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FURTHER EVIDENCE FOR ATP UPTAKE BY RAT TISSUES

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Summary

Intact rat soleus muscles or hemidiaphragms were incubated at 37°C for 1 h in 1.0 ml of Krebs-HCO_3 buffer containing 10 mM glucose, 5 mM (8-^{14}C)-labelled ATP and (α-^{32}P)-labelled ATP together with 5 mM $MgCl_2$ under an atmosphere of 95% O_2—5% CO_2. Samples of the incubation medium and tissue extract were subjected to electrophoretic separation and the radioactivity present as adenine nucleotides was counted. Extensive degradation of the added nucleotides was observed in the presence of both tissues. The concentrations of ^{14}C-labelled and ^{32}P-labelled ATP found in the muscle and diaphragm indicated that ATP was present within the muscle and diaphragm cells. By maintaining higher concentrations of ATP in the medium, ATP uptake in both tissues exhibited a saturation-type kinetics. These results provide further evidence for intracellular uptake of ATP and also suggest that the transport of ATP into the cell could be a carrier-mediated process.

Introduction

It is still a popular belief that cell membranes are impermeable to ATP [1] although there are indications in the literature that ATP might be able to cross the cell membrane. Weidemann et al. [2] have suggested that rat kidney cortical cells are permeable to ATP. The studies of Forrester [3] have shown that ATP was released from active skeletal muscle under conditions in which potassium was not. Silinsky and Hubbard [4] found that ATP is released from motor nerve terminals on indirect stimulation of the mammalian nerve-muscle preparation. Previous work from our laboratory has shown that ATP entered inact skeletal muscle, liver and kidney cells [5,6] and we suggested that this process may be a carrier-mediated process [6]. More recent studies from our

laboratory have shown that the uptake of ATP by tissues during hemorrhagic shock is enhanced [7]. Thus, on the basis of the above mentioned studies [2–6], it would appear that the release and uptake of ATP by the cell or the interaction of ATP with the cell membrane is a physiological process. However, despite the evidence presented in the literature that ATP might be able to cross the intact cell membrane, the theory of 'impermeability' of muscle to ATP has by-and-large tended to prevail. In view of this, we have further studied the uptake of ATP by muscle in vitro using dual labelled ATP. The results below indicate that soleus muscle as well as hemidiaphragm incubated in vitro accumulated both (α-^{32}P)-labelled ATP and ^{14}C-labelled ATP from the external medium. This finding provides additional support to our view that ATP in the extracellular fluid can be transported into the cell.

Methods

Soleus muscles from Holtzman rats weighing approximately 30 mg or hemidiaphragms were placed (2 per beaker) in 1.0 ml Krebs-HCO_3 buffer (pH 7.4) containing 10 mM glucose, 5 mM ATP, 5 mM $MgCl_2$, 2 μmol (8-^{14}C)-labelled ATP and ATP labelled with ^{32}P in the alpha (ribosyl) phosphorus position. Variation to this procedure are described in the text. The 1 : 1 ratio of phosphorus to ^{14}C radioactivity in the incubation medium was kept constant in all experiments. The tissues were incubated in this medium for 1 h at 37°C under an atmosphere of O_2-CO_2 (95 : 5, v/v). At the end of the incubation period, the tissues were removed, rinsed quickly in ice-cold water, blotted on dampened filter paper and frozen between aluminium tongs cooled in liquid N_2. The tissues were then homogenized in 1.0 ml of the solution containing trichloroacetic acid (10%)-HCl (0.1 M) and centrifuged. The supernatant solution was extracted 4 times with water saturated ether and then neutralized with 1 M Tris base. Samples (50 μl) of the muscle and diaphragm extract and incubation medium were separately applied to a Whatman 3 MM paper and over supported with 10 μl of a marker solution containing 0.05 μmol each of ATP, ADP, AMP, IMP, inosine, adenosine and hypoxanthine. Following electrophoretic separation using the system described by Wadkins and Lehninger [8], the individual nucleotide * spots were detected under ultraviolet light, cut from paper and placed in a counting vial together with 15 ml of aqueous scintillation solution. Radioactivity was counted in a Packard scintillation counter. Approximately 90–95% of the radioactivity applied to the electrophoretogram was recovered. Degrees of quenching were estimated by using the external ratio technique based on standards prepared in a manner comparable to the samples.

