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ATP Does Not Regulate the Reconstituted Glucose Transporter[†]

Thomas J. Wheeler
Department of Biochemistry, University of Louisville School of Medicine, Louisville, Kentucky 40292

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ABSTRACT: ATP has been reported to affect glucose transport in human erythrocytes and resealed erythrocyte ghosts [Jacquez, J. A. (1983) *Biochim. Biophys. Acta 727*, 367–378; Jensen, M. R., & Brahm, J. (1987) *Biochim. Biophys. Acta 900*, 282–290]. In more detailed studies, effects of micromolar levels of ATP on transport in ghosts and inside-out vesicles, and on the fluorescence of ghosts and the purified glucose transporter [Carruthers, A. (1986) *Biochemistry 25*, 3592–3602; Hebert, D. N., & Carruthers, A. (1986) *J. Biol. Chem. 261*, 10093–10099; Carruthers, A. (1986) *J. Biol. Chem. 261*, 11028–11037], have been interpreted as supporting a model in which ATP regulates the catalytic properties of the transporter. Both allosteric and covalent effects of ATP were proposed; among the allosteric effects was a 60% reduction in the K_m for zero-trans uptake. In order to test whether allosteric ATP regulation of the transporter occurs, we reconstituted glucose transport activity into liposomes using erythrocyte membranes without detergent treatment. The effects of ATP, present either outside, inside, or both inside and outside the liposomes, on the transport activity were examined. Effects of ATP on trypsin-treated liposomes, which have only a single orientation of active transporters, were also tested. While the model predicts activation by ATP, only inhibition was observed. This was significant only at millimolar concentrations of ATP, in contrast to the previously reported effects at micromolar levels, and was primarily on the extracellular surface of the transporter. In addition, the ATP effects on reconstituted transport were nonspecific, with similar effects produced by tripolyphosphate. The K_m for zero-trans uptake in trypsin-treated liposomes was unaffected by the presence of 4 mM ATP, also in disagreement with the proposed allosteric effects. While these results do not address possible regulatory effects of ATP due to covalent modifications, they argue against allosteric effects of ATP acting directly on the glucose transporter.

A number of studies of glucose transport in human erythrocytes have dealt with the effects of ATP on the transport rate. Jacquez (1983) reported that depletion of erythrocytes of their ATP lowered the V_{max} for zero-trans uptake of glucose at 5 °C by 70–80%, with no effect on the K_m. Hemolysis and resealing in the presence of ATP or ADP reversed the change in V_{max}, with half-maximal effects at about 0.6 mM nucleotide

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[see Figure 5 of Jacquez (1983)].

We later reproduced some of these effects but showed that differences in glucose transport activity between erythrocyte ghosts resealed with or without ATP were not preserved when the membrane proteins were reconstituted in liposomes (Wheeler, 1986a). This argued against a covalent modification of the transporter as the basis of the ATP effect; it was suggested that the effects of changes in ATP levels were general effects on the structure of the erythrocyte membrane. Indeed, Jacquez (1983) noted that there were similar effects of ATP on leucine and urate transport, indicating a general membrane effect.

Effects of cell age on the transport kinetics have been suggested to be related to changes in cellular ATP levels. Jacquez (1983) noted that the uptake rate decreased by about 40% 3 weeks after blood was drawn. Weiser et al. (1983) compared the kinetics of glucose transport in fresh and outdated erythrocytes and found that the outdated cells had a 2-fold higher K_m and a 20% lower V_{max} for equilibrium exchange at 20 °C than fresh cells. Jensen and Brahm (1987) reported that the K_m for exchange at 25 °C was 2-fold lower for outdated than for fresh blood, in conflict with the results of Weiser et al. (1983); the K_m for zero-trans efflux was much higher in the outdated cells. V_{max} values were affected very little by the age of the cells. Jensen and Brahm (1987) also measured the effects of ATP on transport in resealed ghosts. Differences in kinetic parameters for ATP-containing vs ATP-depleted ghosts were consistent with those for fresh blood vs outdated blood (the outdated cells presumably being lower in their ATP content).

The most extensive studies of the effects of ATP on the erythrocyte glucose transporter have been performed by Carruthers and co-workers. A comparison of the kinetic properties of erythrocytes, resealed ghosts, and inside-out vesicles (Carruthers & Melchior, 1983) indicated that the glucose transporter was inherently symmetric in its kinetics but was asymmetric in the presence of low molecular weight intracellular factor(s). A subsequent study (Hebert & Carruthers, 1986) showed that ATP was the factor responsible for this behavior. Fluorescence studies with erythrocyte ghosts (Carruthers, 1986a) and the purified transporter (Carruthers, 1986b) indicated ATP binding in a manner that affected glucose binding to the transporter. ATP also affected the kinetics of glucose efflux from inside-out vesicles (Carruthers, 1986b). Half-maximal ATP effects on both kinetics and fluorescence were produced at concentrations of about 50 μM.

A model was proposed to account for the kinetics in the presence and absence of ATP (Carruthers, 1986a). This model includes the following effects of ATP on the transporter: a 60% decrease in the K_m for zero-trans uptake; an 80% decrease in the V_{max} for zero-trans uptake; a 2-fold increase in the K_m for zero-trans efflux (but no effect on V_{max}); and a 50% decrease in the K_m for equilibrium exchange (again with no effect on V_{max}). The proposed effects of ATP on the uptake K_m and V_{max} thus conflict with those reported by Jacquez (1983), while the effects on the efflux and exchange K_m values conflict with those reported by Jensen and Brahm (1987). The proposed decrease in the exchange K_m is, however, in agreement with the results of Weiser et al. (1983), if outdated cells are lower in their ATP contents.

