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INHIBITION OF THE ADENINE NUCLEOTIDE TRANSLOCATOR BY MATRIX-LOCALIZED PALMITYL-CoA IN RAT HEART MITOCHONDRIA

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The activity of the adenine nucleotide translocator in rat heart mitochondria was quantitatively determined by the rate of [^{14}C]ATP transport at 2°C using the carboxyatractyloside inhibitor-stop technique. Linear uptake was obtained for 15 s, and with differing protein concentrations. The effect of matrix long-chain acyl-CoA esters upon the adenine nucleotide translocator activity was determined in these mitochondria. Incubation with palmitylcarnitine produced an increase in matrix long-chain acyl-CoA esters and decreased the velocity of [^{14}C]ATP transport. Mitochondria isolated in the presence of KCN showed elevated levels of long-chain acyl-CoA esters, decreased transportable nucleotides, and very low adenine nucleotide translocator activity. Addition of potassium ferricyanide to these mitochondria caused a reduction in matrix acyl-CoA esters and partially restored adenine nucleotide translocator activity. Potassium ferricyanide also lowered matrix acyl-CoA in freshly isolated mitochondria and increased [^{14}C]ATP transport. These findings show that the level of long-chain acyl-CoA esters within the mitochondrial matrix affects adenine nucleotide translocator activity and regulates mitochondrial activity.

Introduction

Adenine nucleotide translocator is located on the inner mitochondrial membrane. It catalyzes a molecule-for-molecule exchange of cytosolic ADP for intramitochondrial ATP generated via oxidative phosphorylation [1]. This important transport protein coordinates metabolic processes in the mitochondrial matrix with those of the cytosol, and may be rate-limiting in the overall reaction of oxidative phosphorylation [2,3]. The transport system is specific for ADP and ATP, and is strongly asymmetric in energized mitochondria [4]. The rate of exchange is influenced by the size of the endogenous exchangeable nucleotide pool [5,6], the energy state of the mitochondria [7,8], and the presence of endogenous inhibitor [9,10]. Long-chain acyl-CoA esters are potent inhibitors of adenine nucleotide translocator activity at the outer mitochondrial site [9–12] and in submitochondrial sonicated particles [13–15]. Because long-chain acyl-CoA esters accumulate during myocardial ischemia [16,17], investigators from this laboratory hypothesized that these esters inhibit adenine nucleotide translocator activity and regulate mitochondrial activity during ischemia [18]. The physiological significance of this hypothesis has been questioned by a number of investigators [19–21], largely because: (1) 90% of myocardial tissue CoA is compartmentalized in the mitochondrial matrix [19], and (2) efforts to demonstrate inhibition at the matrix adenine nucleotide translocator site in intact mitochondria have been unsuccessful [20].

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Many previous studies have not used a quantitative measure of adenine nucleotide translocator activity. An accurate and rapid assay of adenine nucleotide translocator in isolated mitochondria is difficult because of the quickness of the ADP-ATP exchange. In the present study, we have developed a fast, quantitative assay which takes advantage of the slower influx rate of ATP [6]. This procedure has allowed us to examine the effects of matrix accumulation of palmityl-CoA upon ATP transport.

Materials and Methods

ADP, ATP, ruthenium red, carboxyatractyloside, and fatty acid free bovine serum albumin were purchased from Sigma Chemical Co. [U-^{14}C]Adenosine triphosphate was obtained from New England Nuclear. Male Sprague-Dawley rats (225–275 g each) were obtained from King Animal Labs., Inc.

Isolation of mitochondria. Each rat was decapitated and bled for 15–30 s. The heart was excised and placed in ice-cold extraction medium (250 mM sucrose/4 mM Tris (pH 7.4)/1 mM EGTA/0.2% fatty acid free bovine serum albumin). It was then rinsed several times, blotted dry, weighed, and minced in ice-cold isolation medium. The tissue was homogenized with a Potter-Elvejhem homogenizer and centrifuged for 25 s in a Damon IEC centrifuge at 15 000 × *g*. The supernatant was poured through cheesecloth and centrifuged at 15 000 × *g* for 3 min. The resulting mitochondrial pellet was resuspended in extraction medium (containing no bovine serum albumin) so that the final protein concentration equaled 10 mg/ml.

