net efflux of glucose was 28 mM and fell to 10 mM when ATP was absent, while in contrast, Jensen and Brahm [3] found that the K_m for net efflux increased from 1.3 mM in freshly drawn red cells to 14 mM in ghosts having $< 200\,\mu M$ ATP. These, and other anomalies, led Wheeler [8] to reinvestigate the effect of ATP using glucose transporters reconstituted in liposomes and he was unable to show any change in transport beyond a generalised inhibition in the presence of ATP. However, there are shortcomings with this approach [7], including the fact that these experiments were done in low ionic-strength media which might have affected the normal ionic interactions between the molecules.

Thus, the weight of evidence supports the view that ATP does modify the behaviour of the glucose transporter, but the precise effect of ATP on glucose transport is still unresolved and if an effect does exist, it raises two obvious questions. Firstly, what is ATP doing to the transporter and, secondly, what is its physiological role?

The properties of the glucose transport system in red cells has been widely studied (for reviews, see Refs. [9,10]). Following the determination of the amino acid sequence [11] and a possible operating mechanism of glucose transporter [12], Widdas and Baker [13] proposed the existence of a cationic shield or gate at the inner end of the transport pathway. This gate consisted of eight highly-conserved arginine residues, arranged in a ring. Each arginine carries a positive charge which was assumed to reside on one of the terminal η-nitrogens. In this position, there would be a strong positive electric field which would repel potassium ions and prevent them from entering the transporter cleft. However, if the charges should move to the ε-nitrogen of the arginines, the electric repulsion would markedly decrease and potassium could pass through the ring with relative ease. Shortly after publication of this idea, Widdas and Baker reported experiments in which an over-shrinkage of red cells, due to a loss of potassium, was recorded following glucose exits [14]. They interpreted these results as indicating the opening of the cationic gate. Recent experiments in this laboratory [15] have confirmed that arginine residues have a role in preventing potassium loss via the glucose transporter.

Calculations of the energy for the transition between the closed and opened states of the gate showed that it was sufficiently low so that some mechanism would be necessary to keep the gate closed [14]. It was, therefore, proposed that during the conformational change in the transport cycle, a strong anion was brought into the vicinity of the ring, thereby pulling the positive charges onto the terminal nitrogens. From a consideration of the size of the ring and the number of charges involved, it was suggested that ATP could be this anion and, thus, it was envisaged that during that half of the transport cycle when the transporter was in the outward-facing conformation, ATP was inserted into the ring and then, as the conformation changed, it was withdrawn, leaving the ring 'closed' to cations but not, of course, to uncharged glucose. Thus, a possible role for ATP in glucose transporter function has been identified.

Studies of the kinetics of the glucose transport system in human red cells is complicated by the apparent multiplicity of operational parameters [16,10] and, at present, there is no consensus as to what the various parameters are measuring. Furthermore, any change in the occupancy of the transporter will lead to redistribution effects, further complicating the kinetics of transfer. It is thus likely to be very difficult to ascribe specific kinetic effects to the binding of ATP. Therefore, rather than trying to investigate the effect of ATP on the transport of glucose, we decided to examine the effect of ATP-depletion on the binding of various glucose transport inhibitors, as this might allow a somewhat easier interpretation. The side of the transporter at which binding occurs is

known for a number of inhibitors. Glucose transport is particularly sensitive to cytochalasin B, which binds to the cytoplasmic surface of the transporter [17] and to phloretin, which binds at the external surface [18]. Baker and Widdas [19] demonstrated that the specific arginine reagent, 1,2-cyclohexanedione, inhibits glucose exits in human erythrocytes and that its reaction is at the cytoplasmic side of the transporter, while Krupka [20] showed that the binding of fluorodinitrobenzene is enhanced during the conformational change. We, therefore, examined the inhibition of glucose transport caused by these inhibitors using the technique described by Sen and Widdas [21] in which the (infinite-cis) exit of glucose from the cells is measured. This technique was applied to normal cells and to metabolically inhibited cells, on the assumption that the effects would be due to the depletion of ATP.

