EFFECT OF EXTERNALLY ADDED ATP ON GLUCOSE UPTAKE BY ISOLATED RAT SOLEUS MUSCLE

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SUMMARY

1. Glucose uptake by isolated rat soleus muscle was stimulated by insulin, anoxia, 2,4-dinitrophenol and salicylate. Anoxia and salicylate both stimulated glucose uptake by increasing the v_{max} of this process.

2. Anoxia, 2,4-dinitrophenol and salicylate, but not insulin, depleted the ATP content. When muscles were incubated at 21° anoxia stimulated glucose uptake, but did not affect the ATP concentration.

3. ATP (5 mM) added to the incubation medium inhibited glucose uptake under anaerobic conditions, but not aerobically. In the presence of 2,4-dinitrophenol, salicylate or insulin, ATP was without effect.

4. Inhibition of anaerobic glucose uptake was specific to ATP; neither GTP, ITP nor the breakdown products of ATP were effective in this respect. ATP inhibited glucose uptake competitively. This inhibition was not due to chelation of divalent cations.

5. From these experiments it is concluded that the effect of anoxia on sugar transport is not directly mediated *via* ATP.

INTRODUCTION

The transport of glucose into the muscle cell is believed to involve a stereo-specific carrier mechanism[1,2], which constitutes the initial rate-limiting step in the overall utilization of glucose by this tissue. RANDLE AND SMITH[3], showed that sugar transport was stimulated by anoxia and certain metabolic inhibitors, and MORGAN *et al.*[4] subsequently demonstrated that this increased sugar uptake was due to acceleration of the monosaccharide transport system and not the result of a damaged cell membrane. As anoxia and these inhibitors all interfered in some way with the process of mitochondrial oxidative phosphorylation, and thus lowered intracellular ATP levels, it was concluded that ATP might serve to control, by a feedback mechanism, the entrance of glucose into the muscle cell[3,5]. However, a cause–effect relationship between stimulation of sugar transport and lowering of ATP levels has yet to be established[6]; in fact ŌZAND *et al.*[7] found that anoxia stimulated glucose uptake in frog sartorius muscle without affecting the level of ATP.

Previous work in this laboratory has shown that rat soleus muscle is particularly suitable for *in vitro* experimentation[8]. As shown below, the addition of ATP to rat

soleus muscle incubated *in vitro*, inhibited anaerobic glucose uptake. This paper explores the nature of this inhibition and its possible relationship to the regulatory system proposed by RANDLE AND SMITH[3].

METHODS

Incubation and basic assay procedures

Soleus muscles weighing approx. 30 mg were incubated in 1.0 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose and other additions as noted below. Incubations were carried out in a Gallenkamp metabolic shaker for 1 h at 37° under an atmosphere of either O_2-CO_2 (95:5, v/v) or N_2-CO_2 (95:5, v/v); shaking rate 110 cycles/min. Glucose uptake, glucose space and raffinose space were all determined as described previously[8].

For the determination of ATP, the muscles were frozen between aluminium plates chilled in dry ice, then homogenized in a trichloroacetic acid (5 %)–HCl (0.1 M) mixture and centrifuged. The supernatant solution was extracted four times with ether and then neutralized with 1 M Tris base. ATP was assayed spectrophotometrically using glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1). Uptake of ^{14}C-labelled ATP was determined using the procedure described in the preceding paper[9].

Determination of extracellular glucose concentration

When soleus muscles were incubated in bicarbonate medium containing glucose at concentrations below 20 mM, the glucose space was less than the extracellular (*i.e.* raffinose) space[8]. This indicated that the concentration of glucose in the extracellular space was less than that of the external medium. Accordingly, from the glucose content of each muscle the mean extracellular concentration of glucose was calculated, using the raffinose space (0.25 ml/g muscle) as the extracellular space. This value has been used for the kinetic experiments presented in Figs. 1 and 2. The theory and application of this procedure have been explained in our previous paper[8].