Assays. Mitochondrial adenine nucleotide levels were determined by the methods of Adam [22] and Stanley and Williams [23] from neutralized perchloric acid extracts. Long-chain acyl-CoA esters were measured from the acid-insoluble fraction using the method of Veloso and Veech [24]. Protein was assayed using the biuret method [25].

Adenine nucleotide translocator assay. Rates of adenine nucleotide transport into isolated rat heart mitochondria were determined by measuring the slower forward transport of [U-^{14}C]ATP at 2°C using the carboxyatractyloside inhibitor-stop technique [6]. Mitochondria (0.5 mg) were added to a 0.5 ml reaction mix containing 35 mM Tris (pH 7.4); 0.88 mM EDTA (pH 7.4); 88 mM KCl; 5 μM Ruthenium red; 1 μCi [U-^{14}C]ATP and various concentrations of unlabeled ATP. The reaction was run for various durations and terminated by addition of 1 μmol carboxyatractyloside. Carboxyatractyloside-sensitive counts were obtained by subtracting the counts in blanks containing carboxyatractyloside prior to mitochondrial addition. Immediately following the assay, the reaction mixture plus mitochondria were pipetted into a microfuge tube containing 0.25 ml silicone oil (density: 1.01–1.02 g/ml) and centrifuged. The reaction mixture was aspirated off the top, the tube was rinsed, and the bottom containing the mitochondria was cut off and incubated with soluene until the tissue was digested. The radioactivity in the soluene extract was counted. The apparent kinetic constants (K_m, V_{max}, K_i) were calculated from the linear portion of the curves by the direct linear plot method of Eisenthal and Cornish-Bowden [26].

Results

Adenine nucleotide translocator kinetics

At a temperature of 2°C and an external ATP concentration of 250 μM, uptake of [^{14}C]ATP followed first-order rate kinetics for the first 10 to 15 s (Fig. 1A). The 10-s time point was chosen for the kinetic analysis. Fig. 1B shows that uptake of external [^{14}C]ATP (250 μM) was linear with varying amounts of mitochondrial protein (0.2–1.2 mg). The effect of external ATP concentration of velocity of [^{14}C]ATP transport is shown in Fig. 2. The apparent K_m and V_{max} values for [^{14}C]ATP transport using 25–250 μM ATP were 58 μM and 4.3 nmol/mg protein per min, respectively. When $K_3Fe(CN)_6$ was added to these mitochondria, the matrix concentration of long-chain acyl-CoA esters decreased (Table I) and the rate of [^{14}C]ATP transport increased (Fig. 2).

Effects of palmitylcarnitine incubation at 22°C

Mitochondrial matrix levels of long-chain acyl-CoA esters were increased by incubating mitochondria with 60 μM palmitylcarnitine for 15 min at 22°C (Table I). Untreated mitochondria (control) were also incubated for 15 min at 22°C,