2. Materials and methods

2.1. Preparation of cells

Blood from normal healthy donors was drawn into heparinised tubes. For most experiments, the blood was divided into two aliquots. One was placed in the refrigerator for use as normal cells on the following day and the other was washed three times with phosphate-buffered saline (NaCl, 120 mM; KCl, 5 mM; NaH_2PO_4, 20 mM; pH 7.4), the plasma and buffy coat being removed. The cells were then treated according to Lew [22] to deplete ATP. Thereafter, 1.3 ml of the washed cells were incubated in 20 ml of phosphate-buffered saline in the presence of 5 mM iodoacetamide and 5 mM inosine at 20°C for 18 h. At the end of this time, the cells were washed again and resuspended in phosphate-buffered saline. The other aliquot of cells were now washed thrice with phosphate-buffered saline after which both normal and ATP-depleted cells were treated similarly.

2.2. Experimental procedure

For glucose exits, two suspensions of cells, one normal and one ATP-depleted, were pre-incubated at 30°C for a minimum of 30 min in 100 mM glucose at an haematocrit of 2%. Exit rates were measured as described by Sen and Widdas [23]. Then, 21 ml of phosphate buffered saline were placed in the cuvette of the photometer [24], the chart recorder started, and 0.15 ml of cell suspension, containing $\sim 3\,\mu l$ cells, injected into the cuvette. The shrinkage of the cells was followed until complete and the temperature and the pH of the cuvette's contents were measured with an Orion 811 pH meter.

The inhibitors of glucose transport, cytochalasin B and phloretin were dissolved in methanol, made up in phosphate-buffered saline to give a final concentration of methanol of $< 1\%$, and added directly to the cuvette before the exit. The irreversible inhibitor, 1-fluoro-2,4-dinitrobenzene (FDNB) was also first dissolved in 1% methanol and incubation of the cells was carried out as described by Bowyer and Widdas [25]. 1,2-Cyclohexanedione was dissolved by adding a small amount of 2N NaOH to the reagent, then adding borate-buffered saline at pH 9.5, titrating with more NaOH to maintain this pH. Incubations with 100 mM 1,2-cyclohexanedione were carried out as described by Baker and Widdas [19].

2.3. Metabolic studies

As already described, 5% haematocrit suspensions of washed red cells were incubated in the presence of iodoacetamide and inosine. Samples were drawn at 0, 3, 6, 12, 24 and 30 h and washed. Aliquots of packed cells were taken for ATP and intracellular potassium determinations and for glucose exits. Control values for ATP and exits were measured in cells incubated in saline. ATP was assayed in neutralized perchloric acid extracts. The extracts were prepared by mixing 0.15 ml of packed red cells with the same volume of 30% perchloric acid. After centrifugation at $4000 \times g$ for 10 min, the supernatant was neutralized with 0.5 M Tris-Cl and 0.5 M KOH and diluted 1 : 10 for subsequent use for ATP determinations. ATP was estimated by the bioluminescent assay described by Summerfield et al. [26] using an LKB/Wallac 1250 Luminometer. Potassium determinations were made by flame photometry of lysed packed cells. Glucose exits in the presence, and in the absence, of 2 μM cytochalasin B, were measured as described, after resuspending the cells in 100 mM glucose for 1 h.

2.4. Potassium experiments

Because ATP-depletion is associated with the loss of potassium from the cells, two sets of preliminary experiments were performed to see if this was likely to affect the results. For one experiment, to reduce potassium loss, both types of cell were suspended in a high potassium phosphate buffer (KCl 125 mM) and incubated with 100 mM of glucose as for ordinary exits. In the other experiment, the possibility that cell shrinkage following potassium loss might have some effect was examined by incubating the cells in hypertonic phosphate-buffered saline (NaCl 210 mM, 5 mM KCl). In both cases, the exit times did not differ significantly from those of the control cells and this approach was not continued.

2.5. Statistical analysis

Statistical analysis of the results was conducted using MINITAB. Results are given as mean ± S.E of the mean.

