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Adenine nucleotide transport and adenosine production in isolated rat heart mitochondria during acetate metabolism

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In view of its vasodilatory effect on the coronary circulation (probably mediated by adenosine) and its metabolic compartmentalization (intramitochondrial activation to form acetyl-CoA), the metabolic effects of acetate were studied in isolated rat heart mitochondria. Acetate caused conversion of adenylates to AMP and the formation of adenosine. Adenylate efflux was inhibited by carboxyatractyloside but not by *N*-ethylmaleimide. The intramitochondrial accumulation of AMP was enhanced by carboxyatractyloside during acetate metabolism and the formation of extramitochondrial adenosine inhibited. A carboxyatractyloside-sensitive unidirectional AMP influx with a K_m of 50 μM and V_{max} of 11 nmol/min per mg mitochondrial protein was also observed. The mitochondrial adenosine content was high and constant during the experiments. The steep apparent concentration gradient of adenosine indicates that most of the mitochondrial adenosine is tightly bound to protein. Adenosine formation was proportional to the extramitochondrial AMP concentration, showing that the 5′-nucleotidase activity of cardiac mitochondrial preparations is extramitochondrial in origin. The data suggest that the mitochondrial ATP/ADP carrier is capable of transporting AMP and that intramitochondrial AMP is recycled during acetate metabolism in the myocardium partially by means of the ATP/ADP translocator, leading to an increase in extramitochondrial AMP and adenosine formation.

Introduction

Adenosine is an effective coronary vasodilator and has been proposed as a local regulator of coronary blood flow [1]. It also increases the atrioventricular conduction time in the heart and antagonizes the inotropic effects of catecholamines [2–4]. Adenosine production increases during ischaemia [5] and increased work load [6,7], being inversely correlated with the decreased cellular energy state. Adenosine production also increases during acetate metabolism [8–10], an effect which may be related to increased tissue AMP generated by acetate activation [11].

Adenosine can be produced in heart muscle by the action of 5′-nucleotidases, *S*-adenosylhomocysteine hydrolase and nonspecific phosphatases. Most of the cardiac 5′-nucleotidase activity is located at the plasma membrane. This *ecto*-5′-nucleotidase has been purified from rat heart [12], while cytosolic 5′-nucleotidase has similarly been purified both from rat heart [13] and to some extent from rabbit heart [14]. Some mitochondrial adenosine production has also been reported to occur [15], but this was shown later to be mostly, if not entirely, due to contamination of the mitochondrial preparation by other organelles [16,17]. It has been demonstrated recently that this contaminating activity in both liver and heart muscle is probably extramitochondrial in origin [14,18].

Adenine nucleotides accumulate in rat liver mitochondria during the first postnatal hours [19] and after glucagon injection [20]. Isolated rat liver mitochondria lose or accumulate adenine nucleotides in vitro depending on the extramitochondrial adenine nucleotide content [21,22], and it has been suggested that this net depletion or accumulation employs a transport system distinct from the ATP/ADP translocator [23], being coupled to a movement of inorganic phosphate in the opposite direction [24].

Myocardial acetate metabolism results in a significant increase in the coronary flow [9,25], a phenomenon

Abbreviations: NEM, *N*-ethylmaleimide; Ap_5A, P^1,P^5-di(adenosine)pentaphosphate; Hepes, *N*-2-dihydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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which may be related to the production of AMP in the fatty acyl-CoA synthase reaction with subsequent formation of adenosine. The effects of acetate are particularly interesting, because its activation in the myocardium is a mitochondrial process [26–28]. Since AMP has been considered to be a poor substrate for the ATP/ADP translocator [29,30], AMP accumulation during acetate metabolism would provide a good model for testing the possibility of mitochondrial adenosine production.

Inhibitors of translocators and adenylate kinase were employed here to investigate further the question of AMP transport and the compartmentation of adenosine production. The present experiments show that carboxyatractyloside potentiates acetate-induced mitochondrial AMP accumulation but inhibits the formation of adenosine, and also that when the intramitochondrial AMP concentration is high, the ATP/ADP carrier is also capable of transporting AMP. The amount of adenosine in the mitochondria remained high and constant, the rate of extramitochondrial adenosine accumulation was proportional to the extramitochondrial concentration of AMP, and the apparent mitochondrial/extramitochondrial concentration gradient of adenosine was high, suggesting that adenosine is tightly bound to the mitochondrial proteins, which may be a major site of adenosine binding in the myocardium. The results are in agreement with the notion that the 5′-nucleotidase activity previously assigned to mitochondria [15] is in fact extramitochondrial in origin. The ATP/ADP carrier is capable of AMP transport under conditions in which adenylate kinase is not operative, although the rate is low.