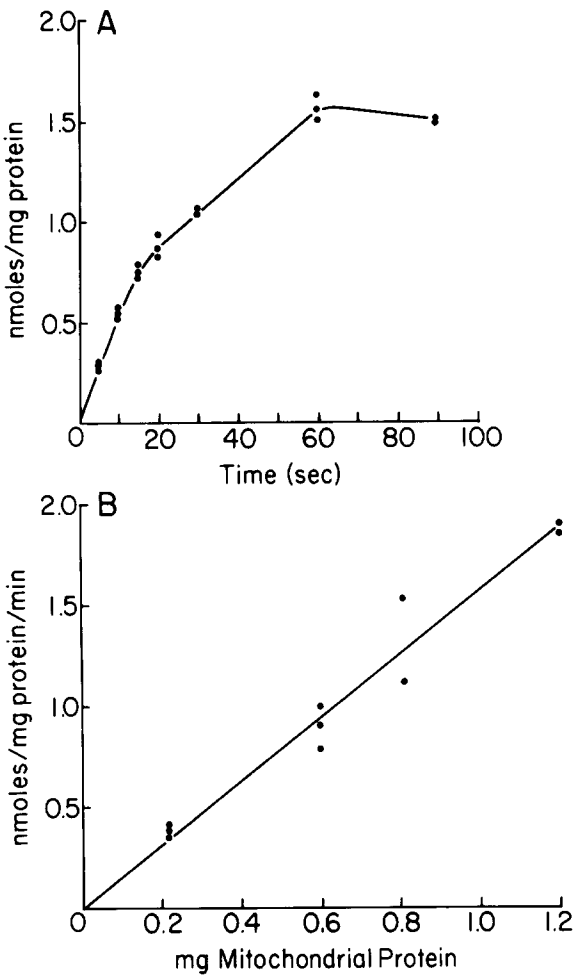

Fig. 1. (A) Time course of [^{14}C]ATP uptake by isolated rat heart mitochondria. The reaction was run at 2°C and started by adding 0.5 mg mitochondria to a reaction mix containing 250 μM ATP and 1 μCi [U-^{14}C]ATP. Carboxyatractyloside (1 μmol) was added after various times to stop the reaction. Triplicate values are shown. (B) Protein concentration curve for [^{14}C]ATP uptake by isolated rat mitochondria. The reaction was started by adding various amounts of mitochondria (0.25–1.2 mg) to a reaction mix containing 250 μM [^{14}C]ATP. The assay duration was 10 s and was done in triplicate.

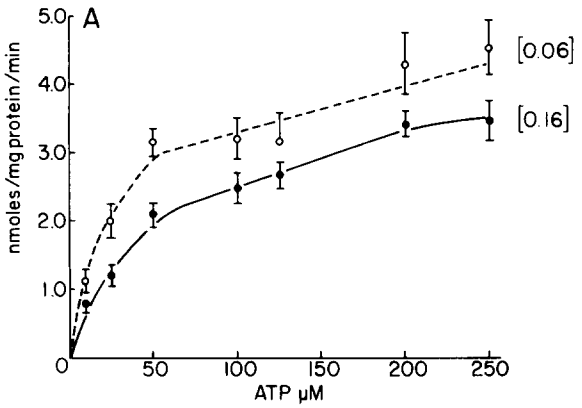

Fig. 2. Effect of external ATP concentration on the velocity of [^{14}C]ATP uptake; effects of potassium ferricyanide. Velocity of [^{14}C]ATP transport was measured using various ATP concentrations (10–250 μM) and a duration of 10 s. Each point for untreated mitochondria (●------●) was derived from five experiments performed in either duplicate or triplicate and represents the overall mean ± S.E. for all datum points. In treated mitochondria (○------○), 1 mM $K_3Fe(CN)_6$ was added (two experiments performed in triplicate). The concentrations of matrix long-chain acyl-CoA (nmol/mg protein) are shown in brackets.

but no palmitylcarnitine was added. Incubation of control mitochondria decreased matrix levels of ATP, ADP, total transportable nucleotides (ATP and ADP), AMP, and long-chain acyl-CoA esters. Addition of 60 μM palmitylcarnitine increased the level of long-chain acyl-CoA esters, but caused no further decrease in adenine nucleotides.

Fig. 3 shows the velocity of [^{14}C]ATP transport in mitochondria incubated with 0 and 60 μM palmitylcarnitine for 15 min at 22°C. The velocity of [^{14}C]ATP transport in control mitochondria incubated at 22°C for 15 min was less than the velocity of freshly prepared mitochondria maintained at 4°C. This decreased activity was most likely caused by depletion of transportable nucleotides during the incubation period (Table I).

