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EVIDENCE FOR THE UPTAKE OF ATP BY RAT SOLEUS MUSCLE *IN VITRO*

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SUMMARY

1. Rat soleus muscle was incubated in the presence of [8-^{14}C]ATP, [8-^{14}C]ADP or [8-^{14}C]adenosine. Samples of incubation medium and muscle extract were subjected to electrophoretic separation and the radioactivity present in ATP, ADP, AMP, IMP, adenosine, inosine and hypoxanthine was counted.

2. Extensive degradation of the added nucleotides was observed. Whereas adenylate kinase and adenosine deaminase activities appeared in the incubation medium, the other enzymes responsible for ATP degradation did not, suggesting that they were located on the exterior surface of the muscle cell.

3. The concentrations of ^{14}C-labelled ATP and ADP found in the muscle indicated that these compounds were present within the fibres. Evidence is presented which suggests that ATP, and to a lesser extent ADP, entered the muscle as such and were not synthesized within the muscle from ^{14}C-labelled adenosine.

INTRODUCTION

It is a commonly held belief that cell membranes are impermeable to ATP[1,2]. On the other hand, there are indications in the literature that ATP might be able to cross the muscle cell membrane. BUCHTHAL *et al.*[3,4] have shown that ATP added to the medium bathing isolated muscle fibres induced their contraction. More recently, BOYD AND FORRESTER[5] found that ATP was released from muscle working *in vitro*, under conditions where K^+ was not. From this, they concluded that the release of ATP was a physiological process, and not simply the result of damaged muscle fibres.

We have recently found that ATP, added to the incubation medium, inhibited anaerobic glucose uptake by isolated rat soleus muscle[6]. It was originally assumed that the added ATP was confined to an extracellular distribution, and thus influenced the glucose transport system at the exterior surface of the cell. However, experiments using ^{14}C-labelled ATP, demonstrated quite the opposite. As reported below, soleus muscle incubated *in vitro* accumulated ATP from the external medium.

METHODS

Soleus muscles weighing approx. 30 mg were incubated (1 per beaker) for 1 h at 37° in 1.0 ml of Krebs–Henseleit buffer (pH 7.4) containing 10 mM glucose and either 5 mM [8-^{14}C]ATP (0.45 μC/μmole), 5 mM [8-^{14}C]ADP (0.05 μC/μmole), or

0.5 mM [8-^{14}C]adenosine (2 μC/μmole); atmosphere, O_2–CO_2 (95:5, v/v). The muscles were homogenized in 1.0 ml of a solution containing trichloroacetic acid (5 %) and HCl (0.1 M) and centrifuged. The supernatant solution was extracted four times with ether and then neutralized with 1 M Tris base. Samples (50 μl) of muscle extract and incubation medium were applied to Whatman No. 3 MM paper and over-spotted with 10 μl of a marker solution containing 0.05 μmole each of ATP, ADP, AMP, adenosine, IMP and inosine. Following electrophoretic separation, using the system described by WADKINS AND LEHNINGER[7], the individual nucleotide* spots were detected under ultraviolet light, cut from the paper and placed in a counting vial together with 15 ml of the aqueous scintillator solution of BRUNO AND CHRISTIAN[8]. Radioactivity was counted in a Nuclear Chicago Unilux liquid scintillation counter. Approx. 90–95 % of the radioactivity applied to the electrophoretogram was recovered.

The concentrations of adenine and hypoxanthine nucleotides in medium (μmoles/ml) and muscle (μmoles/g) were calculated from the radioactivity observed in each fraction. A nucleotide was considered to have an intracellular distribution, when the total muscle content exceeded the extracellular content. Extracellular concentrations were calculated on the assumption that the concentration in the extracellular water was the same as that of the medium. Extracellular space was determined using raffinose, as described previously[9].

Adenine nucleotides were also assayed spectrophotometrically; ATP, using glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1), ADP and AMP, using lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and adenylate kinase (EC 2.7.4.3).

MATERIALS

ATP, ADP, AMP, adenosine, IMP and inosine were all obtained from Sigma Chemical Co., St. Louis. [8-^{14}C]ATP, ammonium salt and [8-^{14}C]ADP, ammonium salt were obtained from the Radiochemical Centre, Amersham. Prior to use, each was purified by electrophoresis using the system described above, and diluted with non-radioactive nucleotide (sodium salts) to the appropriate specific activity. [8-^{14}C]-Adenosine was obtained from International Chemical and Nuclear Corp., Calif., and was purified by electrophoresis before use. All enzymes were obtained from C.F. Boehringer und Soehne, Mannheim.