3. Results

3.1. Depletion of ATP

Initially, cells were depleted for two to four hours before use, as suggested in the literature [1,22]. However, no significant changes in exit times were seen. We, therefore, followed the changes in exit time and ATP concentration over 30 h. The results are shown in Fig. 1(a) and (b). In Fig. 1(a), the logarithm of the ATP concentration is plotted as a function of the time of incubation in the presence of inosine and iodoacetamide. A dramatic decline in ATP levels is seen during the first 6 h of incubation but, thereafter, a much slower loss occurs. Fig. 1(b) shows the corresponding changes in the exit time for glucose. The curve is roughly exponential and significant degrees of inhibition are not seen until after nine-to-twelve hours of incubation. Determinations of intracellular potassium concentrations showed a slow fall over the incubation time. At 24 and 30 h, there was a 15% reduction in potassium within the cell sample, but there was a significant degree of haemolysis also. For this reason, it was decided to use a depletion time of 18 h, drawing blood in the late afternoon and depleting the cells overnight for use the next day. This protocol gave measurable changes in exit times but negligible haemolysis.

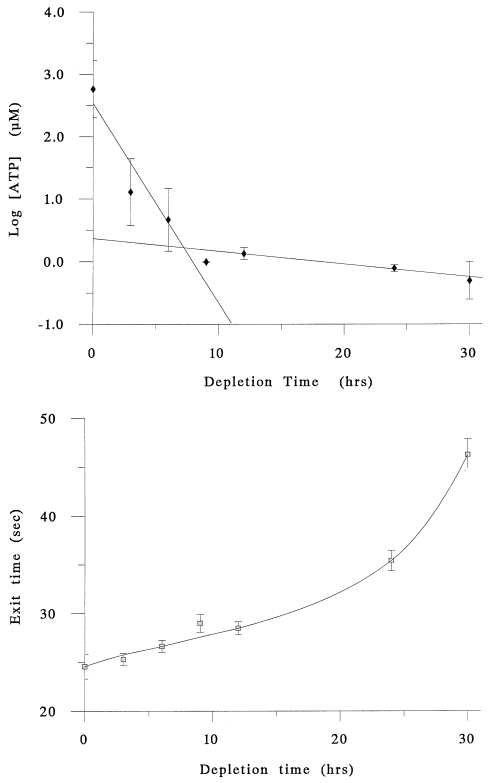

Fig. 1. Effect of incubation with (a) – iodoacetamide and inosine on cellular ATP levels and (b) D-glucose exit times. Points are means and s.e. of a minimum of three determinations at each time point. Incubations were carried out at 20°C and exits at 30°C. The two lines in Fig. 1(a) indicate the possibility of the depletion being from two different pools (see text for further details). Values in cells maintained in saline were not significantly different from the initial values in both experiments.

3.2. Effect on exits

The inhibition of glucose exits by varying concentrations of glucose in the external medium was studied at 30°C. Fig. 2 shows the Sen–Widdas plot of the glucose exits in normal and in ATP-depleted cells. The exit rate was slower in the depleted cells and the apparent K_m for exits increased slightly from 2.5 ± 0.1 mM in normal cells to 3.0 ± 0.1 mM in the depleted cells, ($P < 0.01$). Preliminary experiments in which the cells were loaded with the non-metabolisa-

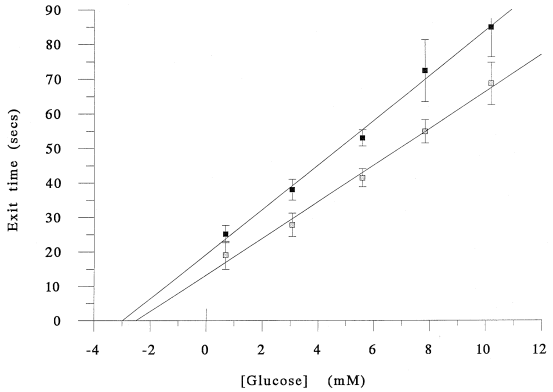

Fig. 2. Sen–Widdas plots of D-glucose exits from normal and ATP-depleted cells into various external concentrations of D-glucose. Cells were depleted for 18 h in 5 mM inosine and 5 mM iodoacetamide at 20°C to give an ATP concentration $<1\,\mu M$, then loaded with 100 mM D-glucose prior to exits. Points are means and s.e. of six determinations at each concentration: (□) – normal cells; and (■) – depleted cells.