Materials and Methods

Reagents. Standard chemicals were purchased from E. Merck, Darmstadt, F.R.G., and Boehringer-Mannheim, Mannheim, F.R.G. *Bacillus subtilis* proteinase 'Nagarse' was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-ethylmaleimide (NEM), *p*-hydroxymercuribenzoate and P^1,P^5-di(adenosine)pentaphosphate (Ap_5A) were from Sigma and carboxyatractyloside from Boehringer-Mannheim. The reagent for the protein measurements was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. [8-^{14}C]Adenosine 5′-monophosphate was from Amersham International, Amersham, U.K.

Isolation of mitochondria. Rat heart mitochondria were isolated by the protease method as modified by Mela and Seitz [31] in a medium consisting of 225 mM mannitol/75 mM sucrose/1 mM EGTA (pH 7.4)/10 mM Hepes (pH 7.4). The respiratory control of the freshly isolated mitochondria was tested before the experiments in the presence of 1 mM malate and 10 mM pyruvate, the ratio being 6.5 ± 0.8 ($n = 50$). The protein content of the mitochondria was measured by the method of Bradford [32].

Mitochondrial incubations. Freshly isolated mitochondria (2 mg protein/ml) were incubated in a medium (A) consisting of 100 mM KCl/30 mM Hepes (pH 7.4)/5 mM KH_2PO_4 (pH 7.4)/1 mM EGTA (pH 7.4) at 30 °C with continuous shaking. Since the mitochondria were found in preliminary studies to become de-energized in incubations without added substrates, the experiments were carried out in the presence of 1 mM malate. If inhibitors were used, these were added to the medium before the mitochondria.

Samples for studying the effects of inhibitors on adenine nucleotide and adenosine transport and distribution were taken from the incubation 1, 3, 6, 10 and 20 min after addition of the mitochondria. When the effects of acetate or pyruvate were studied in the control experiments the mitochondria were preincubated for 10 min with 1 mM malate and samples taken 0, 1, 2, 5 and 10 min after addition of the substrates.

AMP influx. Mitochondria were incubated in medium A in the presence of 1 mM malate and [8-^{14}C]AMP (0–100 μM, spec. radioact. 9 Ci/mol). Since the influx was completely carboxyatractyloside-sensitive, its kinetics were studied by the inhibitor-stop method. In order to study label exchange through the adenylate kinase reaction 100 μM Ap_5A, a specific inhibitor of the enzyme [33], 2 mM glucose and 2 U/l hexokinase were added to the medium. Label distribution in the adenine nucleotides was measured by scintillation counting of fractions collected in HPLC on an anion-exchange column.

Total adenylate label efflux. Isolated mitochondria (20 μg protein) were incubated at 4 °C for 60 min with 2 μCi [^{14}C]ATP (52 Ci/mol) in a total volume of 2 ml of the sucrose-based isolation medium. Thereafter the mitochondria were washed twice with the isolation medium. The final suspension was made immediately before the experiments. The efflux experiments were performed as described under Mitochondrial incubations. When changing the potassium phosphate concentration, the osmolarity was adjusted with KCl.

Sample preparation. 800-μl samples from the incubations were rapidly added to an Eppendorff tube on top of a layer of 300 μl of silicone oil mixture above 400 μl of 7% perchloric acid. After immediate centrifugation, a 500 μl sample of the top layer was acidified with perchloric acid. The acidified samples from the top and bottom layers were neutralized with 2 M KOH/0.5 M triethanolamine hydrochloride and stored at −20 °C until used. Treatment of the samples was similar in experiments on the [^{14}C]AMP influx or [^{14}C]adenylate efflux, except that 20% sucrose (w/v)/0.1% Lubrol PX was used as the bottom phase in the centrifugation through silicone oil. Contamination by extra-matrix

water was detected by [^{14}C]sucrose and the influx data were corrected accordingly.

Metabolite measurements. ATP, ADP, AMP and adenosine in the neutralized samples were measured by HPLC. The nucleotides were measured using a Nucleosil 10 SB anion-exchange column under isocratic elution conditions. ATP and ADP were chromatographed with 275 mM KH_2PO_4/300 mM KCl (pH 4.2) and AMP with 200 mM KH_2PO_4 (pH 3.3). The column was washed with 400 mM KH_2PO_4/400 mM KCl (pH 4.2) between every AMP analysis. Adenosine was measured on a Nucleosil 10 C_{18} reversed-phase column eluted with 200 mM KH_2PO_4 (pH 4.2)/5% methanol. Absorbance of the effluent was monitored at 260 nm. Internal standards were used for peak identification and quantification.