The experimental effects of ATP observed by Carruthers and co-workers included both rapid effects (complete in a few seconds) on the uptake K_m (shown as a decrease in the K_m for efflux from inside-out vesicles) and on fluorescence and a slow effect (requiring 15 min of incubation with ATP) on the uptake V_{max} (again shown for efflux from vesicles). The effect on K_m, but not that on V_{max}, was readily reversed by incubation in the absence of ATP. It was proposed (Hebert & Carruthers, 1986) that the K_m effect could be an allosteric effect of ATP on the transporter, while the V_{max} effect could be the result of phosphorylation of the transporter or of a regulatory protein. The other major feature of the proposed ATP regulation, a 2-fold increase in the K_m for zero-trans efflux (Carruthers, 1986a), was not assigned to one of these categories. Thus, it is not possible to use the model to predict all of the consequences of the allosteric effects of ATP in the absence of the slow (presumably covalent) effects.[1]

We were interested in these proposed regulatory effects of ATP for two reasons. First, while the regulation of glucose transport by insulin and some other factors involves translocation of glucose transporters between an intracellular pool and the plasma membrane (Simpson & Cushman, 1985), certain other regulatory effects have been proposed to occur without translocation [e.g., effects of catecholamines in adipose cells (Joost et al., 1986)]. As pointed out by Carruthers (1986b), the erythrocyte, where translocation cannot occur due to the lack of intracellular organelles, provides the potential for the study of translocation-independent regulation of glucose transport. If such regulation were demonstrated for the erythrocyte glucose transporter, it might be relevant to regulation in insulin-sensitive tissues such as adipose and muscle, since the transporter proteins of these tissues are closely related to those of erythrocytes (Wheeler & Hinkle, 1985).

Second, the proposed regulation by ATP was advanced by Carruthers and co-workers as part of an explanation for the anomalous kinetic behavior of glucose transport in human erythrocytes. A model has been proposed which includes not only modulation of the kinetic properties by ATP (Carruthers, 1986a), but also two sites for glucose (which may be occupied simultaneously) (Helgerson & Carruthers, 1987). However, we (Wheeler, 1986b; Wheeler & Whelan, 1988) and others (Brahm, 1983; Lowe & Walmsley, 1986) have reevaluated some aspects of the kinetics, and our analyses (Wheeler & Whelan, 1988) show that the kinetics can be accounted for by the carrier model. Thus, the two-site model (Carruthers, 1986a; Helgerson & Carruthers, 1987) appears needlessly complex. It does account for certain features of ligand binding reported by Carruthers and co-workers, but some of these conflict with the results of other workers, which have supported a one-site model (such as the carrier model).

It is possible that ATP effects could be incorporated into the one-site carrier model. However, our earlier results with the purified transporter reconstituted into liposomes (Wheeler & Hinkle, 1981) indicated that transport asymmetry was an intrinsic property of the transporter protein. This disagrees with the model of Carruthers, in which the asymmetry arises from regulation of the transporter by ATP. Thus, there are conflicting views concerning the kinetics and the role of ATP which need to be resolved by further studies.

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[1] For a passive transporter in which the transport can be described by the Michaelis–Menten equation, the ratio of V_{max} to K_m must be the same for both directions of transport. Thus, it is not possible for a covalent modification to affect the V_{max} for uptake, or for an allosteric interaction to affect the K_m for uptake, without compensating changes in the other kinetic parameters. If allosteric effects of ATP lower the K_m for zero-trans uptake by 60% with no effect on the V_{max}, then the ratio V_{max}/K_m must increase 2.5-fold for both zero-trans uptake and zero-trans efflux. At concentrations of glucose much less than K_m, the initial rate of uptake is proportional to this ratio. Thus, according to the model of Carruthers, allosteric effects in the absence of covalent effects should increase both zero-trans uptake and zero-trans efflux by 2.5-fold.

In the work presented here, we tested directly the effects of ATP on the activity of the glucose transporter reconstituted into liposomes. Such experiments are free of possible effects of ATP on the erythrocyte membrane, which we proposed (Wheeler, 1986a) could account for activating effects of ATP on glucose transport (Jacquez, 1983). We employed the technique of reconstitution using erythrocyte membranes without detergent treatment (Wheeler, 1986a), which avoids possible changes in the properties of the transporter as a result of detergent extraction and purification. The results show that while ATP does affect the rate of glucose transport in liposomes, the effects are inhibitory, rather than activating, are nonspecific, are significant only at millimolar concentrations of ATP, and appear to be primarily at the extracellular surface of the transporter. Thus, the results argue against direct allosteric effects of ATP on the glucose transporter as proposed by Carruthers and co-workers.

EXPERIMENTAL PROCEDURES

Materials. Outdated human erythrocytes were provided by the American Red Cross, Louisville, KY. D-[U-^{14}C]-Glucose was obtained from ICN, L-[1-^{3}H]glucose was from DuPont NEN, and ATP, GTP, tripolyphosphate, and L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK)[2]-treated trypsin were from Sigma.