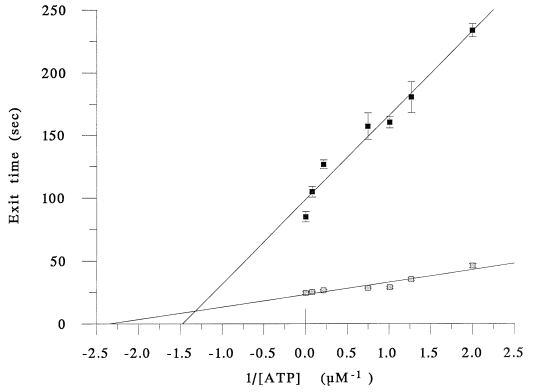

Fig. 4. Lineweaver–Burk plot of the effect of ATP concentration on glucose exit times in the presence, and absence, of 2 μM cytochalsin B. Cells were incubated for various times with 5 mM inosine and 5 mM iodoacetamide, then loaded with 100 mM D-glucose. The external glucose concentration was 0.7 mM. Points are means and s.e. of a minimum of three determinations at each concentration, (□) – control; and (■) – cytochalasin B.

ble sugar, 3-*O*-methylglucose, gave comparable shifts in both, rate and affinity to those seen with glucose. This confirms that the inhibition of glycolysis by iodoacetamide was irreversible and ATP was not being produced when depleted cells were loaded with glucose.

3.3. Effect of transport inhibitors

To gain further information about the effect of ATP-depletion, we examined the inhibition of glucose exit by glucose transport inhibitors. Cytochalasin B is regarded as the most specific inhibitor of glucose transport. It reacts with the transporter at the cytoplasmic face [17] where it binds reversibly with a high affinity. Fig. 3 shows the inhibition of glucose exits by cytochalasin B. The apparent inhibition constant, K_i, decreases from $0.85 \pm 0.09\,\mu M$ in the normal cells to $0.51 \pm 0.06\,\mu M$ in depleted ones ($P < 0.01$). The results suggest that there may be competition between ATP and cytochalasin B for a site on the glucose transporter, contrary to the observation of Carruthers and Helgerson [7].

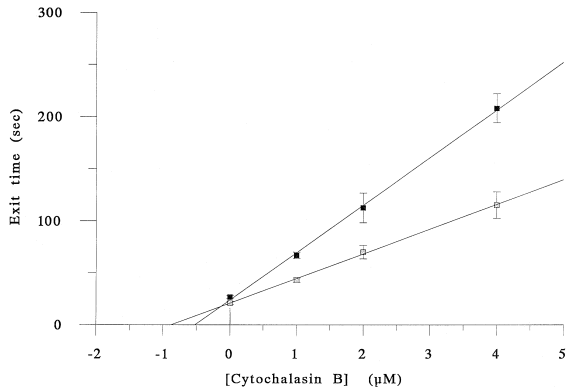

Fig. 3. Effect of ATP-depletion on the inhibition of glucose exits by cytochalasin B. The external D-glucose concentration was 0.7 mM. Points are means and s.e. of six determinations at each concentration: (□) – normal cells; and (■) – depleted cells [ATP] $<1\,\mu M$.

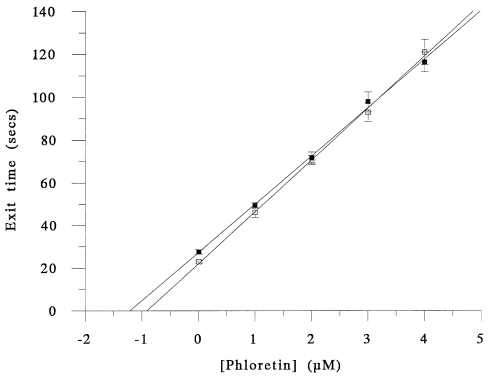

Fig. 5. Effect of ATP-depletion on the inhibition of glucose exits by phloretin. The external glucose concentration was 0.7 mM and the points are means and s.e. of 6 determinations at each concentration, (□) – normal cells; and (■) – depleted cells, [ATP] $<1\,\mu M$.

