EFFECT OF THE DIVALENT CATION IONOPHORE A23187 ON THE TRANSLOCATION OF ADENINE NUCLEOTIDES IN LIVER MITOCHONDRIA

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1. Introduction

Liver mitochondria contain, per mg protein, 20–30 ng atom magnesium [1–3], about half of which is located in the matrix compartment [2]. They also contain about 10 nmol adenine nucleotides per mg protein [4], most of it in the matrix. Although the form of magnesium and adenine nucleotides present in the mitochondrial matrix is not known precisely, it seems likely that most of the nucleotides are complexed by Mg. On the other hand, it has been suggested [5–7] that free ADP and ATP, and not their magnesium complexes, are transported across the mitochondrial membrane by the specific adenine nucleotide translocase [8]. Experimental evidence is a decrease of the translocation rate by increasing external Mg^{2+} concentration [7]. Thus, a very low intramitochondrial concentration of free, non-complexed, ADP and ATP might be one of the factors controlling the rate of the translocation.

One of the intriguing features of the translocase is its preference for external ADP over external ATP [5,9]. An explanation for this can be provided by assuming that the exchange of ATP against ADP is mostly electrogenic [5,10,11] and is therefore driven in one direction only by the mitochondrial transmembrane potential which is negative inside and positive outside. An alternative explanation assumes that, in the energized mitochondrial membrane, the translocase exhibits different affinities towards external ATP and ADP [12,13]. A complication in the first explanation is posed by the fact that no preference has been observed for the export of intramitochondrial ATP over ADP [5].

In view of the problems described above, it seems to us promising to use magnesium-depleted mitochondria as a convenient model to study adenine nucleotide translocation. The ionophore A23187, specific for Mg^{2+} and Ca^{2+} [14,15], was used for that purpose. In a previous article [16] we briefly reported on a stimulation by A23187 of [^{14}C]ADP uptake. In the present paper we show that in coupled mitochondria the increased uptake of ADP is accompanied by an increased efflux of ATP, so that the proportion of exported ATP to ADP is greatly increased and substantially exceeds the intramitochondrial ratio of total ATP to total ADP. These results are interpreted as confirming previous suggestions that free non-complexed adenine nucleotides are transported by the translocase system [5–7] and that the exchange of ATP against ADP is mostly electrogenic and under the control of the transmembrane potential [10,11]. Preliminary results of this study have already been presented [17].

2. Materials and methods

Rat liver mitochondria were isolated in 225 mM mannitol/75 mM sucrose/2 mM Tris–HCl (pH 7.4). Translocation of adenine nucleotides was measured by the inhibitor-stop method using [8-^{14}C]ADP or [8-^{14}C]ATP (Radiochemical Centre, Amersham) and carboxyatractyloside (Boehringer, Mannheim)

Abbreviation: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone

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as described [16]. Both 'forward exchange' and 'back exchange' [5] procedures were used as indicated. The ionophore A23187 (Eli Lilly, Indianapolis, IN) was used as 1 mM stock solution in 25% dimethylformamide–75% ethanol [14].

3. Results

Confirming the results [16], table 1 shows a stimulation of adenine nucleotide translocation by the ionophore A23187 if measured by the uptake of external ADP. Under these conditions, an uncoupler of oxidative phosphorylation CCCP had no effect alone and diminished the stimulation by A23187. In contrast with this, the ionophore was without effect on the uptake of external ATP, whereas the uncoupler exhibited the well known [5] potent stimulation. Although the ionophore results, under certain conditions, in an uncoupling of the energy-conserving mechanism [14], these results clearly demonstrate that its stimulatory effect on the translocase is, by no means, due to its possible uncoupling action.

The uncoupling effect of A23187 has been explained [14] by an energy-consuming recirculation of Ca^{2+} (energy-dependent carrier-mediated uptake and passive ionophore-mediated outflow). This process can be prevented by low concentrations of Mg^{2+} which competes with calcium for the binding site [18]. As shown in fig.1, Mg^{2+} up to 5 mM has little effect on the stimulation by the ionophore of ADP translocation. However, higher concentrations of Mg^{2+} produce a strong decline in the translocation rate

Table 1
Effects of the ionophore A23187 and the uncoupler CCCP on the translocation of adenine nucleotides

Additions	External nucleotides	
	ADP	ATP
	translocation (% control)	
None	100	100
A23187, 3 μM	196	105
CCCP, 2.5 μM	103	197
A23187 + CCCP	138	189

The translocation was measured in the 'forward exchange' system in 2.1 ml medium containing 120 mM KCl, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, about 5 mg mitochondrial protein and 70 μM [^{14}C]ADP or [^{14}C]ATP. After 40 s at 0°C carboxyatractyloside was added to the final concentration of 5 μM. The numbers indicate the rate of uptake of the label as % control (without additions)