Procedures. Erythrocyte ghosts were prepared as described (Dodge et al., 1963). Reconstitution of erythrocyte membranes without detergent treatment (Wheeler, 1986a) was performed by using the freeze–thaw procedure as described (Wheeler & Hauck, 1985) except for the final sonication step, described below. A ratio of 8 μg of ghost protein/mg of soybean lipid was used in the reconstitution. This is within the range of protein to lipid ratios where stereospecific glucose uptake in 2 min is proportional to the amount of added protein [see Figure 2 of Wheeler (1986a)], indicating most transport is into liposomes with only single transporters incorporated. In order to increase the reproducibility of the reconstitutions, the final sonication step (after freezing and thawing) was performed using a probe sonicator (Branson Model 200 with microtip), at a setting of 3.5 for 5 s, rather than a bath sonicator. The volume in each tube (13 × 100 mm) used for the reconstitution was 0.3 mL, and the tip of the probe was placed midway between the top and bottom of the suspension. Stereospecific uptake of glucose was measured as described (Wheeler & Hauck, 1985) except that 40 μL of reconstituted liposomes (1 mg of lipid) was used per assay in a total volume of 300 μL. Unless otherwise noted, assays contained 0.2 mM D-[^{14}C]glucose and tracer L-[^{3}H]glucose and were performed in triplicate or quadruplicate at each time point. The reconstituted ghosts used in this study had a typical initial uptake rate of 0.7 nmol s^{-1} (mg of protein)$^{-1}$ at 0.2 mM glucose, while the stereospecific glucose space equilibrated in 20 min was about 330 μL/mg of protein. In some cases, the internal volume of the liposomes was measured by assaying uptake of 0.2 mM L-[^{3}H]glucose in 24 h. In these assays, no D-glucose was present, and sodium azide (0.2 mg/mL) was included.

In experiments in which the effects of ATP present inside but not outside of the liposomes were tested, liposomes were prepared and reconstituted in the presence or absence of ATP. ATP-containing liposomes were then subjected to chromatography on columns of Sephadex G-50 to remove external ATP; control liposomes were subjected to the same treatment. The absorbance at 650 nm of diluted samples before and after chromatography was used to normalize the amounts of liposomes used in the assays.

Solutions of the sodium salts of ATP, GTP, and tripolyphosphate were adjusted to pH 7.4. The biological activities of the ATP solutions were confirmed in assays containing glucose, NADP, hexokinase, and glucose-6-phosphate dehydrogenase.

RESULTS

Effects of External ATP on Glucose Uptake into Reconstituted Liposomes. We tested the effects of various concentrations of ATP on the stereospecific uptake of glucose by liposomes reconstituted with human erythrocyte membrane proteins. In some experiments, the ATP was added along with glucose at the initiation of the assays, and thus was present only outside of the liposomes. Two points are important in interpreting the results of such experiments. First, we previously showed that transporters are reconstituted with a scrambling of orientations (Wheeler, 1986a). The uptake therefore initially corresponds to a mixture of zero-trans uptake and zero-trans efflux in the erythrocyte, but at low glucose concentrations, these must have equal rates in the case of a passive transporter. Because of the scrambling, only about half of potential allosteric regulatory sites (presumably on the cytoplasmic domain of the transporter as oriented in erythrocytes) are exposed to the external ATP, which would reduce the magnitude of regulatory effects of ATP by about half. Second, the added ATP will increase the osmolarity of the external solution relative to that inside of the liposomes (made in 10 mM Tris·HCl), which would be expected to make the liposomes shrink.

Table I lists results of these experiments, with data expressed as uptakes relative to controls for the same time points. Uptakes were measured both at 20 s, to give initial rates, and at 20 min, by which time the stereospecific uptake has reached a maximum [see Figure 1 of Wheeler (1986a) for the time course of uptake in the first 10 min]. At later times, the continued nonspecific uptake of L-glucose is greater than the additional D-glucose uptake, so that the stereospecific uptake is less at 60 min than at 20 min (not shown). The final equilibrated volume, or glucose space, measured at 20 min, is a function of both the number of functional transporters reconstituted and the internal volume of the liposomes incorporating them.

The data in Table I show that external ATP at 0.1 (twice the apparent K_d reported by Carruthers and co-workers) and 1 mM had no effect on the initial rate of glucose uptake. Higher concentrations (2 and 4 mM) produced slight (13% and 17%, respectively) inhibitions of the uptake rate. The final uptake, measured at 20 min, was unaffected by 1 mM ATP and reduced by 14% and 30% by 2 and 4 mM ATP, respectively. These results are consistent with osmotic effects of ATP; in fact, an even larger decrease (about 45%) in the internal volume would be expected in the presence of 4 mM ATP if the liposomes shrank ideally in response to osmotic changes.[3]

In order to test further the changes in response to added ATP, we measured the penetration of L-glucose in 24 h with

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[2] Abbreviations: TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PPP, tripolyphosphate.

[3] Using a pK_a of 6.95, it can be calculated that at pH 7.4, a solution of 4 mM ATP would contain 2.96 mM ATP^{4-} and 1.04 mM ATP^{3-}. Since the solution was prepared from the sodium salt and the pH adjusted with NaOH, there must be 14.96 mM Na^{+} present, giving a total osmolarity of 18.96 mOsm/L. The added ATP was from a 20 mM stock solution, and therefore reduced the amount of 10 mM Tris·HCl (18.3 mOsm/L, based on a pK_a of 8.1) by 20%, or 3.66 mOsm/L, for a net increase in osmolarity of 15.3 mOsm/L.