Table 1
The effect of treating normal and ATP-depleted red cells with fluorodinitrobenzene in the presence, and absence, of D-glucose. The D-glucose exit times and % inhibitions are shown. Normal and depleted red cells were incubated in 1 mM FDNB with, or without 100 mM glucose for 90 min at 25°C. After treatment, cells were loaded with 100 mM glucose by incubation at 36°C for 1 h. Values are the means and standard errors of 10 determinations; % inhibitions are relative to controls

	Control exit time (s)	FDNB exit time (s)	Inhibition (%)	FDNB + Glucose exit time (s)	Inhibition (%)
Normal	23.0 ± 0.7	51.8 ± 5.6	50.6 ± 4.9	135.2 ± 23.0	75.9 ± 4.0
ATP-Depleted	27.6 ± 1.1	72.1 ± 9.0	56.4 ± 4.5	158.9 ± 21.0	79.5 ± 2.51

Table 2
The effect of treatment with 1,2-cyclohexanedione on D-glucose exit from red cells and on the inhibition produced by 2 μM cytochalasin B. Glucose exit times in seconds determined on normal and ATP-depleted cells treated with 100 mM 1,2-cyclohexanedione for 30 min at 25°C. Values are means and standard errors of 10 determinations; % inhibitions are relative to control cells, the values in brackets are relative to dione-treated cells

	Control exit times (s)	Cytochalasin 2 μM	Dione treated	Dione treated + cytochalasin 2 μM
Normal	26.25 ± 1.1	104 ± 3.2	44.12 ± 4.1	169.33 ± 13.7
Inhibition	—	75%	40%	85% (74%)
ATP-Depleted	31.25 ± 1.4	170 ± 7.3	39.0 ± 1.9	43.37 ± 3.8
Inhibition	—	82%	20%	28% (10%)

This view is further supported by the results of the ATP determination experiments. Fig. 4 shows a Lineweaver–Burk plot relating the concentration of ATP in the cells to the glucose exit time in the presence, and absence, of cytochalasin B. The $K_{d(app)}$ for ATP in the normal cells was $0.43 \pm 0.05\,\mu M$, but in the presence of 2 μM cytochalasin B, the $K_{d(app)}$ was increased to $0.68 \pm 0.08\,\mu M$, ($P < 0.05$) confirming a reduced affinity for ATP.

Fig. 5 shows the inhibition of glucose exits by phloretin. The inhibition constant for phloretin increased from $0.82 \pm 0.09\,\mu M$ in the normal cells to $1.09 \pm 0.09\,\mu M$ in depleted cells, but the effect was small and not significant ($P > 0.05$).

Fluorodinitrobenzene (FDNB) is an irreversible inhibitor of glucose transport. It was found by Bowyer and Widdas [25] to be more effective when reacted in the presence of glucose, and Krupka [20] extended this observation to demonstrate that binding was enhanced by the conformational change that occurs with transfer. The effect of FDNB on glucose exits from control and depleted cells is shown in Table 1. Although the percentage inhibition is marginally greater in depleted cells, both in the presence and in the absence of glucose, the differences are not statistically significant.

1,2-Cyclohexanedione reacts with, and modifies arginine residues in proteins [27] and inhibits glucose efflux from human red cells when incubated with cells at high pH [19]. Table 2 shows the effect of 1,2-cyclohexenadione treatment on glucose exit from control and ATP-depleted red cells. In both control and ATP-depleted cells the exit rate was inhibited after the treatment of cells with dione. However, the ATP-depleted cells were less affected by dione and although, as already shown, they had a higher affinity for cytochalasin B when untreated, after dione treatment their affinity for cytochalasin B was dramatically reduced.

4. Discussion

4.1. Predicted effects of ATP-depletion

To our knowledge, this is the first study where the effects of ATP-depletion on inhibitor binding to the glucose transporter have been studied in intact human erythrocytes. Although, there have been a number of reports in the literature suggesting an interaction between the glucose transporter and ATP, it is not

known if this represents a physiological role for the nucleotide.