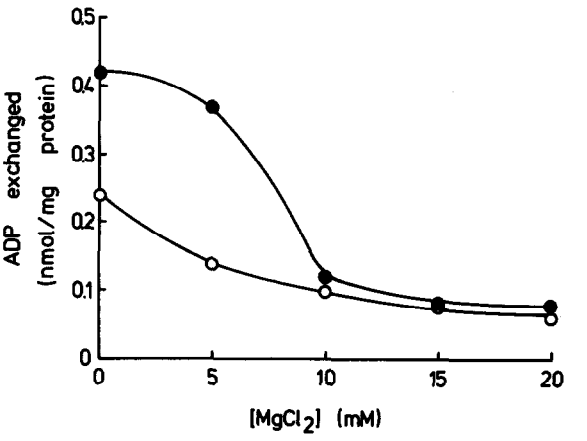

Fig.1. Effect of Mg^{2+} on the translocation of ADP and the effect of A23187 thereupon. Experimental conditions as in table 1 but with 0.25 mM 2,4-dinitrophenol. (○) Control; (●) in the presence of 3 μM A23187.

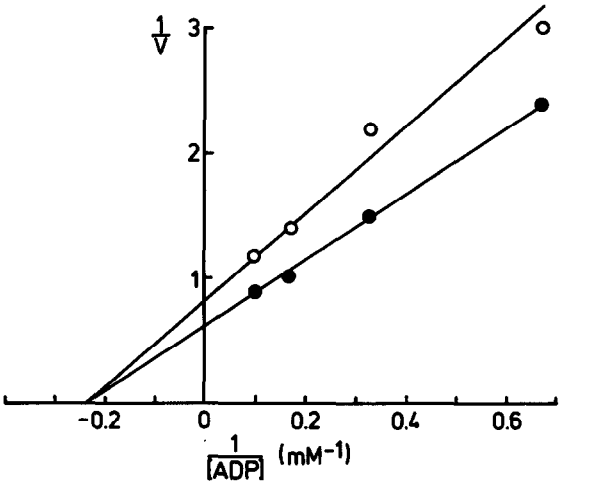

Fig.2. Effect of A23187 on the translocation of ADP. Lineweaver-Burk plot. Experimental conditions as in table 1. (○) Control; (●) in the presence of 3 μM A23187. $1/V$ is expressed in arbitrary units.

in the presence of A23187. At 10 mM $MgCl^{2+}$ the rate in the presence of the ionophore becomes equal to the rate in its absence. It is to be noted that, in the absence of the ionophore, Mg^{2+} also produces a decrease in the translocation rate, as described [7]. Since the experiment shown in fig.1 was performed in the presence of 2,4-dinitrophenol, i.e., under conditions where no energy-dependent accumulation of Mg^{2+} in mitochondria was possible, it could be assumed that intramitochondrial concentration of free Mg^{2+} in the presence of the ionophore was equal to Mg^{2+} concentration in the medium.

As shown in fig.2, the ionophore increased V_{max} of the uptake of external ADP, but had no effect on the K_m value.

To follow the profiles of adenine nucleotides exported from mitochondria in exchange for external ADP or ATP, mitochondrial nucleotides were labelled by preincubation in the presence of [^{14}C]ADP [5] and such mitochondria were used in the back exchange system. After further preincubation with phosphate, malate and glutamate in order to convert as much as possible mitochondrial AMP into ADP and ATP, oligomycin was added to stop further phosphorylation and dephosphorylation of adenine nucleotides and the translocation was started by adding unlabelled ADP or ATP. The amounts of labelled nucleotides liberated to the external medium were corrected for unspecific leakage, as calculated from samples to which carboxyatractyloside was added before the nucleotide and, in particular, from the amount of [^{14}C]AMP found in the external medium.

Values obtained in this way are shown in table 2. It is evident (expt A) that intramitochondrial ATP and ADP were exchanged for external ADP in proportion of 1:3, although the proportion of these nucleotides inside mitochondria was slightly over 1:1. De-energization of the membrane by CCCP resulted in a further shift of the proportion of exported nucleotides towards ADP. In contrast with this, the ionophore A23187 greatly increased the proportion of exported ATP, so that the ratio of exported ATP/ADP became higher than the intramitochondrial ATP/ADP ratio. After de-energization in the presence of A23187 the exported ATP/ADP ratio was decreased to a value which was not much different from the intramitochondrial ATP/ADP ratio.

A similar tendency in the action of A23187 could be observed when ATP was the external substrate of the translocase (table 2, expt B). In this case internal ATP and ADP were exported in a proportion approximately equal to that inside mitochondria, and again ADP became the predominant nucleotide exported in the presence of uncoupler. The ionophore A23187 increased the proportion of ATP to ADP exported in both energized and de-energized mitochondria in a similar way as with external ADP.