Table I: Effects of ATP and Other Compounds on Stereospecific Glucose Uptake in Reconstituted Liposomes[a]

		relative uptake		
compound, concn (mM)	time	external	internal	external plus internal
		Untreated Liposomes		
ATP (0.1)	20 s	1.02 (1.02, 1.03)		1.10 (1.01, 1.20)
	20 min			1.10 (1.02, 1.19)
ATP (1)	20 s	0.94 (1.00, 0.88)		0.74
	30 s			0.83
	5 min			0.90
	20 min	0.97 (0.98, 0.96)		0.76
ATP (2)	20 s	0.87 (0.88, 0.86)		0.75
	30 s			0.76
	5 min			0.91
	20 min	0.86 (0.84, 0.88)		0.77
ATP (4)	20 s	0.83 ± 0.03 (9)	0.67 ± 0.05 (3)	0.65 ± 0.04 (5)
	30 s			0.73
	5 min			0.73
	20 min	0.70 ± 0.10 (3)	0.80 (0.72, 0.87)	0.60 ± 0.05 (4)
	60 min	0.59	0.63	0.54 (0.55, 0.54)
MgCl$_2$ (4)	20 s	1.02 ± 0.16 (3)		
ATP (4) + MgCl$_2$ (4)	20 s	0.82 ± 0.15 (3)		
CaCl$_2$ (0.1)	20 s	1.00 (1.08, 0.92)		
ATP (0.1) + CaCl$_2$ (0.1)	20 s	1.00 (1.02, 0.98)		
ATP (4) + CaCl$_2$ (0.1)	20 s	0.94 ± 0.05 (3)		
GTP (4)	20 s	0.97 ± 0.09 (3)		
PPP (4)	20 s	0.84 ± 0.08 (4)		0.81 ± 0.07 (3)
	20 min	0.70 (0.87, 0.52)		0.80 ± 0.03 (3)
ATP (4), preincubated	20 s	0.72 (0.72, 0.72)		
PPP (4), preincubated	20 s	0.72 (0.72, 0.71)		
ATP (4), added NaCl[b]	20 s	0.80 ± 0.07 (3)		0.86 ± 0.16 (3)
	20 min	0.74 ± 0.07 (3)		0.70 ± 0.09 (3)
		Trypsin-Treated Liposomes		
ATP (4)	20 s	0.47 ± 0.07 (3)	0.77 ± 0.07 (5)	0.72 (0.71, 0.74)

[a] Human erythrocyte membrane proteins were reconstituted into lipsomes, and the stereospecific uptake of glucose was determined. For compounds present externally only, liposomes were prepared and reconstituted in the absence of the compounds, and the compounds were added at the time of the assays (except for those designated "preincubated", in which the reconstituted liposomes were exposed to the compounds for at least 15 min before assay). For ATP present internally only, liposomes were prepared and reconstituted in the presence of ATP, and the external ATP was removed by gel filtration. For ATP and PPP present internally and externally, the liposomes were prepared, reconstituted, and assayed in the presence of the compounds. Trypsin-treated liposomes were incubated after reconstitution with 10 μg of TPCK-treated trypsin/mL for at least 15 min before assays. Assays contained 0.2 mM D-[^{14}C]glucose and tracer L-[^{3}H]glucose, except for one experiment with trypsin-treated liposomes and external plus internal ATP, which was performed at 0.4 mM D-glucose. Stereospecific uptake was determined at the indicated times. Results are single values, means with both values given (for $n = 2$), or means ± SE (for $n > 2$) and expressed as uptakes relative to control uptakes at the same time points. [b] Reconstitutions and assays contained 10 mM NaCl in addition to the usual 10 mM Tris buffer.

Table II: Effects of ATP and Tripolyphosphate on Nonspecific Glucose Uptake[a]

		relative uptake		
compound, concn (mM)	time	external	internal	external plus internal
ATP (4)	20 s	1.05 ± 0.08 (9)	1.15 ± 0.29 (3)	1.11 ± 0.14 (5)
	20 min	0.68 ± 0.10 (3)	0.80 (0.83, 0.76)	0.98 ± 0.02 (4)
	60 min	0.66	0.83	0.81 (0.89, 0.73)
	24 h	0.55 (0.53, 0.57)	0.56 (0.45, 0.66)	0.69 ± 0.09 (3)
PPP (4)	20 s	0.96 ± 0.20 (4)		1.04 (1.08, 1.01)
	20 min	0.66 (0.93, 0.40)		1.08 ± 0.07 (3)
	24 h	0.71 (0.77, 0.66)		
ATP (4), added NaCl[b]	24 h	0.71 ± 0.06 (3)		0.69 ± 0.07 (2)

[a] Reconstitutions were performed as in Table I. For assays at 20 s –60 min, the data are the L-glucose components of the same experiments listed in Table I. For uptakes in 24 h, assays contained L-[^{3}H]glucose but no D-glucose. Results are expressed as uptakes relative to control uptakes at the same time points. [b] Reconstitutions and assays contained 10 mM NaCl in addition to the usual 10 mM Tris buffer.

and without 4 mM ATP. Results are listed in Table II, along with the L-glucose components of the assays for earlier time points. While 4 mM ATP had no effect on the initial rate of L-glucose uptake, it produced about a 30% decrease in the uptake measured in 20 min, in agreement with the decrease in stereospecific uptake. At 24 h, a 45% decrease was observed, in agreement with the calculated shrinking. These measurements do not, however, distinguish between liposomes with and without transporters incorporated; the latter comprise about 40–50% of the total intraliposomal volume.

We also tested whether increasing the osmolarity of the solutions decreased these presumed osmotic effects. Preliminary experiments indicated that performing the reconstitutions and assays in the presence of 10 mM NaCl and 10 mM Tris buffer reduced the activity by about 30% compared to reconstitutions and assays with buffer alone; 25 mM NaCl reduced the activity by 50%. In three experiments in which the reconstitutions and assays contained 10 mM NaCl in addition to buffer, the stereospecific uptake was inhibited an average of 20% at 20 s and 26% at 20 min by 4 mM ATP (Table I). These are not significantly different than the effects in the absence of NaCl. The nonspecific uptake in 24 h was reduced by 29% (Table II). This value is the predicted shrinking of the liposomes in response to the added ATP and is smaller than the effect observed without the NaCl (45%). Thus, the liposomes appear to be shrinking when ATP is added externally, which may be why ATP reduces the stereospecific uptake at relatively late time points (e.g., 20 min).