MATERIALS

ATP, ADP, AMP, adenosine, ITP, IMP, inosine, cyclic adenosine 3',5'-phosphate and GTP were obtained from Sigma Chemical Co., St. Louis. All enzymes used were obtained from C. F. Boehringer und Soehne, Mannheim. Crystalline beef insulin was a gift from the Commonwealth Serum Laboratories, Melbourne.

RESULTS

Intracellular ATP content and glucose uptake

In Table I, the effects of insulin, anoxia, 2,4-dinitrophenol and sodium salicylate on glucose uptake and several associated parameters, are shown. From this it can be seen that all of these agents stimulated glucose uptake. Glucose space was unaffected by insulin or anoxia and, although increased by treatment with 2,4-dinitrophenol or salicylate, it was only in the 2,4-dinitrophenol-treated muscles that this value exceeded the extracellular space, indicating an intracellular accumulation of free glucose.

TABLE I

EFFECT OF INSULIN, ANOXIA, 2,4-DINITROPHENOL AND SALICYLATE ON VARIOUS PARAMETERS ASSOCIATED WITH GLUCOSE UPTAKE

Soleus muscles were incubated for 1 h at 37° in 1.0 ml of Krebs–Henseleit buffer (pH 7.4) containing glucose (10 mM) and other additions as shown; insulin (0.1 unit/ml), 2,4-dinitrophenol (0.5 mM), or sodium salicylate (5 mM). For details concerning the determination of the various parameters shown, refer to METHODS. Values are mean of four determinations ± S.E.

| Incubation conditions		Glucose uptake (μmoles/g per h)	Glucose space (ml/g)	Raffinose space (ml/g)	ATP (μmoles/g)
Atmosphere	Additions				
O_2–CO_2	—	12.7 ± 1.2	0.18 ± 0.05	0.26 ± 0.01	2.4 ± 0.15
O_2–CO_2	Insulin	22.4 ± 2.1	0.19 ± 0.05	0.25 ± 0.03	2.3 ± 0.12
O_2–CO_2	—	12.6 ± 1.0	0.16 ± 0.01	0.26 ± 0.01	2.0 ± 0.04
N_2–CO_2	—	21.0 ± 1.1	0.15 ± 0.02	0.25 ± 0.01	1.0 ± 0.04
O_2–CO_2	—	10.8 ± 0.5	0.17 ± 0.01	0.25 ± 0	2.4 ± 0.04
O_2–CO_2	2,4-Dinitrophenol	15.3 ± 1.2	0.73 ± 0.03	0.30 ± 0.01	<0.2
O_2–CO_2	—	9.5 ± 1.0	0.18 ± 0	0.26 ± 0.01	2.4 ± 0.04
O_2–CO_2	Salicylate	16.6 ± 1.0	0.26 ± 0.01	0.27 ± 0.01	0.4 ± 0.05
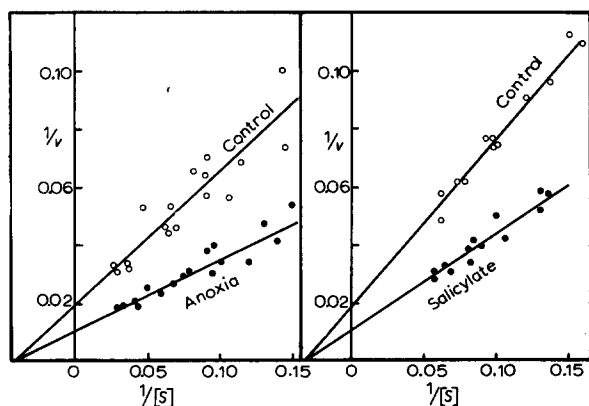
Fig. 1. Effect of anoxia and sodium salicylate on the kinetics of glucose uptake. Glucose uptake was determined at various concentrations of glucose ranging from 10 to 36 mM, using the system described in Table I. The results are presented here in the form of a Lineweaver–Burk plot where v = glucose uptake (μmoles glucose per g muscle per h) and $[S]$ = the concentration of glucose (mM) in the extracellular space, calculated as described under METHODS. Control, aerobic incubation; Anoxia, anaerobic incubation; Salicylate, aerobic incubation + 5 mM sodium salicylate. Each line has been drawn using the method of least squares. As the muscles accumulated glucose in the presence of salicylate when the initial external glucose concentration exceeded 20 mM, results above this concentration have not been included in the figure.