From the data of table 2 it can easily be concluded that the increased translocation rate produced by the ionophore in the presence of external ADP, as shown in table 1, was mainly due to an increased export of internal ATP.

4. Discussion

The present results provide explanations for the two following features of the translocase:

(i) The first order kinetics of the translocase with respect to intramitochondrial nucleotides [6], in spite of its very low K_m value [6] and a high apparent concentration of adenine nucleotides in the matrix compartment [4,8].

(ii) The lack of preference for internal ATP observed so far [5] (see also [21]).

(i) Taking into account high stability constants of Mg.ATP and Mg.ADP, amounting to $10^{4.2}$ M^{-1} and $10^{3.2}$ M^{-1}, respectively [22,23], one can expect very low concentrations of free ATP and ADP in the presence of Mg^{2+}. Assuming the concentration of free Mg^{2+} in the matrix compartment as 10 mM and the concentration of total (free plus complexed) ATP and ADP as 5 mM each, it can be calculated that the concentration of free ATP and ADP should be 31 μM and 297 μM, respectively. These values are probably still overestimated because neither binding of adenine nucleotides to proteins nor complexing by calcium was taken into account. It seems therefore likely that the translocase may not be saturated from the inside and can therefore exhibit the first order kinetics with respect to internal nucleotides [6]. Depletion of mitochondrial Mg^{2+} and Ca^{2+} in the presence of the ionophore A23187 increases the translocation rate

Table 2
Profiles of adenine nucleotides exported from mitochondria in exchange for external ADP or ATP – effects of the ionophore A23187 and the uncoupler CCCP

Additions	Intramitochondrial nucleotides			Exported nucleotides	
	AMP	ADP	ATP	ADP	ATP
	(% total)			(% total)	
A. Exchange for external ADP					
None	4	42	54	75	25
CCCP, 2.5 μM	5	42	53	90	10
A23187, 3 μM	5	45	50	37	63
CCCP + A23187	5	47	48	62	38
B. Exchange for external ATP					
None	6	48	46	43	57
CCCP, 2.5 μM	6	46	48	99	1
A23187, 3 μM	6	44	50	37	63
CCCP + A23187	6	46	48	64	36

The experiment was performed in the 'back exchange' system. Intramitochondrial adenine nucleotides were labelled by incubating mitochondria for 1 h at 0–4°C in 160 mM sucrose/40 mM KCl/7 mM Tris–HCl (pH 7.4)/serum albumin, 0.5 mg/ml/ 0.3 mM [^{14}C]ADP. After isolation and resuspension in the same medium but without ADP and serum albumin, mitochondria (1.3 mg protein) were added to 2.0 ml samples of the incubation medium containing: 120 mM KCl, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.5 mM phosphate, 1 mM L-malate and 5 mM glutamate. After preincubation at 0°C during 10 min, 3 μg oligomycin and the additions indicated in the table were added and the translocation was started by adding 70 μM unlabelled ADP or ATP and stopped after 40 s by 5 μM carboxyatractyloside. Then, the samples were passed through millipore filters (0.65 μm pore size), the filtrates acidified with perchloric acid and the nucleotides adsorbed on charcoal. After elution with a water/ethanol/ammonia mixture [19], the nucleotides were separated by paper chromatography [20] and the spots counted for radioactivity. Profiles of intramitochondrial nucleotides were determined in aliquots which were removed and extracted with perchloric acid after preincubation and addition of oligomycin and A23187 and/or CCCP, but before adding external nucleotides. Control samples for unspecific leakage of the nucleotides were run in such a way that carboxyatractyloside was added before the nucleotide

even at saturating concentrations of external nucleotides (fig.2).

(ii) Because of a 10-fold difference between the stability constants of Mg.ATP and Mg.ADP, the proportion between free ATP and free ADP in the matrix compartment is about 10-times lower than the proportion between total ATP and total ADP (see preceding paragraph). This provides a logical explanation for the effect produced by the ionophore A23187 on the proportion of exported nucleotides and may also explain why no preference for ATP has been observed in non-depleted mitochondria even at elevated intramitochondrial ATP_{total}/ADP_{total} ratios [21]. Since depletion of mitochondrial divalent cations increases the concentration of free ATP much more than of free ADP, an increase of the export of internal ATP is to be expected (table 2). It can thus be concluded that the preference of the translocase for intramitochondrial ATP over ADP is normally counteracted and masked by very low concentration of free internal ATP and can be visualized only after depletion of mitochondrial divalent cations and the

concomitant increase of the concentration of the free nucleotides. This argumentation also provides a further support to the postulation of the electrogenic nature of the translocation [5,10,11].

From the observation (fig.1) that, at external 10 mM Mg^{2+}, the stimulatory effect of A23187 on the translocation rate in uncoupled mitochondria disappears it may be concluded that this is the concentration of free Mg^{2+} normally present in the matrix compartment of liver mitochondria.

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