Effects of Mg^{2+}, Ca^{2+}, GTP, and Tripolyphosphate on Uptake. Allosteric effects of ATP on the glucose transporter might be influenced by the presence of Mg^{2+} or Ca^{2+}. Carruthers (1986b) noted that the presence or absence of 6 mM $MgCl_2$ had no effect on ATP modulation of fluorescence measurements and of glucose transport in vesicles. We nevertheless tested the effects of externally added MgATP (4 mM) on the uptake in reconstituted liposomes. Results are listed in Table I. The effect of ATP was not significantly different in the presence of Mg^{2+}, while $MgCl_2$ (4 mM) alone had no effect. In the presence of 0.1 mM $CaCl_2$, 0.1 mM ATP again had no effect, while the inhibition produced by 4 mM ATP was lower than in the absence of Ca^{2+}.

We also examined the specificity of the ATP inhibition. Hebert and Carruthers (1986, Table III) reported that GTP, UTP, and ITP at 0.5 mM had no effect on glucose exit from inside-out vesicles, while Carruthers (1986b, Figure 2b) observed that the fluorescence of the transporter was unaffected by 10 μM to 1 mM tripolyphosphate. These results indicated that the proposed regulatory effects were specific for ATP. We tested the effects of externally added GTP and tripolyphosphate on the initial rate of reconstituted transport (Table I), using concentrations (4 mM) at which ATP produced about 20% inhibition. While GTP produced no significant inhibition in these experiments, tripolyphosphate produced an inhibitory effect similar to that of ATP. This suggests that the ATP inhibition of reconstituted transport is a nonspecific effect unrelated to allosteric regulation of the transporter. Tripolyphosphate also produced an effect (30% inhibition) similar to that of 4 mM ATP on the stereospecific uptake in 20 min (Table I) and had effects similar to those of ATP on the nonspecific uptake at various time (Table II).

Effects of Preincubation with ATP and Tripolyphosphate. Because some of the effects of ATP observed by Carruthers and co-workers required several minutes to reach their maximum level, we tested the effects of preincubation of reconstituted liposomes with ATP, even though it seemed actions of protein kinases would be unlikely after reconstitution. In the case of externally added ATP, preincubation with the nucleotide (4 mM) increased the level of inhibition slightly, to about 30% (Table I). However, preincubation with 4 mM tripolyphosphate produced the same degree of inhibition, indicating that the enhanced inhibition with prolonged exposure to ATP is a nonspecific effect. Possibly the effect could be due to slow changes in the structure of the liposomes as a result of the increased osmolarity in the extraliposomal solution when ATP or tripolyphosphate is added.

Effects of Internal ATP. We also carried out experiments in which 4 mM ATP was present during the preparation and reconstitution of the liposomes and was therefore incorporated inside of them. To ensure greater reproducibility of the reconstitutions, since different batches of liposomes were being compared, a probe sonicator was used for the final step of the reconstitution, and two or more separate tubes (0.3 mL) of liposomes were reconstituted for each condition and then combined. The external ATP was then removed by gel filtration, and the rate of uptake was compared to liposomes made and reconstituted without ATP. Results are listed in Table I. The initial rate of uptake was reduced by 33%, somewhat more than by external ATP. If the transporters were scrambled in their orientations, internal ATP should have the same effect as external ATP; however, in the former case, the presence of ATP during the reconstitution might also produce an effect on the efficiency of incorporation of the transporters.

The stereospecific uptake at 20 min was reduced by about 20%, consistent with an effect of ATP on the number of transporters reconstituted and/or their internal volume. The uptake of L-glucose in 20 s was unaffected by the ATP, but uptakes in 20 min to 24 h were reduced (Table II), indicating that part of the effect on the 20-min uptake may be due to a change in the size distribution of the liposomes or a reduction in the number of intact liposomes that are formed.[4]

Effects of External and Internal ATP. In another series of experiments, ATP was included in the preparation of the liposomes, in the reconstitutions, and in the assays. Thus, it was present on both sides of the liposomal membranes and able to interact with all potential regulatory sites. Unlike the experiments with only external ATP, constant osmolarity was maintained during reconstitutions and assays for each condition, so shrinking of the liposomes during the assays should not occur. However, as in the experiments where ATP was present only internally, the added ATP present during the reconstitution could change the efficiency of the reconstitution and/or the size of the reconstituted liposomes.

Effects of ATP on the stereospecific uptake are listed in Table I. Initial rates of uptake (measured at 20 or 30 s) were unaffected by 0.1 mM ATP but were reduced by 17–35% at 1–4 mM ATP. These levels of inhibition were greater than those produced by the corresponding levels of ATP when present only externally. Such increased inhibition could result from an allosteric effect of ATP, now able to modulate all of the transporters instead of just half. However, the inhibition produced by 4 mM ATP (35%) was about the same when ATP was present only internally, indicating that some of the increased inhibition (compared to ATP present externally only) could be due to changes in the reconstitution efficiency. In support of effects on the reconstitution, the stereospecific equilibrated volumes (assayed by the uptake at 5 or 20 min) were affected to the same extent as the initial rates.

The nonspecific uptake was unaffected by 4 mM ATP at 20 s and 20 min but was reduced by 20% at 60 min and by 40% at 24 h. Since the liposomes should not be shrinking during the assays, the result demonstrates that ATP has produced nonspecific effects on the reconstitution, resulting in fewer intact liposomes and/or smaller liposomes.