In 1991, Widdas and Baker [15] suggested that ATP might be responsible for maintaining the integrity of a cationic shield at the cytoplasmic side of the transporter and, thus, preventing leakage of potassium from the cells. Given this hypothesis is correct, what effect would we expect ATP-depletion to have on glucose transport? Since ATP is presumed to alter the position of electrical charges within the transporter, it would not be expected to have a significant effect on the transport of a non-electrolyte like glucose. However, it might alter the distribution of inward and outward facing transporters since the electrical attraction between the ATP and the arginine residues would increase the energy requirements of the conformational change. Thus, in the normal situation, when ATP is present we would expect that more transporters would be facing outwards than inwards, since we envisage ATP associating with the arginines when the transporter is in the outward-facing conformation. In depleted cells, we would expect relatively more transporters to be facing inwards.

In agreement with most of the literature (e.g. Refs. [1,2,6]) we find that cells containing ATP have a high rate of glucose transport and that depletion of the ATP content of the cells is associated with an increase in the exit time. However, the effect is small and intracellular ATP levels must be reduced to nanomolar concentrations in order to significantly affect glucose transport rates. In fact, we find that the $K_{d(app)}$ of the transporter for ATP is only 430 nM. This value is a hundred times lower than Carruthers's [6] estimate of $\sim 45\,\mu$M. One explanation for this difference may lie in the suggestion of Hoffman [28] that there is a membrane-associated compartment of ATP and it is with this that the glucose transporter is equilibrating. Carruthers's estimate was made using inside-out vesicles exposed to known concentrations of the nucleotide, but in our experiments we measured the ATP in the whole cell. As Fig. 1(b) shows, the concentration of ATP in the depleted cells declined with time as a double exponential suggesting the existence of two pools. The values of the intercepts at zero time will give the relative amounts of ATP in those pools. If the fast rate constant corresponds to the depletion of the cytoplasmic pool and the slow constant is for the membrane pool, the intercepts suggest that the latter contains $\sim 1/150$th the ATP of the former, in which case our value would be in approximate agreement with Carruthers's estimate.

4.2. Effect of ATP-depletion on reversible inhibitors

As well as lowering the rate of glucose exits, our experiments showed that ATP-depletion lowered the affinity of the transporter for glucose (Fig. 2). ATP has been demonstrated to modify the K_m for D-glucose, but the effect depends on the flux that is measured and there are disagreements between authors. Thus, Jensen and Brahm [3] showed that the K_m for exchange increased with ATP, while that for net efflux (zero-trans exit) decreased. Hebert and Carruthers [5], on the other hand, found that the K_m for net efflux increased when ATP was added to red cell ghosts. Carruthers [6], has interpreted this result as indicating that ATP reduces the K_m for D-glucose binding to the external site and increases it for binding to the internal site. Our findings agree with this interpretation since the Sen–Widdas K_m is a measure of the affinity of the external modifier site [29]. The shift in affinities implies a redistribution of the transporter with relatively more sites in the outward-facing conformation when ATP is present. This redistribution is consistent with a greater energy requirement for transformation from the outward to the inward-facing position, as we predict. It explains Hebert and Carruthers's [5] suggestion that the transporter becomes asymmetrically in the presence of ATP since the attraction between the electrical charges of the ATP and arginines would reduce the work required to face the transporter outwards and increase the work necessary to return it to face the cytoplasm.

The distribution in the presence of ATP presumably represents an optimal arrangement for exits and any shift from this reduces the number of carriers returning empty. Since it is the return of empty carriers that determines net transfers, when the numbers fall there is a slowing of the exit rate.

In contrast to glucose, cytochalasin B showed a significant increase in its affinity for the transporter in depleted cells. Cytochalasin B acts at the cytoplasmic side of the glucose transporter and this result, again, indicates that there are more transporters facing inwards in depleted cells. However, the relative

change in affinity with cytochalasin B is twice as great as that seen with inhibitors at the exofacial side and the effect can also be interpreted as the result of direct competition between ATP and cytochalasin B. As Fig. 4 indicates, the rate of glucose exit is inversely related to the intracellular ATP concentration and estimates of the $K_{d(app)}$ for ATP in the presence, and absence, of cytochalasin B give values of 0.68 μM and 0.43 μM, respectively. Thus, cytochalasin B does appear to interfere with ATP binding to the transporter and vice versa. Clohery et al. [30] showed a reduction in the affinity of cytochalasin binding to red cell ghosts in the presence of ATP but the effect was not significant. The reason for this difference between ghosts and intact cells is not clear.