Statistics. The group data are expressed as means ± S.D. or S.E. (Fig. 5). The correlation between extramitochondrial AMP concentration and adenosine production was calculated by the least-squares method, and a regression method which takes into account the errors in both dimensions was employed when the data necessitated the use of mean values [34]. Weighted regression analysis for the Michaelis-Menten kinetics was performed according to Wilkinson [35].

Results

Net efflux of ATP, ADP and AMP

More than 80% of the total nucleotides in the first sample, taken 1 min after the beginning of the mitochondrial incubation in the presence of malate were intramitochondrial and 70% of the total consisted of ATP (Fig. 1). Under control conditions, a constant rate of net efflux continued for 10 min (0.5 nmol/min per mg mitochondrial protein), during which time the intramitochondrial total adenine nucleotides had decreased by 64%. Thereafter the efflux rate remained low.

Carboxyatractyloside (20 μg/ml) totally inhibited the net efflux of nucleotides. The adenylate kinase inhibitor Ap_5A (100 μM) reduced the initial efflux rate by 60% to a constant rate of 0.2 nmol · min^{-1} · mg^{-1}. NEM (100 μM) did not affect the total efflux of adenine nucleotides, although the ATP/ADP ratio decreased from 3.05 to 0.48 in 20 min.

Although the mitochondrial ATP/ADP ratio decreased under control conditions, both ATP and ADP were probably exported, as their extramitochondrial ratio remained almost constant (3.42–3.65).

In the presence of Ap_5A the experiment was complicated by an increase in total adenine nucleotides, mainly in the form of ADP, most probably caused by hydrolysis of Ap_5A. Even so, an appreciable concentration gradient existed from the matrix to the extramitochondrial space. One reason for the Ap_5A-induced decrease in adenylate efflux could still be the decrease in the concentration gradient.

Under control conditions (i.e., in the presence of malate) the amount of AMP in the system was low, so that the evaluation of AMP transport was difficult. Therefore experiments with acetate were designed for the estimation of AMP efflux (see below). The only situation where appreciable amounts of AMP were found was in the presence of NEM, which after a lag of about 6 min caused an accumulation of AMP in the matrix and a corresponding change in its extramitochondrial concentration (Fig. 1).

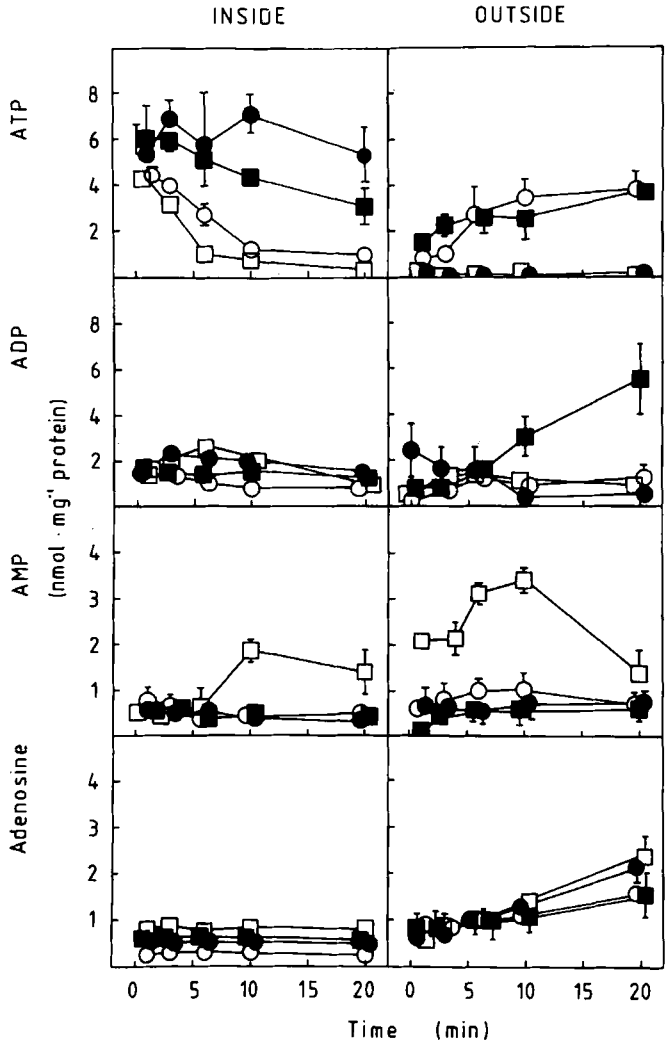

Fig. 1. Adenylate and adenosine content inside and outside mitochondria incubated with malate. Isolated rat heart mitochondria were incubated with 1 mM malate in an isosmotic medium. Inhibitors, when used, were added to the incubation medium before the mitochondria. Samples were withdrawn from the incubation medium at the times indicated. The mitochondria were separated from the medium and intramitochondrial and extramitochondrial adenylate and adenosine analyzed by HPLC. Values (nmol/mg mitochondrial protein) represent means ± S.D. from four independent experiments. ○, no inhibitor; ●, carboxyatractyloside (20 μg/ml); □, NEM (100 μM); ■, Ap_5A (100 μM).