RESULTS

Penetration of muscle by adenine and hypoxanthine nucleotides

When soleus muscles were incubated for 1 h in medium containing 5 mM ^{14}C-labelled ATP, extensive degradation of ATP occurred (Fig. 1A). The principal breakdown products appearing in the medium were ADP, AMP and IMP, together with smaller amounts of adenosine and inosine. No radioactivity was found on the electrophoretogram in the areas where adenine or hypoxanthine, if present, would have been located. As anticipated, AMP and IMP were restricted to an extracellular distribution, while adenosine and inosine entered the muscle. What was not anticipated,

* To avoid repeated qualifications, 'nucleotide' will also include adenosine and inosine where applicable.

however, was the clear demonstration that externally added ATP was now present intracellularly, together with labelled ADP.

Intracellular ATP and ADP are interconvertible, and so it was not clear whether the labelled ATP and ADP within the muscle had entered as ATP, ADP, or even as adenosine or inosine. In order to resolve these questions, further experiments were undertaken, (a) using a regenerating system to maintain a high ATP/ADP ratio (Fig. 1B), and (b) using ^{14}C-labelled ADP and adenosine (Figs. 1C and 1D). [^{14}C]-Adenosine was used at a concentration of 0.5 mM. This was less than the concentration of ATP and ADP, but still much greater than the concentration of adenosine formed as a result of ATP or ADP breakdown. Despite the increased concentration, the ATP synthesized by the muscle from labelled adenosine amounted to only 2 % of that found in muscles incubated with ^{14}C-labelled ATP, thus eliminating the synthetic pathway as a major source of this ATP. In the presence of a regenerating system (creatine phosphate: creatine kinase (EC 2.7.3.2)) about 80 % of the added ATP remained at the end of the incubation period. Intracellular ^{14}C-labelled ATP was doubled, while ^{14}C-labelled ADP was barely detectable within the cell. This seems to indicate that ATP entered the muscle cell as such. Using ^{14}C-labelled ADP, external degradation of the added nucleotide was also observed. One of the products of ADP breakdown was a small amount of ATP produced presumably through the action

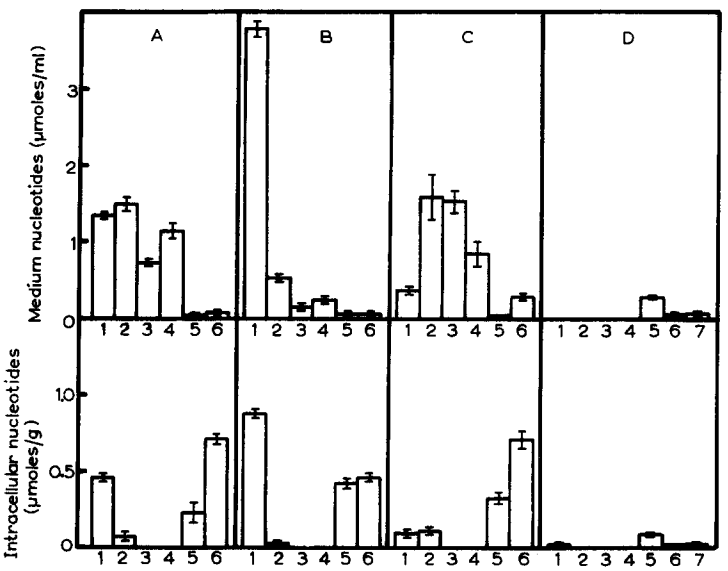

Fig. 1. External degradation and intracellular accumulation of adenine nucleotides. Soleus muscles were incubated for 1 h at 37° under O_2–CO_2 (95:5, v/v) in 1.0 ml of glucose–bicarbonate medium containing (A) 5 mM ^{14}C-labelled ATP (0.45 μC/μmole); (B) 5 mM ^{14}C-labelled ATP (0.45 μC/μmole), 20 μmoles creatine phosphate, 4 units creatine kinase (1 unit = the amount of enzyme which phosphorylates 1 μmole ADP per min at 37°); (C) 5 mM ^{14}C-labelled ADP (0.05 μC/μmole); (D) 0.5 mM [^{14}C]adenosine (2 μC/μmole). The radioactivity present as adenine and hypoxanthine nucleotides in medium and muscle was counted following electrophoretic separation. Intracellular nucleotides were calculated as described under METHODS. The height of each column represents the mean value of three determinations ± S.D. 1, ATP; 2, ADP; 3, AMP; 4, IMP; 5, adenosine; 6, inosine; 7, hypoxanthine.