Anoxia, 2,4-dinitrophenol and salicylate are all believed to influence sugar transport by the same mechanism. As shown in Fig. 1, salicylate and anoxia affected the kinetics of glucose uptake in like fashion. Both increased the maximum velocity of this process without altering the apparent K_m. This is in marked contrast to the effect of insulin, which was shown to decrease the apparent K_m of the transport process[8]. As argued in our previous paper, only if sugar transport remains rate-limiting so that free glucose

does not accumulate within the cell, is it possible to apply Michaelis–Menten kinetics to the process of glucose uptake in soleus muscle. For this reason we have not attempted to determine the kinetics of 2,4-dinitrophenol stimulation.

The residual content of ATP in the muscles at the end of the incubation is also shown in Table I. Although insulin did not affect the ATP concentration, the increased glucose uptake induced by anoxia, 2,4-dinitrophenol and salicylate was accompanied by decreased levels of ATP. It has been proposed that the stimulation of glucose uptake is a direct result of this lowering of ATP concentrations. However, in contrast to the effect of anoxia seen here, stimulation of glucose uptake in frog sartorius muscle during extended periods of anaerobiosis was not accompanied by depletion of intracellular ATP[7]. This difference appears to be a consequence of the lower temperatures used for the incubation of frog muscle, rather than any inherent difference between amphibian and mammalian muscle. When soleus muscle was incubated at 21° anoxia still stimulated glucose uptake but now did not affect the level of intracellular ATP (Table II). Thus we have confirmed, in a mammalian muscle, the separation of glucose transport from ATP depletion, first observed in the frog by ÖZAND et al.[7]. On first appraisal, this result would appear to render invalid the suggestion that glucose transport was controlled by the level of ATP. However, as discussed below, if the source of the ATP is considered, it is still possible that the ATP produced by either glycolysis or oxidative phosphorylation may have a specific regulatory function.

TABLE II

EFFECT OF ANOXIA ON GLUCOSE UPTAKE AND ATP CONTENT OF SOLEUS MUSCLE INCUBATED AT 21°

Soleus muscles were incubated for 1 h at 21° in 1.0 ml of Krebs–Henseleit buffer (pH 7.4) containing glucose (10 mM), under an atmosphere of either O_2-CO_2 (95:5, v/v) or N_2-CO_2 (95:5, v/v). Glucose uptake and muscle ATP content were determined as described under METHODS. Values are mean of four determinations ± S.E.

Incubation conditions	Glucose uptake ($\mu moles/g$ per h)	ATP ($\mu moles/g$)
Aerobic	4.3 ± 0.7	3.1 ± 0.04
Anaerobic	12.8 ± 0.4	3.1 ± 0.06

Effect of externally added ATP

Although increased glucose uptake in the presence of anoxia, 2,4-dinitrophenol and salicylate was associated with decreased ATP levels, the problem remained of establishing a direct link between ATP and the regulation of sugar transport. According to the feedback theory, ATP acts by phosphorylating a carrier molecule, thereby rendering it inactive. As this occurs at the cell membrane, it seemed reasonable to assume that external ATP might influence the carrier system in a manner similar to that proposed for internal ATP. The addition of ATP to the incubation medium decreased glucose uptake under anaerobic conditions, but was without effect under aerobic conditions (Table III).

This observation lead to a re-examination of the generally held belief that muscle was impermeable to ATP, and, as reported in the preceding paper[9] it was established that externally added ^{14}C-labelled ATP actually entered soleus muscle. As shown in Table III anoxia increased the uptake of labelled ATP from the medium.

Thus the effect of ATP on glucose uptake reported here, need not necessarily be considered an external effect as originally thought.