Acetate-induced AMP production and transport

Acetate activation in the mitochondrial matrix induces trapping of the adenylates as AMP. Experimentation with acetate was complicated by the fact that acetate also causes a change in the mitochondrial energy state in the absence of other substrates except malate, so that the AMP accumulation was superimposed upon changes in the concentration, distribution and transmembrane transport of ADP and ATP. The acetate effects were therefore evaluated by comparing mitochondria supplemented with malate in the presence of acetate or pyruvate (Figs. 2 and 3). The preincubation

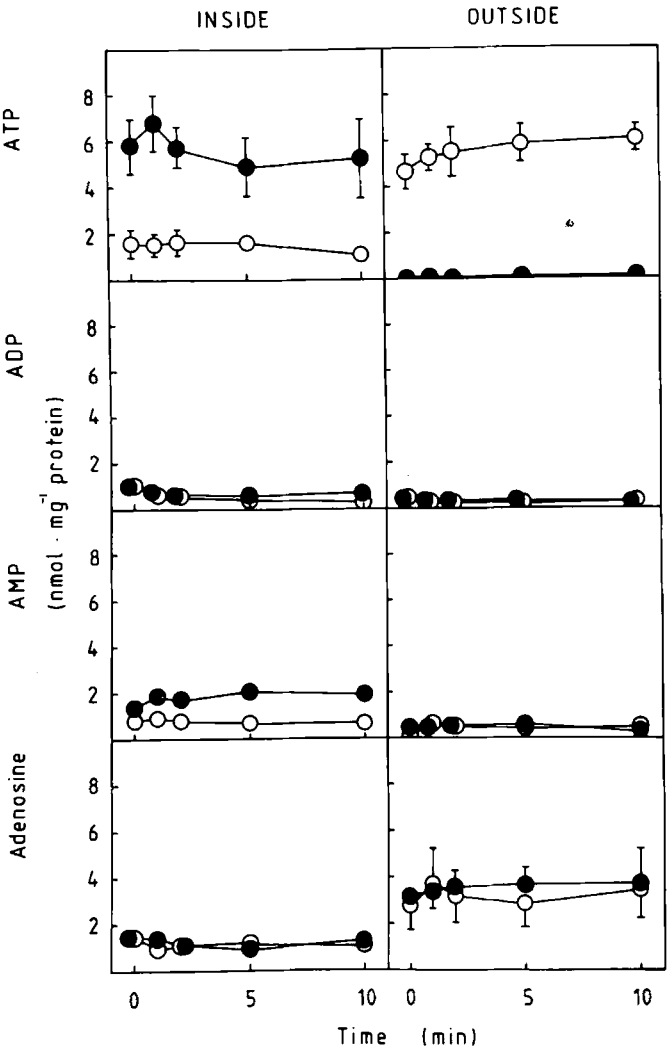

Fig. 2. Effect of pyruvate on adenine nucleotide and adenosine concentrations inside and outside mitochondria. Isolated rat heart mitochondria were preincubated with 1 mM malate for 10 min in an isosmotic medium. 20 μg/ml carboxyatractyloside, when used, was added to the incubation medium prior to the mitochondria. 10 mM pyruvate was added at time zero. The mitochondria were separated from the medium by centrifuging through a silicone oil layer. The intramitochondrial and extramitochondrial adenine nucleotides and adenosine were analyzed as described in Methods. Values (nmol/mg mitochondrial protein) represent means ± S.D. from six independent experiments. The concentration of mitochondria was 2 mg protein/ml. ○, no inhibitor; ●, carboxyatractyloside (20 μg/ml).