The concentration of adenine nucleotides in the incubation medium (μmol/ml) and tissues (μmol/g) were calculated from the radioactivity observed in each fraction. A nucleotide was considered to have intracellular distribution when the total tissue content exceeded the extracellular content. Excellular concentrations were calculated on the assumption that the concentrations of

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

the extracellular water was the same as that of the medium. Extracellular space was determined by measuring the raffinose space as described previously [9].

Materials

ATP, ADP, AMP, adenosine, IMP, inosine were all obtained from Sigma Chemical Company, St. Louis, MO. (8-^{14}C)-labelled ATP (tetrasodium salt) and (α-^{32}P)-labelled ATP (triethyl-ammonium salt) were obtained from New England Nuclear. Prior to use, each was purified by electrophoresis using the system described above, and diluted with non-radioactive nucleotide (sodium salt) to the appropriate specific activity.

Results

Extracellular spaces of muscle and diaphragm

The extracellular space of muscles and diaphragm was measured in a separate set of experiments using raffinose as a marker [9]. This space was found to be 0.24 ± 0.02 ml/g and 0.27 ± 0.03 ml/g, respectively for muscle and diaphragm (mean of 8 experiments). The extracellular content of nucleotides was calculated on the assumption that the concentration of each nucleotide in the extracellular water was the same as that of plasma. A nucleotide was considered to have an intracellular distribution when the total tissue content exceeded the extracellular content.

Penetration of muscle and diaphragm by ATP

Extensive degradation of ATP occurred when soleus muscles (Table IA) were incubated for 1 h in a medium containing (α-^{32}P)-labelled and ^{14}C-labelled ATP. The major breakdown products in the medium were ADP, AMP, and IMP together with smaller amounts of adenosine and inosine. No radioactivity was found in the electrophoretogram in the areas where adenine and 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. As reported in our previous studies [5] externally added (α-^{32}P)-labelled ATP as well as ^{14}C-labelled ATP were also present intracellularly in equal amounts in the muscles, together with dual labelled ADP.

Extensive degradation of ATP also occurred when 'intact' rat hemidiaphragms [10] were incubated and the extent of ATP degradation was approximately the same as that in the presence of muscle. Moreover, the diaphragm intracellular values of radioactive ATP were also the same as that of the muscle. Since the ATP uptake values as well as ATP degradation by hemidiaphragm were the same as that of the muscle, the absolute ATP uptake values for hemidiaphragm are not reported here for the sake of conciseness.

When the concentration of ATP in the medium was increased from 5 mM to 10 mM, intracellular ATP level in muscles increased by approximately 75%, but intracellular ADP, AMP and IMP did not increase proportionally (Table IB). In the presence of an ATP regenerating system (phospho*enol*pyruvate-pyruvate kinase (EC 2.7.3.2), about 80% of the initially added ATP remained at the

TABLE I

EXTERNAL DEGRADATION AND INTRACELLULAR ACCUMULATION OF ADENINE NUCLEOTIDES BY MUSCLE

Rat soleus muscles were incubated for 1 h at 37°C under 95% 0—5% CO_2 in 1.0 ml Krebs-HCO_3 medium containing (A) 5 mM (8-^{14}C)-labelled and (α-^{32}P)-labelled ATP; (B) 10 mM (8-^{14}C)-labelled and (α-^{32}P)-labelled ATP, (C) 5 mM (8-^{14}C)-labelled and (α-^{32}P)-labelled ATP, 20 μmol phospho*enol*pyruvate, 5 I.U. pyruvate kinase (I.U. = the amount of enzyme which phosphorylates 1 μmol ATP/min at 37°C). 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. Value are means of 8 animals ± S.E.