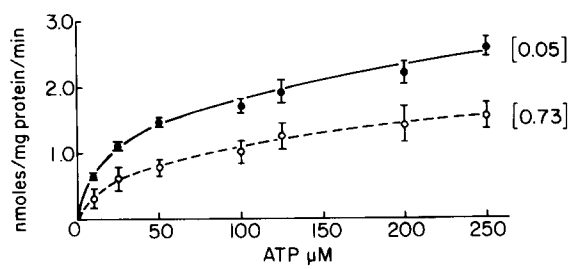

Fig. 3. Effects of palmitylcarnitine incubation on the velocity of [^{14}C]ATP uptake. Mitochondria were incubated at 22°C for 15 min with no (●------●) or 60 (○------○) μM palmitylcarnitine. The brackets contain the matrix concentration (nmol/mg protein) of long-chain acyl-CoA produced by this incubation. Each point of zero palmitylcarnitine was derived from six experiments performed in either duplicate or triplicate and represent overall mean ± S.E. For 60 μM palmitylcarnitine, each point was derived from four experiments.

TABLE I

MITOCHONDRIAL MATRIX LEVELS OF ADENINE NUCLEOTIDES AND LONG-CHAIN ACYL-CoA

Levels (expressed as nmol/mg mitochondrial protein) were assayed in freshly prepared mitochondria (control), after the addition of 1 mM $K_3Fe(CN)_6$, after incubation at 15 min at 22°C with and without 60 μM palmitylcarnitine (PC), in mitochondria isolated with $1 \cdot 10^{-4}$ M KCN in extraction medium, and after the addition of $K_3Fe(CN)_6$ to KCN-isolated mitochondria. Values are mean ± S.E. for *n* shown in parentheses.

Group (*n*)	ATP	ADP	AMP	Long-chain acyl-CoA
Control untreated (10)	3.2 ± 0.3	5.5 ± 0.3	3.1 ± 0.4	0.18 ± 0.03
Control + $K_3Fe(CN)_6$ (2)	–	–	–	0.06
Control incubation: 15 min, 22°C (4)	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.05 ± 0.03
Incubation + 60 μM PC (4)	0.9 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	0.73 ± 0.05
Isolated with $1 \cdot 10^{-4}$ M KCN (5)	0.9 ± 0.1	4.2 ± 0.3	8.7 ± 0.4	1.07 ± 0.07
KCN + $K_3Fe(CN)_6$ (4)	–	–	–	0.26 ± 0.05

The K_m and V_{max} values of these control-incubated mitochondria were 43 μM and 2.5 nmol/mg per min, respectively. Incubating mitochondria with 60 μM palmitylcarnitine resulted in inhibition of [^{14}C]ATP transport at several different external concentrations of ATP. Since many studies have shown that palmitylcarnitine has no effect on adenine nucleotide translocator activity [9,10], we believe that the inhibition shown in Fig. 3 was caused by accumulation of long-chain acyl-CoA esters within the mitochondrial matrix (Table I). The apparent K_i was calculated by converting nmol/mg mitochondrial protein to μM using the conversion factor of 1 μl mitochondrial matrix space per mg mitochondrial protein [27]. The apparent K_i value for matrix long-chain acyl-CoA inhibition of ATP transport equaled 0.38 mM. However, because of binding of long-chain acyl-CoA esters to mitochondrial proteins [12,28], this value is probably an overestimation of the actual K_i concentration.