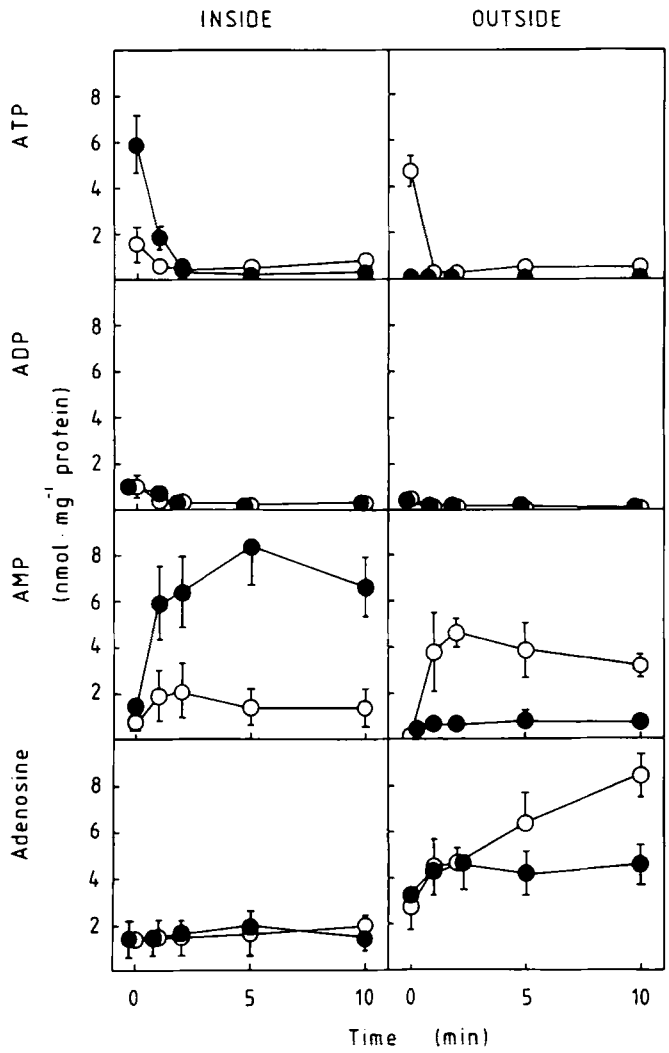

Fig. 3. Effect of acetate on adenine nucleotide and adenosine concentrations inside and outside mitochondria. Isolated rat heart mitochondria were preincubated with 1 mM malate for 10 min in an isosmotic medium. 20 μg/ml carboxyatractyloside, when used, was added to the incubation medium prior to the mitochondria. 10 mM acetate was added at time zero. The mitochondria were separated from the medium by centrifuging through a silicone oil layer. The intramitochondrial and extramitochondrial adenine nucleotides and adenosine were analyzed as described in Methods. Values (nmol/mg mitochondrial protein) represent means ± S.D. from 6 independent experiments. ○, no inhibitor; ●, carboxyatractyloside (20 μg/ml).

with malate lasted for 10 min, so that the starting point of the acetate or pyruvate metabolism (Figs. 2 and 3) corresponded to the 10 min point in Fig. 1.

Pyruvate did not have any significant effect on the distribution of the adenylates in the absence or presence of carboxyatractyloside (Fig. 2), whereas acetate caused an accumulation of intramitochondrial and extramitochondrial AMP and a precipitous decrease in the amount of ATP + ADP in the system (Fig. 3). The changes in the amounts of AMP in both the matrix and the extramitochondrial spaces corresponded to the amount of ATP + ADP. Considering that the total amounts of the nucleotides were higher in the extrami-

tochondrial space, one could argue that acetate activation occurs largely in the extramatrix space, but the concentration of ATP there is too low. The carboxyatractyloside experiments (Fig. 3) indicate that the activation occurs in the matrix space. When adenylate transport is inhibited, a large accumulation of intramitochondrial AMP takes place, with little change in the extramitochondrial nucleotides. Moreover, the experiments show that adenylate translocator is involved in the transport of AMP. The extramitochondrial AMP accumulation during acetate metabolism was not sensitive to Ap_5A (experiment not shown), which indicates that adenylate kinase is not involved, i.e., AMP is transported as such, not as ADP.

Unidirectional AMP influx

It was shown using ^{14}C-labelled AMP that AMP penetrates the mitochondrial inner membrane in a carboxyatractyloside-sensitive manner. To eliminate the possibility of the influx occurring in the form of ATP/ADP with the mediation of adenylate kinase, further experiments were conducted in the presence of Ap_5A and glucose plus hexokinase. The latter were used to keep the extramitochondrial ATP concentration low enough to prevent any residual adenylate kinase reaction, even though the Ap_5A concentration used (100 μM) was sufficient to inhibit the reaction by 97%.