	A		B		C	
	^{14}C	^{32}P	^{14}C	^{32}P	^{14}C	^{32}P
Medium nucleotides (μmol/ml)						
ATP	1.20 ± 0.08	1.27 ± 0.06	2.50 ± 0.14	2.43 ± 0.15	3.95 ± 0.24	3.86 ± 0.25
ADP	1.48 ± 0.10	1.41 ± 0.09	1.16 ± 0.09	1.68 ± 0.11	0.50 ± 0.08	0.62 ± 0.09
AMP	0.81 ± 0.07	0.88 ± 0.08	2.01 ± 0.12	2.01 ± 0.14	0.11 ± 0.01	0.13 ± 0.01
IMP	1.02 ± 0.08	0.92 ± 0.07	1.45 ± 0.13	1.40 ± 0.12	0.19 ± 0.01	0.24 ± 0.02
Inosine	0.11 ± 0.01	0.14 ± 0.01	0.72 ± 0.12	0.84 ± 0.11	0.06 ± 0.01	0.08 ± 0.01
Adenosine	0.18 ± 0.02	0.23 ± 0.02	1.30 ± 0.15	1.19 ± 0.13	0.07 ± 0.01	0.08 ± 0.01
Intracellular nucleotides (μmol/g)						
ATP	0.44 ± 0.03	0.45 ± 0.04	0.77 ± 0.06	0.80 ± 0.07	0.90 ± 0.08	0.88 ± 0.07
ADP	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
AMP	0	0	0	0	0	0
IMP	0	0	0	0	0	0
Inosine	0.30 ± 0.02	0.27 ± 0.02	0.62 ± 0.09	0.55 ± 0.08	0.43 ± 0.04	0.44 ± 0.04
Adenosine	0.69 ± 0.06	0.64 ± 0.04	0.86 ± 0.10	0.92 ± 0.09	0.52 ± 0.06	0.58 ± 0.06

end of the incubation period (Table IC). Intracellular ^{14}C- as well as ^{32}P-labelled ATP was doubled, while ^{14}C- and ^{32}P-labelled ATP was barely detectable under these conditions. Thus, there was a preponderance of both ^{32}P- and ^{14}C-labelled ATP in the muscles when an ATP-regenerating system was added to the incubation medium.

Discussion

The 'impermeability' of cell membrane to ATP appears to have arisen as a corollary to a general theory that cell membranes are impermeable to cations [11]. Although subsequent studies indicated that certain anions could, in fact, enter and leave the muscle cell [12,13], ATP was not considered to behave in the similar manner. The studies of Maxild [14] have shown that externally added ATP caused an elevation of the concentration of ATP in the kidney and he concluded that there was intracellular uptake of ATP. Recent studies of Pant et al. [15] have yielded results consistent with the view that ATP in the extracellular fluid could be transported into the squid axoplasm. More recent studies of Williams et al. [16] have shown that exogenous ATP increased the ATP contents of cultured myocardial cells. Their studies also showed that the increase in tissue ATP elicited by exogeneous ATP was not due to breakdown products of ATP since neither adenosine nor ADP altered ATP levels of myocardial cells. Despite the abundance of evidence presented by various investigators [2—6,14—16], the notion that tissue cells are impermeable to ATP has

tended to prevail. In the present study, a 'double isotope' method was employed to follow the nucleotide radioactivity. Muscles and diaphragms were incubated with ATP labelled with 8-^{14}C and with ^{32}P in alpha phosphorus position. The results presented above indicate that the ratio of phosphorus to ^{14}C in the muscle was the same as that as in the initial incubation medium. The fact that the ^{14}C : ^{32}P ratio in the muscle was the same as that of the medium suggests that the ribosyl phosphorus did not undergo cleavage or transfer.

In this study, we have used an intact soleus muscle preparation [9] and hemidiaphragms and have proposed that the distribution of 'double isotope' between the medium and muscle shown in Table I is an indication that external ^{32}P-labelled and ^{14}C-labelled ATP entered the muscle cell. It would appear appropriate, however, to discuss the evidence that radioactive ATP has an intracellular distribution. If one assumes that the concentration of ^{32}P-labelled and ^{14}C-labelled ATP in the extracellular water is the same as that in the incubation medium, then, on the basis of the concentration in the medium shown in Table I, it is possible to calculate the amount of labelled nucleotide present in the extracellular space. If the total amount of radioactive nucleotide found in the tissue exceeds that in the extracellular space, one may assume either that the excess radio labelled ATP is present intracellularly or that there is an accumulation of the nucleotide in the extracellular space. Because of the extensive degradation of external ATP, it is unlikely that the observed accumulation of ^{14}C-labelled and ^{32}P-labelled ATP occurred in the extracellular space. If the ratio of ^{14}C : ^{32}P in the cell was not the same as that in the incubation medium, it would have raised doubts concerning intracellular uptake of ATP. However, since the ratio of ^{32}P : ^{14}C was the same in the medium and muscle it provides further evidence that both ^{14}C-labelled and ^{32}P-labelled ATP had entered the tissue to the same extent.