of adenylate kinase. Both ^{14}C-labelled ATP and ADP were found within the cell, in approximately equal amounts. This contrasts with the preponderance of labelled ATP observed when ^{14}C-labelled ATP served as substrate and suggests that both ATP and ADP have entered the cell.

Extracellular degradation of ATP

A series of experiments was undertaken in order to establish whether the breakdown of externally added ATP, shown in Fig. 1 was due to leakage from the muscle of the respective enzymes involved. Soleus muscles were incubated for 1 h at 37° in glucose–bicarbonate medium an under atmosphere of O_2–CO_2 (95:5, v/v). At this point the muscles were removed and either ATP (5 mM), ADP (5 mM), AMP (2 mM) or [^{14}C]adenosine (0.5 mM, 0.05 μC/μmole) was added. After incubation for a further

TABLE I

EXTERNAL DEGRADATION OF ATP

Soleus muscles were incubated for 1 h at 37° under O_2–CO_2 (95:5, v/v) in 1 ml of glucose–bicarbonate medium. The muscles were removed and ATP (5 mM), ADP (5 mM) or AMP (2 mM) added to the final concentrations shown in parenthesis. After removing a zero-time sample, the medium was incubated for a further 1 h. ATP, ADP and AMP were assayed enzymatically as described under METHODS. Values are mean of four determinations ± S.D.

Substrate	*Incubation (min)*	*Adenine nucleotides (μmoles/ml)*		
		ATP	*ADP*	*AMP*
ATP	0	4.8 ± 0	0	0
	60	4.9 ± 0.08	0	0
ADP	0	0	5.2 ± 0.15	0
	60	0.7 ± 0.05	3.7 ± 0.08	0.7 ± 0.05
AMP	0	0	0	2.0 ± 0
	60	0	0	2.1 ± 0

TABLE II

EXTERNAL DEGRADATION OF [^{14}C]ADENOSINE

Soleus muscles were incubated for 1 h at 37° under O_2–CO_2 (95:5, v/v) in 1 ml of glucose–bicarbonate medium. The muscles were removed and 0.5 μmole [8-^{14}C]adenosine (0.05 μC/μmole) added. After removing a zero-time sample, the medium was incubated for a further 1 h. Muscles were also incubated for 1 h in medium containing 0.5 μmole [8-^{14}C]adenosine. Adenosine, inosine and hypoxanthine in 50-μl samples of medium were separated electrophoretically and their radioactivity counted as described under METHODS. Values are mean of three determinations ± S.D.

Incubation		*Products of [^{14}C]adenosine metabolism (μmole/ml)*		
Conditions	*Time (min)*	*Adenosine*	*Inosine*	*Hypoxanthine*
Muscle removed	0	0.50 ± 0.01	0	0
Muscle removed	60	0.20 ± 0.02	0.18 ± 0.01	0.10 ± 0.01
Muscle present	60	0.23 ± 0.01	0.18 ± 0.01	0.06 ± 0.01

60 min, ATP, ADP and AMP were determined enzymatically, while the conversion of adenosine to inosine was measured by radioactive counting following electrophoretic separation.

Neither ATP nor AMP levels changed during this second incubation period (Table I), indicating that none of the enzymes responsible for their metabolism had leaked into the medium. On the other hand, from the results shown in Tables I and II, it is clear that both adenylate kinase and adenosine deaminase (EC 3.5.4.4) activities were present in the incubation medium following removal of the muscle. From these experiments, we may conclude that ATPase (EC 3.6.1.3), AMP deaminase (EC 3.5.4.6) and 5'-nucleotidase (EC 3.1.3.5) activities were all located on the muscle surface. As the depletion of ATP in the presence of glucose was never accompanied by the appearance of detectable amounts of glucose 6-phosphate, hexokinase does not appear to be involved in the degradation of external ATP.