Rapid AMP influx occurred under these conditions (Fig. 4). The initial velocities revealed an extrapolated V_{max} of 11 ± 2 (S.E.) nmol/min per mg mitochondrial protein and a K_m value of 50 ± 17 (S.E.) μM. Since the influx was totally carboxyatractyloside-sensitive, the inhibitor stop method was used when measuring the time course of the reaction. Label redistribution into extramitochondrial ADP and ATP was extremely slow, reaching 11% of the total label in 5 min. At 10 s, the sampling time for the kinetic measurements (Fig. 4), only 0.2% of the label was found in the extramitochondrial ADP + ATP. This label redistribution was totally eliminated by carboxyatractyloside. The influx of [^{14}C]AMP was not sensitive to 10 μM *p*-hydroxymercuribenzoate under conditions which caused 91% inhibition of the translocation of inorganic phosphate.

Phosphate as a counter-ion

The insensitivity of the AMP transport to inhibitors of the phosphate translocator does not necessarily rule out phosphate as a counter-ion. This could better be evaluated in a phosphate-depleted system, but attempts at total depletion of intramitochondrial P_i would probably be only partially successful. We therefore tested the effects of extramitochondrial P_i on the efflux of total adenylates (intramitochondrial AMP cannot be labelled specifically). It was found that the label efflux (as percentage of the total label) in 5 min (still in the linear

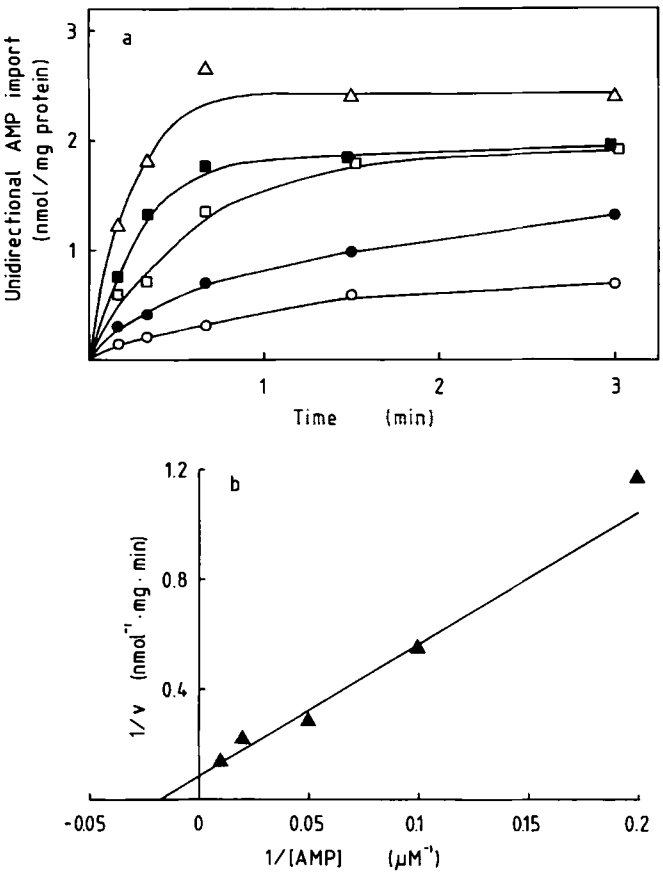

Fig. 4. Concentration dependence of unidirectional AMP influx in rat heart mitochondria. (a) Time-course of influx. (b) Double-reciprocal plot of the initial velocities measured at 10 s. The experimental details are as described under Materials and Methods. The plot yields a K_m value of 50 ± 17 (S.E.) μM and V_{max} of 11 ± 2 (S.E.) nmol/min per mg mitochondrial protein. Symbols: ○, 5 μM AMP; ●, 10 μM AMP; □, 20 μM AMP; ■, 50 μM AMP; △, 100 μM AMP.

range) was 42 in the presence of 5 mM P_i and 15 in its absence. In the presence of carboxyatractyloside (20 μg/ml) these figures were 14 and 19 in the presence and absence of 5 mM P_i, respectively.

Adenosine production

The adenosine content of the mitochondria was below 1 nmol/mg protein under all conditions and remained constant throughout the experiments (Figs. 1–3). Use of a mitochondrial matrix volume of 1.8 μl/mg protein [36] gives an intramitochondrial adenosine concentration of about 0.5 mM, a surprisingly high value. The adenosine content of the extramitochondrial space showed an almost constant rate of increase under all conditions tested (Figs. 1–3). The highest rate of adenosine production, 0.45 $nmol \cdot mg^{-1} \cdot min^{-1}$ was observed during the metabolism of acetate (Fig. 3), but this was again sensitive to carboxyatractyloside.