It could be argued that the extracellular space values observed in the present experiments may have been an underestimation of the true extracellular space. If this were so, then externally added ATP could have been confined to the extracellular compartment, rather than having an intracellular distribution. This possibility, however, is extremely unlikely, since the values for extracellular space observed in this study are similar to the values we, as well as others, have reported previously [9]. In addition, the extracellular space would have to be 0.8 ml/g of tissue if ATP was to be confined to an extracellular compartment. An extracellular space of 0.8 ml/g is extremely hard to accept, particularly for an intact muscle preparation, such as the soleus muscle. In view of this, it could be concluded that the ATP present in these tissues was indeed intracellular. Evidence for ATP uptake intracellularly by kidney, was also provided by the recent studies of Maxild [14].

It is well known that some substances such as phlorizin and certain hormones which bind to cell membrane without entering the cells have larger distribution volumes than extracellularly water. It is extremely unlikely that the same is the case with ATP due to the fact that extensive degradation of external ATP occurred in the presence of the tissues. Moreover, previous experiments [5] have shown that the value of distribution of ADP was much less than that of ATP and that externally added AMP failed to have an intracellular distribution [5]. In view of the above observations, it is highly unlikely

that the ATP present in the cell in excess of extracellular space was simply bound to the cell surface. Although these experiments clearly indicate that ATP enters the intact tissue cell, the precise localization and distribution of ATP within the cell remains to be determined.

When the concentration of ATP in the incubation medium was increased from 5 mM to 10 mM ATP, the ratio of double labelled ATP to ADP in the muscle increased from 7 : 1 to 15 : 1 (Table IA and B). When an ATP regenerating system was used to maintain the ATP levels in the incubation medium, more labelled ATP was found within the muscle cell and ATP : ADP ratio increased 90 : 1. This would rule out the possibility that the ATP arose from synthesis from adenosine and the studies of Williams et al. [16] support this notion. Moreover, previous results have shown that the contribution of adenosine to the ATP synthetic pathway was negligible [5].

Since intracellular ATP and ADP are interconvertible, it is necessary to study ADP uptake and determine the contribution of this towards ATP formation. However, (α-^{32}P)-labelled ADP is not available commercially and, thus, we did not conduct such studies. Using ^{14}C-labelled ADP as a substrate, we have previously shown that the muscle cells were more permeable to ATP than ADP [5]. Thus, although ADP is capable of entering the cell, this was not the nucleotide responsible for intracellular ATP values when muscles were incubated with ATP.

The present study demonstrates that ATP can enter muscle cells and that after 1 h, approximately 0.5 μmol ATP/g was present as labelled ATP when muscles were incubated in the presence of 5 mM ATP. In a static system this would suggest that only 10% of the added ATP was taken up by the muscle cells as ATP. However, since intracellular ATP is subjected to utilization for reaction in various intracellular energy-requiring processes, more ATP could have entered the muscle cells from the external medium and been subjected to utilization, and hence, degradation.

If the medium ATP levels at the end of the incubation period (shown in Table I) are plotted, it becomes evident that by maintaining higher medium ATP levels, intracellular ATP levels increased. However, the relationship between the medium ATP levels and intracellular ATP, i.e. ATP uptake, is not linear. There was an increase of approximately 75% in intracellular ATP when medium ATP levels were maintained ranging from 1.2 to 2.5 μmol/ml. By further maintaining the medium ATP at levels extending to 3.9 μmol/ml, intracellular ATP only increased by 13%. These results suggest that ATP uptake activity was at a near saturation level when medium ATP levels were maintained above 2 μmol/ml. Since the occurrence of saturation in uptake activity can be interpreted to indicate the involvement of membrane carrier in the uptake process, the results presented above further suggested that ATP uptake in muscle could also be a carrier-mediated process. We have previously presented experimental evidence to indicate that ATP uptake in liver and kidney could be a carrier-mediated process similar to that in muscle observed in the present study.

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