We next tested the effect of differing incubation times and temperatures on matrix long-chain acyl-CoA and [^{14}C]ATP transport. Incubation of mitochondria for 15 min at 4°C had no effect on levels of long-chain acyl-CoA esters or [^{14}C]ATP transport (Table II). When mitochondria were incubated with 60 μM palmitylcarnitine at 4°C for 15 min, long-chain acyl-CoA esters increased 2-fold and [^{14}C]ATP transport was depressed. Incubating control mitochondria without palmityl CoA at 22°C for 15 min decreased long-chain acyl-CoA levels and [^{14}C]ATP transport. As discussed earlier, the decreased ATP transport was caused by depletion of transportable nucleotides. Palmitylcarnitine (60 μM) incubation at 22°C for 15 min produced a 5-fold increase in long-chain acyl-CoA and severely inhibited [^{14}C]ATP transport. Addition of $K_3Fe(CN)_6$ to these mitochondria oxidized and lowered the levels of long-chain acyl-CoA. Since $K_3Fe(CN)_6$ only allows oxidation of matrix acyl-CoA [20], this finding indicates that accumulation of long-chain acyl-CoA produced by palmitylcarnitine incubation occurred in the mitochondrial matrix compartment. Addition of $K_3Fe(CN)_6$ also reversed inhibition of [^{14}C]ATP transport. This reversal by $K_3Fe(CN)_6$ was probably the result of lowered levels of matrix long-chain acyl-CoA esters.

Mitochondria isolated with 10^{-4} M KCN in extraction medium

The concentration of long-chain acyl-CoA esters within the mitochondrial matrix was also in-

TABLE II

EFFECT OF TEMPERATURE, INCUBATION TIME AND PALMITYLCARNITINE ON MITOCHONDRIA LONG-CHAIN ACYL-CoA AND ADENINE NUCLEOTIDE TRANSLOCATOR ACTIVITY

Mitochondria were incubated with and without palmitylcarnitine for the durations and temperatures shown. Long-chain acyl-CoA esters were assayed as described in Materials and Methods. Adenine nucleotide translocator activity was determined by measuring the uptake of 250 μM [^{14}C]ATP for 10 s as described in Materials and Methods. 1 mM $K_3Fe(CN)_6$ were used in the final group.

Palmitylcarnitine concentration (μM)	Incubation time (min)	Temp. (°C)	Long-chain acyl-CoA (nmol/mg protein)	Adenine nucleotide translocator activity (nmol/mg protein per min)
0	0	4	0.18	3.52
0	15	4	0.16	3.04
60	15	4	0.33	2.25
0	15	22	0.09	2.51
60	15	22	0.86	1.63
60 + $K_3Fe(CN)_6$	15	22	0.06	3.95

creased by isolation of mitochondria with a respiratory inhibitor in the extraction medium. This prevented oxidation of fatty acids during the isolation procedure. Addition of KCN ($1 \cdot 10^{-4}$ M) to our extraction medium produced about a 70% inhibition of State 3 respiration [11]. It also caused a 5-fold increase in long-chain acyl-CoA esters (Table I), but decreased the matrix level of transportable nucleotides by 50%. Both results could lead to a decrease in adenine nucleotide transport [5,6,9–12].

To distinguish between these two inhibitory conditions, we decreased the matrix content of long-chain acyl-CoA by adding $K_3Fe(CN)_6$ (1 mM) to the incubation medium [20]. It was impossible to measure the nucleotide concentration in these mitochondria because $K_3Fe(CN)_6$ interfered with the assay. Fig. 4 shows the effect on velocity of [^{14}C]ATP transport by isolating mitochondria with $1 \cdot 10^{-4}$ M KCN in the extraction medium. Transport was severely inhibited, but was partially reversed by addition of $K_3Fe(CN)_6$.