Effects of External and Internal Tripolyphosphate. We also tested the specificity of the above effects produced by ATP on both sides of the membrane by performing similar experiments with 4 mM tripolyphosphate. The stereospecific uptake at 20 s was inhibited 19% (Table I); this was less than the effect of external plus internal ATP at 4 mM (35% inhibition) and similar to the inhibition produced by tripolyphosphate added externally only (16%). The uptake at 20 min using tripolyphosphate on both sides of the membrane was reduced 20%. While less than the 40% reduction produced by ATP under similar conditions, these results indicate that tripolyphosphate also produces effects on the reconstitution efficiency and/or the volume of the reconstituted liposomes. Thus, all of the observed effects of ATP on the reconstituted glucose

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[4] Electron microscopy of negatively stained liposomes reconstituted with and without 4 mM ATP showed them to be similar in appearance. Both preparations were quite heterogeneous in their size distributions, as noted in previous studies using the freeze–thaw procedure (Kasahara & Hinkle, 1977; Sase et al., 1982). The liposomes appeared to be predominantly unilamellar. Since the important conclusions of this study deal with initial rates, which are affected little by the sizes of the liposome, and since the size distributions appeared similar with or without ATP, the heterogeneity should not affect those conclusions.

transport activity are mimicked at least in part by tripolyphosphate, indicating that they are nonspecific. External plus internal tripolyphosphate at 4 mM had no effect on the nonspecific uptake in 20 s or 20 min (Table II), the same result as was observed with 4 mM ATP.

ATP Inhibition of Glucose Uptake in Trypsin-Treated Liposomes. In order to distinguish between the effects of ATP on the two membrane surfaces of the transporter, we incubated the reconstituted liposomes with TPCK-treated trypsin (10 μg/mL) for at least 15 min. Trypsin inactivates glucose transport in erythrocytes only when present at the cytoplasmic surface of the membrane (Masiak & LeFevre, 1977). The sites of trypsin action have recently been characterized (Cairns et al., 1987). We previously showed that trypsin treatment of the purified transporter (Wheeler & Hinkle, 1981) and of ghost proteins (Wheeler, 1986a) after reconstitution results in about a 40% decrease in transport activity, indicating a scrambling of orientations. After treatment of the reconstituted liposomes with external trypsin, the remaining active transporters are therefore oriented as in the erythrocyte, and zero-trans uptake in the liposomes corresponds to zero-trans uptake in erythrocytes. Effects of the initial rate of glucose uptake produced by 4 mM ATP present externally, internally, and on both sides of the membrane were tested (Table I).

When the ATP was added externally, the inhibition (compared to the same trypsin-treated liposomes in the absence of ATP) was about 50%, greater than for liposomes without trypsin. For ATP present internally only, and present on both sides, slightly less inhibition was seen than in the corresponding experiments without trypsin treatment. These results suggest that the effect of ATP is stronger on the extracellular domain of the transporter, since greater inhibition is seen with external ATP when trypsin treatment leaves active transporters oriented only as in erythrocytes.

Effects of ATP on the K_m for Glucose Uptake in Trypsin-Treated Liposomes. According to the model of Carruthers and co-workers, ATP produces, through an allosteric effect, a 60% decrease in the K_m for zero-trans uptake with no effect on the V_{max}. In order to test this prediction with the reconstituted liposomes, we prepared and reconstituted liposomes with and without 4 mM ATP, a concentration which produced modest though significant effects on transport after reconstitution (Table I), and which is 80-fold higher than the concentration producing half-maximal effects in the studies of Carruthers and co-workers. The liposomes were then treated with trypsin, as in the above experiments. In one pair of experiments, ATP was included on both sides of the membrane; in another pair of experiments, liposomes were subjected to gel filtration such that ATP was present only internally. For both of these conditions, ATP was present internally and potentially able to exert allosteric effects at the cytoplasmic domains of those transporters remaining active after the trypsin treatment.

We determined the zero-trans uptake K_m for the trypsin-treated liposomes with and without ATP. The initial rate of glucose uptake at the lowest glucose concentration tested was, for liposomes not containing ATP, reduced 35–40% by the trypsin treatment, verifying that trypsin had produced the expected effect. Table III lists the kinetic parameters obtained in the two pairs of experiments.

In the first pair of experiments, in which ATP was present both inside and outside of the liposomes, the mean K_m values (0.52 and 0.57 mM for control and for ATP, respectively) were not significantly different. In the second pair of experiments, in which ATP was present only internally, more variation in the K_m values was observed, but again the mean K_m values were not significantly different for control and for ATP-containing liposomes. These results are inconsistent with the 60% decrease in the K_m in the presence of ATP which the model of Carruthers and co-workers predicts.

Table III: Effects of ATP on Kinetic Parameters for Zero-Trans Uptake in Trypsin-Treated Reconstituted Liposomes[a]

		kinetic parameters[b]		
[ATP] (mM)	parameter	expt I	expt II	mean
		External plus Internal ATP		
0	K_m	0.62 ± 0.13	0.42 ± 0.07	0.52
	V_{max}	2.33 ± 0.22	1.05 ± 0.11	1.69
4	K_m	0.54 ± 0.15	0.60 ± 0.10	0.57
	V_{max}	1.47 ± 0.14	1.04 ± 0.10	1.26
		Internal ATP		
0	K_m	0.63 ± 0.14	0.91 ± 0.27	0.77
	V_{max}	1.73 ± 0.20	1.96 ± 0.40	1.84
4	K_m	1.40 ± 0.23	0.63 ± 0.20	1.02
	V_{max}	1.90 ± 0.21	1.09 ± 0.20	1.50

[a] Liposomes were prepared and reconstituted in the presence of 0 or 4 mM ATP, and after reconstitution were incubated with 10 μg of TPCK-treated trypsin/mL for at least 15 min before assays. In the first pair of experiments, ATP-containing liposomes were also assayed in the presence of 4 mM ATP. In the second pair of experiments, control and ATP-treated liposomes were passed through gel filtration columns and assayed in the absence of ATP. Stereospecific uptake in 20 s of glucose at four concentrations (0.4, 0.8, 1.5, and 3 mM for the first experiment with and without external plus internal ATP; 0.2, 0.4, 0.8, and 1.5 mM for the other three experiments) was measured. Relative uptakes at the lowest glucose concentration in each experiment are included in the data of Table I. [b] The kinetic parameters and their standard errors were determined by a nonlinear regression program (Duggleby, 1981). Units of K_m, millimolar; of V_{max}, nanomoles per second per milligram.