Extramitochondrial adenosine production showed a correlation with extramitochondrial AMP concentration ($r = 0.78$ in a semilogarithmic plot) (Fig. 5). This sug-

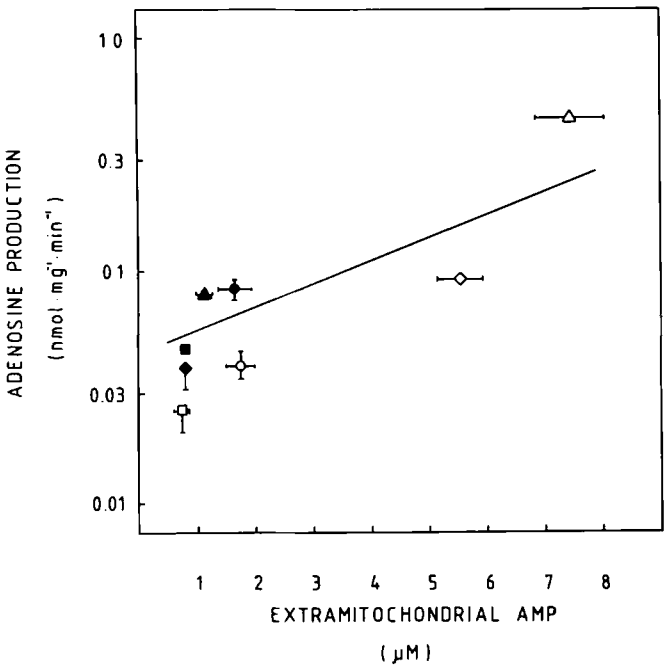

Fig. 5. Correlation between extramitochondrial AMP concentration and adenosine production in isolated rat heart mitochondria. Freshly isolated mitochondria were incubated under isosmotic conditions in the presence of different substrates and metabolic inhibitors as described in Methods. The mitochondria were separated from the medium by centrifuging through a silicone oil layer and extramitochondrial AMP and adenosine measured by HPLC. Values represent means ± S.E. from 4–6 independent experiments. The regression line was calculated by the least-squares method taking into account standard errors of the mean values. Log $y = 0.0985 \times - 1.347$ gives $r = 0.78$. ○, malate only; ●, malate + carboxyatractyloside; ◇, malate + NEM; ◆, malate + Ap_5A; □, malate + pyruvate; ■, malate + pyruvate + carboxyatractyloside; △, malate + acetate; ▲, malate + acetate + carboxyatractyloside.

gests that the 5′-nucleotidase activity in the system is nonmitochondrial in origin.

Discussion

The initial scope of the present investigation was to study the sites of adenosine production during the metabolism of acetate in the light of discrepant reports on the existence of a mitochondrial 5′-nucleotidase [15,17]. The rapid, extensive intramitochondrial formation of AMP during the metabolism of acetate can be exploited to investigate not only the substrate availability of 5′-nucleotidase but also the modes of AMP recycling to ADP, the substrate for oxidative phosphorylation. The mitochondrial recycling of AMP can be effected through a GTP/AMP transphosphorylase [37,38], as has been suggested for liver mitochondria. Extramitochondrial recycling of mitochondrial AMP is dependent on the transmembrane transport of AMP, a system which has remained largely unknown.

It is generally held that in liver mitochondria, at least, ATP/ADP translocase is specific to ATP and ADP and does not transport AMP, the low rates of AMP exchange being interpreted as occurring through the mediation of adenylate kinase in conjunction with the ATP/ADP translocase [29]. A phosphate-linked, NEM and mersalyl-sensitive, atractyloside-insensitive system for the net transport of adenylates has been described in the liver [24], and an atractyloside-sensitive adenylate exchange with inorganic pyrophosphate capable of net transport has also been observed [39].

The present data demonstrate, however, that the ATP/ADP carrier is capable of transporting AMP, as inferred from the carboxyatractyloside sensitivity of the latter process. The system is capable of a net efflux of AMP and a unidirectional influx with the same inhibitor sensitivity. Since the transport of AMP was not sensitive to the SH-blocking agents NEM or *p*-hydroxymercuribenzoate at concentrations which block the P_i/OH translocator, it is unlikely that this AMP carrier is the net transporting system described by Austin and Aprille [24]. It also unlikely that AMP transport occurs through the rather nonspecific anion channels described by Garlid et al. [41] and Selwyn et al. [42], because, although reminiscent of the adenine nucleotide translocator, the nonspecific anion channel is not sensitive to atractyloside.

During the course of the present investigation, Wilson and Asimakis [40] reported on an atractyloside-sensitive adenylate efflux system in rat heart mitochondria. They did not test AMP transport, however, measuring only total (labelled) nucleotide efflux, and they also confirmed the insensitivity of the system to SH-blocking agents, in contrast to liver mitochondria [24].