4.3. Effect of ATP-depletion on irreversible inhibitors

In the FNDB experiments, the slowing of glucose efflux was greater in the depleted cells than in normal cells, but the degree of inhibition relative to controls was not significantly greater (Table 1). Thus, ATP does not appear to have an effect on the events of the conformational change.

In experiments with 1,2-cyclohexanedione, the inhibition of glucose exits reported by Baker and Widdas [19] is confirmed but the effect of 1,2-cyclohexanedione on the glucose exits from ATP-depleted cells gave unexpected results, which are shown in Table 2. In normal cells the percentage inhibition by cytochalasin B is the same before, and after dione treatment and the results suggest that there is no interaction between the inhibitors. The total inhibition with dione and cytochalasin is simply the sum of the fraction of transporters modified by dione plus the fraction of those unmodified which are blocked by cytochalasin B. In ATP-depleted cells, the inhibition by cytochalasin B is raised relative to that in normal cells, as we have already shown, while the inhibition due to dione is reduced. If the two inhibitors acted independently of one another, the total inhibition in cells treated with dione and cytochalasin B would be about the same as that in normal cells. However, we see that, in the ATP-depleted dione-treated cells, the inhibition by cytochalasin B is dramatically reduced. This indicates a reduction in cytochalsin B affinity and, therefore, an interaction between the binding of the two inhibitors.

In normal cells, it is assumed that the dione reacts with the guanido groups of the arginines at the cytoplasmic side of the transporter from within the transport cleft. The resultant reaction products obstruct the movement of sugar. Plotting the data given by Baker and Widdas [19] indicates that inhibition increases with the square of the dione concentration and that, therefore, two dione molecules are needed to block the cleft. In this situation, access to the cytochalasin B binding site is also prevented so that cytochalasin can only bind to the unmodified transporters. If the withdrawal of ATP, as occurs in the depleted cells, simply lowered the transporter's affinity for dione (perhaps by reducing the number of positive charges on the η-nitrogens of the arginines), then it would be expected that more transporters would be available for cytochalasin B inhibition. But this is clearly not the case.

One possible explanation is that ATP-depletion permits cyclohexanedione to displace the arginine residues from their normal position. This would allow the reaction products to move away from the cleft so that the channel was no longer obstructed. However, this also implies that the position of the arginine residues is important for cytochalasin B binding and that, perhaps, as Baker and Widdas have suggested, the ring itself is the binding site [31]. Holman and Rees [32] have proposed that cytochalasin B binds between domains 10 and 11. This region includes R400 which is one of the arginines that could be in the ring. The possibility that the integrity of the ring is necessary for cytochalasin B binding is supported by an observation of Baker et al. [15]. Treating human red cells with 1,2-cyclohexanedione produces a slow leak of potassium which is prevented if glucose, maltose or phloretin is present during the reaction. However, the leak cannot be prevented by reacting with cyclohexanedione in the presence of cytochalasin B.

Clearly ATP affects the binding of inhibitors to the cytoplasmic face of the glucose transporter and, therefore, must bind as well. Carruthers and Helgerson [7] have identified three potential binding sites in the glucose transporter on the basis of consensus sequences. Of these, two are of interest. One of the sites (residues 332–338) is thought to associate with the triphosphate chain and contains the conserved RRGXR sequence. Widdas and Baker [13] have pos-

tulated that this sequence forms part of the ring and the negatively charged triphosphate chain is that part of ATP which is inserted into the ring to close it. It is perhaps these arginines which are responsible for the interaction between ATP and cyclohexanedione.