TABLE III

EFFECT OF EXTERNALLY ADDED ATP ON GLUCOSE UPTAKE

Glucose uptake was determined using the system described in Table I. Where ATP (5 mM) was added to the incubation medium, an equimolar amount of $MgCl_2$ was also added. Uptake of ^{14}C-labelled ATP was determined in a parallel series of experiments. Two soleus muscles were incubated for 1 h at 37° in 1.0 ml of glucose–bicarbonate medium containing 5 mM [8-^{14}C]ATP (0.05 $\mu C/\mu mole$). The ATP present in 50-μl samples of medium and a trichloroacetic acid extract of muscle was separated electrophoretically and the radioactivity counted. Intracellular ^{14}C-labelled ATP was determined by subtracting from the total ^{14}C-labelled ATP content of the muscle, the amount calculated to be present in the extracellular water. For details of these procedures refer to the preceding paper[9]. Values are mean ± S.E. of the number of observations shown in parentheses.

Incubation conditions	Glucose uptake ($\mu moles/g$ per h)		Intracellular [^{14}C]ATP ($\mu mole/g$)
	No ATP	+ATP	
Aerobic	9.6 ± 0.5 (4)	10.3 ± 0.6 (4)	0.44 ± 0.01 (2)
Anaerobic	25.4 ± 1.9 (4)	15.4 ± 1.6 (4)	0.63 ± 0.04 (2)

Specificity of ATP inhibition

When soleus muscles were incubated in medium containing ATP, extensive degradation of the nucleotide occurred[9]. ADP, AMP, IMP, adenosine and inosine were all detected in the medium at the end of the incubation period. This raised the possibility that these products of ATP breakdown might be the inhibitory agent(s), rather than ATP itself. However, when tested, ADP (2 mM), AMP (2 mM), adenosine (2 mM), IMP (2 mM), inosine (2 mM), P_i (10 mM) and NH_4Cl (3 mM) all proved ineffective. GTP (5 mM) and ITP (5 mM) had no effect anaerobically, and contrary to the claim of EDELMAN et al.[10], cyclic adenosine 3′,5′-phosphate (5 mM) was without effect either

TABLE IV

EFFECT OF DIVALENT CATIONS AND EDTA ON GLUCOSE UPTAKE

Glucose uptake was determined using the system described in Table I. Additions: $MgCl_2$ (20 mM), $CaCl_2$ (10 mM), EDTA (5 mM), ATP (5 mM). Values are mean of four determinations ± S.E.

Additions	Glucose uptake ($\mu moles/g$ per h)	
	Aerobic	Anaerobic
—	15.8 ± 1.7	22.8 ± 2.1
+ $MgCl_2$	13.0 ± 0.9	20.7 ± 0.9
+ $CaCl_2$	—	23.5 ± 1.9
+ EDTA	5.7 ± 0.4	15.5 ± 1.3
+ EDTA + $MgCl_2$	11.9 ± 0.1	22.5 ± 0.7
+ ATP	—	15.4 ± 1.6
+ ATP + $MgCl_2$	—	12.7 ± 0.9
+ ATP + $CaCl_2$	—	13.9 ± 1.0

aerobically or anaerobically. From this we may conclude, that the inhibitory effect on glucose uptake was confined to ATP itself.

Role of chelation

Before postulating a direct metabolic effect of ATP on the sugar transport system, it was necessary to eliminate the possibility that the effect seen here was due simply to chelation of divalent cations. We had anticipated this possibility, and so, in all experiments where ATP was added to the incubation medium, an equimolar amount of $MgCl_2$ was also added. However, as this might not have been enough to overcome the capacity of ATP for chelation, this point was studied further and these experiments are shown in Table IV. Glucose uptake was inhibited by EDTA (5 mM). In contrast to ATP which inhibited only under anaerobic conditions, EDTA inhibited uptake aerobically and anaerobically. Furthermore, whereas inhibition by EDTA could be relieved by increasing the Mg^{2+} content of the medium, neither Mg^{2+} nor Ca^{2+} affected inhibition by ATP. These experiments suggest that the effect of ATP on this system was not due to its chelating activity.