Although unidirectional or net transport rates were measured in the present case to ascertain the specificity for AMP, the results do not imply a uniport system for translocation. It was found that although the system is not sensitive to inhibitors of the phosphate translocator, phosphate is a potential counter ion for transport. A pyrophosphate-linked adenylate efflux has been described and assigned to the adenylate translocator. This system probably exchanges ADP for PP_i and its capacity is lower (V_{max} 2 nmol/min^{-1} per mg protein in the absence of an uncoupler in rat liver mitochondria) than the system described here for the transport of AMP [39]. Nevertheless, a physiological role of the ADP/PP_i exchange has been suggested in the regulation of the size of the mitochondrial adenylate pool [43].

The contribution of adenylate kinase to AMP transport by converting it to ADP, a known substrate for ATP/ADP translocase, was eliminated here by the use of Ap_5A, glucose plus hexokinase, but this did not affect the translocation of AMP. This means that AMP is a true substrate of the ATP/ADP translocase.

Although the present experiments demonstrate mitochondrial transport of AMP, its physiological significance must be evaluated in quantitative terms. Here

the rate of net efflux of AMP during mitochondrial acetate metabolism was found to be 3.6 nmol/min per mg mitochondrial protein. When this is converted to correspond to the situation in the intact myocardium, a value of 2.2 μmol/min per g dry tissue weight is obtained. Acetate oxidation in heart muscle would typically yield AMP at a rate of 12.3 μmol/min per g dry tissue weight [44], however. The calculated V_{max} (for the AMP influx) of 11 nmol/min per mg mitochondrial protein (Fig. 4) is equivalent to 6.5 μmol/min per g dry tissue weight, and this is again lower than the value necessary for extramitochondrial cycling in the myocardium. In this context, one could also compare the efficiency of the adenylate translocator to transport AMP with that for the exchange of its main substrates, ATP and ADP. In beef heart mitochondria the latter rate has been determined to be 1200 nmol/min per mg protein at 30 °C [45].

Although adenosine was produced by the mitochondrial suspension during AMP accumulation (Figs. 1–3), the amount within the mitochondria remained constant. Expressed in relation to the matrix water space of rat heart mitochondria [36], the apparent concentration in the mitochondria (0.5 mM) was high, corresponding to a 120- to 180-fold concentration gradient across the mitochondrial membrane. This gradient may be only apparent, however, being due to tight binding to mitochondrial components. The measurement method does not distinguish between free and total concentrations. Cytosolic binding of adenosine to *S*-adenosylhomocysteine hydrolase has been suggested [42], but the mitochondrial binding sites are largely unknown.

The observed initial adenosine content of the mitochondria (0.23 nmol/mg protein) is equivalent to 142 nmol/g dry wt. in tissue, higher than the figure of about 50 nmol/g dry wt. reported for the isolated perfused rat heart [7], but it is known that adenosine may be produced during the isolation of mitochondria in heart homogenate containing active 5′-nucleotidase. If the steep apparent concentration gradient is interpreted as being due to high-affinity binding, the results also offer an alternative for adenosine binding in the myocardium, namely the mitochondria.

Since the formation of adenosine was proportional to the concentration of AMP in the extramitochondrial space (Fig. 5), the 5′-nucleotidase activity of the preparation is most probably extramitochondrial. Although extramitochondrial AMP accumulation in the presence of NEM was appreciable, the increase in adenosine production was only moderate (Fig. 5). This is not due to an inhibition of 5′-nucleotidase, however, as NEM did not inhibit 5′-nucleotidase activity in isolated rat heart mitochondria when measured in terms of formation of P_i from exogenous AMP (data not shown). We have recently shown that the distribution of 5′-nucleotidase activity in a density gradient centrifugation of rat heart homogenate coincides with that of the plasma membrane marker, radioactive α-bungarotoxin (Kiviluoma, K.T., Hiltunen, J.K., Hassinen, I.E. and Peuhkurinen, K.J., unpublished observations), although positive proof of the absence of an enzyme in an organelle is difficult to obtain by cell fractionation. The present data may be used as more direct evidence of the absence of 5′-nucleotidase in the mitochondrial matrix, since the formation of adenosine was not correlated with the AMP concentration in the matrix space but with its concentration in the extramitochondrial space.

In summary, previous evidence and the results presented here lead us to conclude that rat heart mitochondria are unable to produce adenosine during intramitochondrial AMP loading. It is highly probable that the observed carboxyatractyloside-sensitive transport of AMP is mediated by the ATP/ADP carrier protein. Full characterization of transmembrane AMP transport awaits further experimentation.

Acknowledgement

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