The other potential binding site of interest (residues 225–229), which is proposed to react with the adenosine ribose moiety of ATP, is located on the cytoplasmic loop between domains 6 and 7. This part of the transporter is sufficiently long and flexible to be able to move in and out during the conformational change, and could provide the point of attachment for the nucleotide.

Thus, the possible binding sites for ATP are consistent with its mechanism of action as proposed in the model of Widdas and Baker [14], but in any case the evidence of this study supports the view that ATP binds to the cytoplasmic face of the GLUT1 transporter and, in doing so, alters its distribution across the membrane.

Acknowledgements

We would like to thank Professor W.F. Widdas for his helpful comments during this work.

References

[1] J.A. Jaquez, Biochim. Biophys. Acta 727 (1983) 367–378.
[2] M.B. Weiser, M. Razin, W.D. Stein, Biochim. Biophys. Acta 727 (1983) 379–388.
[3] M.R. Jensen, J. Brahm, Biochim. Biophys. Acta 900 (1987) 282–290.
[4] A. Carruthers, D. Melchior L, Biochim. Biophys. Acta 728 (1983) 254–266.
[5] D.N. Hebert, A. Carruthers, J. Biol. Chem. 261 (1986) 10093–10099.
[6] A. Carruthers, J. Biol. Chem. 261 (1986) 11028–11037.
[7] A. Carruthers, A.L. Helgerson, Biochem. 28 (1989) 8337–8346.
[8] T.J. Wheeler, Biochem. 28 (1989) 3413–3420.
[9] W.F. Widdas, Biochim. Biophys. Acta 947 (1988) 385–404.
[10] A. Carruthers, R.J. Zottola, in: W.N. Konings, H.R. Kaback, J.S. Lolkema (Eds.), Handbook of Biological Physics. Vol. 2, 1996, pp. 311–342.
[11] M. Mueckler, C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard, H.F. Lodish, Science 229 (1985) 941–945.
[12] W.F. Widdas, G.F. Baker, Cytobios 66 (1991) 179–204.
[13] W.F. Widdas, G.F. Baker, Cytobios 68 (1991) 71–76.
[14] W.F. Widdas, G.F. Baker, Cytobios 68 (1991) 131–152.
[15] G.F. Baker, R. O'Gorman and P. Baker, J. Physiol. 499.P (1997) 51P.
[16] G.F. Baker, R.J. Naftalin, Biochim. Biophys. Acta 550 (1979) 474–484.
[17] D.A. Basketter, W.F. Widdas, J. Physiol. 278 (1978) 389–401.
[18] R.M. Krupka, J. Membr. Biol. 83 (1985) 71–80.
[19] G.F. Baker, W.F. Widdas, J. Physiol. 467 (1993) 107.
[20] R.M. Krupka, Biochem. 10 (1971) 1143–1148.
[21] A.K. Sen, W.F. Widdas, J. Physiol. 160 (1962) 404–416.
[22] V.L. Lew, Biochim. Biophys. Acta 233 (1971) 827–830.
[23] A.K. Sen, W.F. Widdas, J. Physiol. 160 (1962) 392–403.
[24] W.F. Widdas, J. Physiol. 120 (1953) 20P–21P.
[25] F. Bowyer, W.F. Widdas, J. Physiol. 141 (1958) 219–232.
[26] G.P. Summerfield, J.P. Keenan, N.J. Brodie, A.J. Bellingham, Clin. Lab. Haematol. 3 (1981) 259–271.
[27] R.L Lundblad, C.M. Noyes, Chemical Reagents for Protein Modification. Vol. 2, CRC Press Inc., Boca Raton, Florida, 1984.
[28] J.F.Hoffman, F. Proverbio, J.P.Geibel, J. Physiol. 489.P (1995) 10S.
[29] G.F. Baker, W.F. Widdas, J. Physiol. 395 (1988) 57–76.
[30] E.K. Cloherty, D.L. Diamond, K.S. Heard, A. Carruthers, Biochem. 35 (1996) 13231–13239.
[31] G.F. Baker, W.F. Widdas, J. Physiol. 446 (1992) 589P.
[32] G.D. Holman, W.D. Rees, Biochim. Biophys. Acta 897 (1987) 395–405.