DISCUSSION

As far as can be ascertained, the "impermeability" of muscle to ATP appears to have arisen as a corollary to a general theory that muscle is impermeable to anions[10]. Although the experiments of FENN AND COBB[11], and BOYLE AND CONWAY[1] subsequently indicated that certain anions could, in fact, enter and leave the muscle cell, ATP was not considered to be one of these exceptions, and in the absence of evidence to the contrary, this attitude has tended to prevail. Following their demonstration that externally added ATP induced contraction in isolated muscle fibres, BUCHTHAL *et al.*[3] suggested that ATP had penetrated the cell membrane, but this observation seems to have been ignored. We have proposed that the distribution of ^{14}C-labelled ATP between medium and muscle, shown in Fig. 1, is an indication that external ATP entered the muscle cell. It would seem appropriate to review the evidence for this; firstly, that radioactive ATP has an intracellular distribution, and secondly that the radioactive ATP within the cell entered as such, and was not synthesized from adenosine or inosine.

If it is assumed that the concentration of isotopically labelled nucleotide in the extracellular water is the same as that in the external medium, then, on the basis of the concentrations in the medium shown in Fig. 1, it is possible to calculate the amount of labelled nucleotide present in the extracellular space. When the amount of labelled nucleotide found in the muscle exceeds this value one may assume either that this excess is present intracellularly, or that there is an accumulation of the nucleotide in the extracellular space. In view of the extensive degradation of external ATP, it is difficult to accept that the observed accumulation of ATP occurred in the extracellular space.

Depending on whether ^{14}C-labelled ATP or ADP was used, the ratio of labelled ATP to labelled ADP within the muscle varied from 6.5:1 to 0.9:1. When a regenerating system was used to maintain the ATP level, more labelled ATP was found within the cell and the ATP:ADP ratio increased to 89:1. This would tend to rule out the possibility that the ATP arose by synthesis from adenosine. Using ^{14}C-labelled adenosine it was shown that the contribution of this synthetic pathway was negligible. Thus ATP, and, on the basis of the observed ATP:ADP ratios, ADP as well, appear to have entered the muscle.

Although peripheral to the main theme of these studies, the observation that ATP was degraded by enzymes located on the suface of the muscle cell deserves further comment. SHAW AND STADIE[13] claimed that in rat hemidiaphragm there were two cytologically distinct, Embden–Meyerhof enzyme systems, one located intracellularly and the other on the surface of the cell. While this may very well be so, their data might simply reflect the fact the muscle fibres were cut in this preparation. Thus the part of each fibre nearest the cut ends would appear to function as an 'extracellular' site, whereas normal permeability barriers and insulin sensitivity, (*i.e.* the 'intracellular' pathway) would prevail elsewhere. More substantial evidence for extracellular enzymic activity has come from WILLIAMSON AND DI PIETRO[14], who have shown that glucosephosphate isomerase (EC 5.3.1.9), triosephosphate isomerase (EC 5.3.1.1) and aldolase (EC 4.1.2.13) were all capable of reacting with extracellular substrates perfused through the isolated rat heart. In their system, both ATP and AMP were rapidly hydrolyzed by extracellular enzymes.

Our results extend this observation. Enzymic and electrophoretic examination of the incubation medium revealed that ATP was converted into ADP, AMP, adenosine, IMP and inosine, and indicated that, of the enzymes responsible for these transformations, ATPase, adenylic deaminase and 5'-nucleotidase were all present on the surface of the cell.

Similar results have been reported by MANERY *et al.*[15], who also showed leakage of adenylate kinase. They considered that adenylate kinase was also located on the muscle surface and that the observed leakage simply indicated that this enzyme was bound more loosely than the others. We would agree that leakage need not necessarily rule out the possibility of an extracellular location, but this is an aspect which we have not pursued further. Nor have we attempted to establish whether the enzymes of the Embden–Meyerhof pathway were also present on the muscle surface as claimed by SHAW AND STADIE[13], however, there are a few experimental observations pertinent to this question. From the experiments described above, it is clear that hexokinase was not active externally. As ATP, incubated in the presence of soleus muscle, could not be rephosphorylated from added phosphoenolpyruvate unless crystalline pyruvate kinase was also added (I. H. CHAUDRY AND M. K. GOULD, unpublished data), this would also exclude pyruvate kinase. Thus it is unlikely that the conversion of glucose to lactate can be effected extracellularly.

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