Another way of considering the data is to compare the effects of ATP at various glucose concentrations. The model predicts that at low glucose concentrations ATP should activate by reducing the K_m, but the effect should decrease with increasing glucose concentration. However, in these experiments, ATP inhibited at all concentrations of glucose tested with no apparent trend in the magnitude of the effect as the glucose concentration was increased.

Since the ATP effect on the V_{max} for uptake (experimentally determined from exit from inside-out vesicles) was slow and was attributed to phosphorylation (Hebert & Carruthers, 1986), the model predicts that there should be no effect of ATP on V_{max} in reconstitution experiments such as these. In both pairs of experiments described in Table III, the mean V_{max} values were slightly (20–25%) lower for ATP-containing liposomes, consistent with the effects observed in other experiments at 0.2 mM glucose (Table I). These differences in V_{max} values were not, however, statistically significant.

DISCUSSION

The technique of reconstitution of membrane proteins into liposomes allows the testing of some aspects of their mechanisms and regulation independent of possible complicating factors in intact cells and native membranes. We used this technique to test whether ATP regulates glucose transport via direct interactions with the glucose transporter. Such regulation has been proposed by Carruthers and co-workers. Other workers have also noted effects of ATP and/or cell age on the kinetics of glucose transport in erythrocytes, though they did not attribute the effects to allosteric interactions with the transporter.

In the experiments reported here, we observed small but significant effects of ATP on reconstituted glucose transport (Table I). When ATP was present only externally, small decreases (only 17% at 4 mM ATP) in the initial rates of uptake were produced. This effect was nonspecific, however, with similar inhibition being produced by tripolyphosphate. Larger decreases were observed in the final stereospecific equilibrated volume, estimated by the uptake in 20 min, but these were likely due to shrinking of the liposomes in the presence of the increased extraliposomal osmolarity. This interpretation is supported by the decrease in L-glucose uptake at 20 min and later times (Table II). Preincubation with ATP increased the inhibition, but this effect was also duplicated by tripolyphosphate.

When ATP was present only internally, a larger degree of inhibition of the initial rate of glucose uptake was produced (33% by 4 mM ATP, Table I). Since ATP was present during the reconstitution, there could be effects of ATP on the reconstitution efficiency and on the size distribution of the reconstituted liposomes. A reduction in the uptake of L-glucose in 24 h (Table II) is consistent with such effects.

When ATP was present both inside and outside of the liposomes, such that it could interact with all transporters at both membrane surfaces, the inhibition of the initial rate of uptake was also larger than in the case of ATP present only externally. However, it was not significantly greater than for ATP presently only internally. This suggests that much of the increased inhibition (compared to external ATP) resulted from changes in the reconstitution efficiency, rather than from the increased exposure of potential regulatory sites to ATP.

Tripolyphosphate, when present both inside and outside of the liposomes, was somewhat less inhibitory than ATP. It is possible that larger effects of ATP than of tripolyphosphate result from interactions of the adenosine portion of ATP with the transporter. Adenosine itself, at millimolar levels, inhibits glucose transport in erythrocytes (Plagemann & Woffendin, 1987; May, 1988), prevents photolabeling of the transporter (Jarvis et al., 1986), and blocks the binding of cytochalasin B (Jarvis, 1988). However, more likely the greater effect of ATP compared to tripolyphosphate results from the ATP effect on the reconstitution efficiency, since when tripolyphosphate was present only externally, it inhibited as strongly as ATP.

The scrambling of the transporters after reconstitution complicates the interpretation of the experiments described above. It is possible that allosteric effects of ATP acting at the cytoplasmic domain of the transporter could be masked by opposing effects of ATP acting on the extracellular surface. Therefore, additional experiments were carried out using trypsin to generate populations of active reconstituted transporters oriented as in erythrocytes (Wheeler & Hinkle, 1981). Using a simple model for the effects of ATP when it is present on only one side of the membrane, it is possible to estimate the contributions of effects at the two surfaces of the transporter and on reconstitution (see the Appendix). When applied to the data obtained with 4 mM ATP, this analysis indicates that the effect at the extracellular surface of the transporter (30–50% inhibition) is greater than that at the cytoplasmic surface (5% inhibition to 20% activation). This can be seen readily by noting that external ATP inhibited more strongly (50%) after trypsin treatment (when the effects are only at the extracellular domains) than before treatment (20%), when half of the transporters have their cytoplasmic domains outward.

The major effect of ATP after reconstitution is therefore unlikely to be of physiological significance, since the extracellular domain of the transporter would not be exposed to cellular ATP. Furthermore, the analysis reveals that ATP acting at the cytoplasmic domain produces only weak effects, if any; there is not a strong activation (such as 2.5-fold predicted by the model of Carruthers and co-workers) which is masked by a strong inhibition at the opposite surface (note that for scrambled transporters without trypsin treatment, even if *all* of the transport activity were blocked by ATP acting on the extracellular domain, the remaining half of the transporters, if activated 2.5-fold, would give a net 25% increase in rate).