Kinetics of ATP inhibition

As shown in Fig. 1, the kinetics of anoxia- and salicylate-stimulated glucose uptake indicated that these two agents acted by increasing the transport v_{max}. From this, one might expect that the inhibition of anaerobic glucose uptake by ATP would exhibit non-competitive kinetics, however, as shown in Fig. 2, ATP was found to inhibit competitively. Thus, kinetically, the inhibitory effect of ATP resembles more the stimulatory effect of insulin, than that of anoxia[8].

Effect of externally added ATP in the presence of 2,4-dinitrophenol, salicylate and insulin

Whereas 2,4-dinitrophenol and salicylate stimulate glucose uptake in a manner considered to be similar to that of anoxia, this stimulation was unaffected by the addition of ATP to the incubation medium (Table V). Muscles incubated in the presence of these uncouplers accumulated less radioactive ATP from the medium than corresponding control muscles (*cf.* Table III), however, at this stage we cannot say
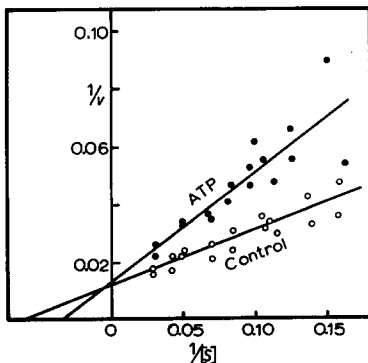

Fig. 2. Effect of ATP on the kinetics of glucose uptake. Glucose uptake was determined under anaerobic conditions in the presence and absence of ATP (5 mM). An equimolar amount of $MgCl_2$ was added along with the ATP. The glucose concentration was varied from 10 to 36 mM. The results are shown in the form of a Lineweaver–Burk plot as described under Fig. 1.

TABLE V

EFFECT OF EXTERNALLY ADDED ATP ON GLUCOSE UPTAKE IN THE PRESENCE OF 2,4-DINITRO-PHENOL, SALICYLATE AND INSULIN

The uptake of glucose and ^{14}C-labelled ATP was determined as described in Table III. Additions:: 2,4-dinitrophenol (0.5 mM), sodium salicylate (5 mM), insulin (0.1 unit/ml). Values are mean $\pm$ S.E. and the number of observations shown in parentheses.

Incubation conditions		Glucose uptake (μmoles/g per h)		Intracellular [^{14}C]ATP (μmole/g)
Atmosphere	Additions	No ATP	+ ATP	
O_2–CO_2	2,4-Dinitrophenol	17.9 $\pm$ 1.0 (4)	18.7 $\pm$ 1.2 (4)	0.12 $\pm$ 0.01 (2)*
O_2–CO_2	Salicylate	19.4 $\pm$ 0.7 (4)	19.5 $\pm$ 0.5 (4)	0.09 $\pm$ 0.01 (2)*
O_2–CO_2	Insulin	23.6 $\pm$ 2.4 (4)	—	—
N_2–CO_2	Insulin	35.5 $\pm$ 0.5 (4)	37.0 $\pm$ 3.1 (4)	0.62 $\pm$ 0.04 (2)*

* For corresponding control values, refer to Table III.

with certainty whether this was due to a lowering of ATP uptake or increased break-down of labelled ATP subsequent to its entry. It is very likely that the failure of added ATP to affect glucose uptake in the presence of 2,4-dinitrophenol or salicylate reflects secondary effects of these two agents, e.g. their stimulation of mitochondrial ATPase activity[11].

When glucose uptake was stimulated by insulin, anoxia evoked a further in-crease (Table V). Despite this, ATP had no effect anaerobically in the presence of insulin, even when the muscles were first preincubated with ATP before exposure to the hormone. In contrast to the results observed in the presence of 2,4-dinitrophenol and salicylate, insulin did not affect the uptake of labelled ATP from the medium.