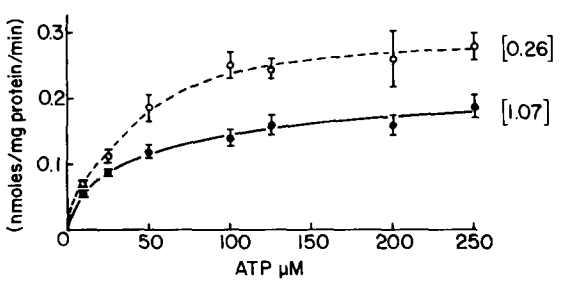

Fig. 4. Uptake of [^{14}C]ATP by mitochondria isolated in the presence of potassium cyanide; effects of potassium ferricyanide. Uptake of [^{14}C]ATP was measured in mitochondria isolated with medium containing $1 \cdot 10^{-4}$ M KCN (●------●) (four experiments in triplicate). In other similarly treated mitochondria, 1 mM $K_3Fe(CN)_6$ was added (○------○) (two experiments in triplicate). The concentrations (nmol/mg protein) of matrix long-chain acyl-CoA are shown in brackets. Each point represents overall mean ± S.E.

Discussion

Recent studies have established that the inhibitory effects of long-chain acyl-CoA esters on adenine nucleotide translocator [9–12] and other transport systems [29] occur at levels well below the critical micelle concentration [30]. These findings rule out a nonspecific detergent type of interaction; however, many questions remain about the possible physiological significance of long-chain acyl-CoA inhibition of adenine nucleotide translocator activity [4,19,20,28,31]. Some investigators have argued that nonspecific binding of long-chain acyl-CoA esters to intracellular protein or competitive interaction with endogenous ADP or ATP may prevent inhibition of adenine nucleotide translocator by long-chain acyl-CoA esters [12,20]. Others have suggested that inhibition of heart mitochondrial adenine nucleotide translocator could occur only at the matrix site because long-chain acyl-CoA esters accumulate in the mitochondrial matrix during ischemia [19,20,31].

Although many studies have shown long-chain acyl-CoA inhibition of adenine nucleotide translocator at the outer mitochondrial site [9–12], inhibition at the matrix loci has not been clearly demonstrated in isolated mitochondria [4,20,21]. Because of this, the possible regulation of mitochondrial function by acyl-CoA-adenine nucleotide translocator inhibition remains in question.

In the present study, we developed a simple and rapid technique to measure adenine nucleotide translocator activity by the slower forward transport of [^{14}C]ATP at 2°C. The assay followed first-order kinetics for the first 15 s and was linear with protein concentration. Our results show conclusively that matrix long-chain acyl-CoA esters can inhibit the exchange of external [^{14}C]ATP for internal adenine nucleotides, although it is not certain whether there is a similar inhibition by matrix long-chain acyl-CoA esters of the exchange of external ADP for internal ATP. We found that incubation of mitochondria with palmitylcarnitine caused matrix long-chain acyl-CoA esters to increase, which in turn inhibited [^{14}C]ATP transport. Addition of $K_3Fe(CN)_6$ to these mitochondria or freshly prepared mitochondria decreased matrix long-chain acyl-CoA levels and increased [^{14}C]ATP transport. In other experiments, mitochondria isolated in the presence of $1 \cdot 10^{-4}$ M KCN showed an approximate 5-fold increase in matrix long-chain acyl-CoA esters in addition to a 50% decrease in transportable nucleotides. [^{14}C]ATP was severely inhibited in these mitochondria. Addition of $K_3Fe(CN)_6$ to these mitochondrial preparations markedly reduced matrix long-chain acyl-CoA and partially restored [^{14}C]ATP transport.

During myocardial ischemia, long-chain acyl-CoA esters accumulate primarily in the mitochondrial matrix and reach levels of about 1.06 nmol/mg mitochondrial protein [19]. In the present study, we were able to demonstrate significant inhibition of the adenine nucleotide translocator at considerably lower matrix levels (0.73 nmol/mg mitochondrial protein) of long-chain acyl-CoA than which occur during ischemia. Thus, these findings offer strong support of our previous studies, which proposed that long-chain acyl-CoA esters play an important metabolic role in the regulation of adenine nucleotide translocator activity during the early phase of myocardial ischemia [9,16,18,29,32].

Acknowledgement

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