Finally, the analysis suggests that the presence of 4 mM ATP produces about a 20% decrease in the transport rate as a result of decreasing the efficiency of reconstitution.

The results presented here argue against the model of Carruthers and co-workers. According to that model, if only allosteric effects of ATP occur, at low glucose concentrations ATP should cause a 2.5-fold activation (by increasing the ratios of $(V_{max}/K_m)^1$ of zero-trans flux when all transporters are exposed to ATP. In the case of ATP present only externally, when only half of the transporters have their cytoplasmic domains accessible to ATP, a 1.75-fold activation would be expected. Instead, we observed only weak inhibition. This was nonspecific and was significant only at ATP levels much higher than the apparent K_d (50 μM) reported by Carruthers and co-workers. Finally, the model predicts a 60% decrease in the K_m for zero-trans uptake in the presence of ATP; for the reconstituted trypsin-treated liposomes with internal ATP, no effect on K_m was observed (Table III).

The absence of activating effects of ATP on the reconstituted transporter also argues against effects of ATP on the V_{max} for zero-trans uptake (Jacquez, 1983) and on the K_m for zero-trans efflux (Jensen & Brahm, 1987) being allosteric effects on the transporter. Previously we had shown that activating effects of ATP in ghosts were not preserved after reconstitution and thus were unlikely to be due to covalent regulation (Wheeler, 1986a). Since the results reported here do not support allosteric regulation, the ATP effects observed by Jacquez (1983) and by Jensen and Brahm (1987) are most likely indirect effects on the structure of the membrane, as suggested previously (Wheeler, 1986a).

It is possible that during the reconstitution the transporter is altered such that allosteric effects can no longer be observed. However, the procedure employed in our experiments involves a minimum amount of manipulation of samples and avoids possible changes in the protein as a result of detergent treatment and/or further purification procedures. It should be noted that ATP effects on the fluorescence of the transporter, which were thought to be related to allosteric effects on transport, were observed after the protein was extracted with Triton X-100 and purified (Carruthers, 1986b), and thus was subjected to conditions potentially more injurious than those which we employed. We cannot, however, rule out the possibility that when reconstituted into the new environment of the liposomal membrane, the transporter is altered so that it becomes insensitive to ATP regulation. Since the reconstituted transporter resembles the transporter in the erythrocyte in several respects (asymmetry of kinetic parameters, faster exchange than net flux, sensitivity to glucose transport inhibitors) (Wheeler & Hinkle, 1981), such a selective effect of the change in environment seems unlikely.

Our experiments did not address the time-dependent effects observed by Carruthers and co-workers; these presumably require the action of erythrocyte enzymes (such as protein kinases) and would not necessarily be expected to be retained after reconstitution. The experiments reported here do, however, strongly argue against the existence of allosteric effects of ATP on glucose transport in erythrocytes.

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APPENDIX

The effect of ATP on the two membrane surfaces of the glucose transporter can be estimated by considering experiments in which ATP is present on only one side of the membrane and by comparing results obtained with and without trypsin treatment of the liposomes. This analysis assumes that the observed effects of ATP on initial rates either are directly on the transporter or are on the efficiency of reconstitution; possible osmotic effects are not considered.

Let the effects of a given concentration of ATP on initial rates, relative to rates in the absence of ATP, be defined as follows:

a = effect produced at the extracellular surface of the transporter

b = effect produced at the cytoplasmic surface of the transporter

c = effect on the reconstitution efficiency

(a and b refer to the respective surfaces of the transporter as it is oriented in erythrocytes; after reconstitution, each of these may be present at either surface of the liposomes.)

If the transporters are reconstituted with a scrambling of orientations, such that approximately equal amounts of each transporter surface are oriented outward, then the relative rate in the presence of external ATP will be

$$0.5a + 0.5b$$

When trypsin treatment is used to inactivate the transporters having their cytoplasmic domains oriented on the outside of the liposomes, the effect of external ATP (relative to trypsin-treated liposomes without ATP present) will be solely at the extracellular surface of the remaining active transporters, and be equal to a.

When the transporters are reconstituted in the presence of ATP, there may be an effect on the efficiency of reconstitution (c). After the external ATP is removed by gel filtration, so that ATP acts only internally, the effect on the scrambled liposomes will be

$$c(0.5a + 0.5b)$$

assuming that the effect c is equal for both orientations. With trypsin treatment, only transporters with their cytoplasmic domains oriented inside the liposomes and accessible to ATP will remain active. Then the relative rate will be bc.

This analysis can be applied to the data of Table I for 4 mM ATP. Using external ATP only for control and for trypsin-treated liposomes, respectively:

$$0.5a + 0.5b = 0.83 \pm 0.03$$

$$a = 0.47 \pm 0.07$$

Then $b = 1.19 \pm 0.09$.

Comparing external ATP to internal ATP (both without trypsin treatment), respectively:

$$0.5a + 0.5b = 0.83 \pm 0.03$$

$$c(0.5a + 0.5b) = 0.67 \pm 0.05$$

Then $c = 0.81 \pm 0.07$.

Using this value of c in the case of trypsin-treated liposomes with ATP present internally:

$$bc = 0.77 \pm 0.07$$

Then $b = 0.95 \pm 0.12$. This value of b, when substituted into the expression $0.5a + 0.5b$ above, yields a higher estimate of a (0.71) than estimated from the rate obtained with external ATP and trypsin-treated liposomes (0.47).

Registry No. ATP, 56-65-5; PPP, 14127-68-5; glucose, 50-99-7.

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