DISCUSSION

According to the theory proposed by RANDLE AND SMITH[3], ATP regulates the transport of glucose into the muscle cell by functioning as a feedback inhibitor; anoxia, 2,4-dinitrophenol and salicylate all stimulate sugar transport by lowering the intracellular concentration of ATP. Although our kinetic experiments have shown that anoxia and salicylate affected glucose uptake in like fashion, from the results presented in Tables I and II, it is clear that there was no simple relationship between depletion of ATP and stimulation of glucose uptake. Thus enhanced uptake was ob-served under conditions where the residual level of ATP varied from 0 to 100 % of that found in control muscles. The most striking example of this was the complete separation of glucose uptake from ATP depletion seen when soleus muscles were incu-bated under anaerobic conditions at 21°. This result, however, does not preclude the possibility that ATP is involved in the regulation of sugar transport.

WU AND RACKER[12] have shown that intracellular compartmentation can limit the availability of ATP produced by glycolysis and by oxidative phosphorylation, so that a system may have a specific requirement for the ATP produced by one or other of these two sources. Although, at 21°, aerobic and anaerobic muscle contained the same amount of ATP, in aerobic muscle this would have been produced largely by oxidative phosphorylation, whereas in anaerobic muscle the ATP present would have been produced exclusively by glycolysis. If these qualitative differences are con-

sidered, then the experiment shown in Table II is still consistent with the regulation of sugar transport by ATP. However, if ATP is directly involved in this process, one must now differentiate between two very different systems, each with a specific requirement for either mitochondrial or glycolytic ATP. If mitochondrial ATP were the regulator, then it would indeed be acting as a feedback inhibitor. On the other hand, if regulation were mediated *via* glycolytic ATP, this would mean that sugar transport was an active process requiring specifically the ATP produced by glycolysis.

The failure of added ATP to stimulate glucose uptake under any of the conditions used for these experiments would appear to refute this second possibility. Corroborative evidence for this comes from experiments which studied the effect of iodoacetate on the uptake of [1-^{14}C]xylose by soleus muscle (M. K. GOULD AND E. RACKER, unpublished data). The ATP content of muscles incubated for 30 min in the presence of iodoacetate (5 mM) and 2,4-dinitrophenol (0.5 mM) was completely depleted, as determined by spectrophotometric assay using glucose-6-phosphate dehydrogenase and hexokinase; nevertheless, maximal rates of xylose uptake were observed in these ATP-depleted muscles. This would appear to eliminate the possibility that glycolytic ATP served to activate the monosaccharide transport system.

Under anaerobic conditions, the addition of ATP to the incubation medium inhibited glucose uptake. This inhibition was not due to chelation of divalent cations, and was specific to ATP; GTP, ITP and the breakdown products of ATP were all without effect. In two respects, the effects of ATP, reported above, run counter to those which might be expected, if ATP were to regulate glucose transport in the manner proposed by RANDLE AND SMITH[3]. In the presence of insulin, anoxia effected a further stimulation of glucose uptake. If this stimulation were mediated *via* ATP as has been suggested, then it is difficult to understand why added ATP was without effect. Insulin did not affect the penetration of ^{14}C-labelled ATP (Table V), and so, the inability of added ATP to influence glucose uptake in the presence of the hormone cannot be explained on this basis. Furthermore, the kinetic experiments shown in Figs. 1 and 2, indicate that ATP did not act by simply reversing the stimulation of glucose uptake produced by anoxia. It is difficult, therefore, to reconcile these results with the second alternate proposed above, namely that mitochondrial ATP serves as a feedback inhibitor to regulate sugar transport.

Our consideration of separate, specific regulatory roles for glycolytic and mitochondrial ATP was prompted by the lack of any correlation between stimulation of glucose uptake and the extent to which ATP levels were depleted by anoxia and certain uncoupling agents. The evidence presented above leads us to reject both alternates, and to conclude that there is no cause–effect relationship between these two events. Thus, the mechanism whereby anoxia stimulates glucose transport does not appear to involve an ATP-mediated feedback system.

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