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ADENINE NUCLEOTIDE TRANSLOCATION IN JERUSALEM-ARTICHOKE MITOCHONDRIA

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SUMMARY

1. Atractyloside does not inhibit State-3 respiration in coupled Jerusalem-artichoke mitochondria.

2. Bongkrekic acid inhibits State-3 respiration in artichoke mitochondria in a similar way to that described for rat-liver mitochondria.

3. The exchange of a small endogenous adenine nucleotide pool (0.5–3.0 nmoles/mg protein) is extremely slow and unspecific, and is insensitive to atractyloside and bongkrekic acid, indicating the absence of a translocator similar to that known for animal mitochondria.

INTRODUCTION

The nucleotide specificity of the oxidative phosphorylation reactions of animal mitochondria has been shown to be imposed by the specificity of the adenine nucleotide translocator situated within the cristae membrane[1,2]. Two invaluable tools used to investigate the translocation process are atractyloside and bongkrekic acid, which inhibit the exchange of adenine nucleotides in rat-liver mitochondria presumably by interacting with the translocator[3–6]. In the absence of any reports in the literature on adenine nucleotide translocation in plant mitochondria, it was considered to be of interest to examine the effects of these inhibitors on artichoke mitochondria and to measure the rates of nucleotide exchange.

METHODS

Jerusalem-artichoke tubers were obtained from Covent Garden, London, and stored at 0–4 °C until required. Mitochondria were isolated using methods and media previously described[7]. In some cases 1–2% bovine serum albumin and polyvinylpyrrolidone were included in the grinding medium. 1% bovine serum albumin was also included in the suspending medium. Respiratory control values, with malate as substrate, were between 3 and 7, indicative of mitochondrial integrity. Oxygen up-

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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take was measured polarographically using a Clark electrode. Measurement of the exchange of ^{14}C-labelled nucleotide was based on the filtration technique described by Winkler *et al.*[8]. ATPase activity was measured by following the decrease in pH accompanying ATP hydrolysis[9]. Protein was determined by the biuret procedure[10].

RESULTS

Fig. 1 illustrates the effect of bongkrekic acid and atractyloside on the oxidation of malate by artichoke mitochondria. Preincubation of mitochondria with 1.33 μM bongkrekic acid for 2 min prevents the stimulation of the rate of O_2 uptake by ADP.

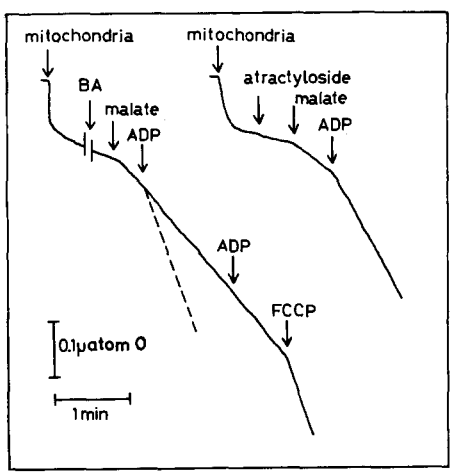

Fig. 1. The effect of atractyloside and bongkrekic acid on the oxidation of malate by Jerusalem-artichoke mitochondria. O_2 uptake was measured using a Clark electrode. The reaction medium contained, in a total volume of 1.5 ml, 0.35 M sucrose, 0.04 M Tris–HCl buffer, 7 mM P_i at pH 7.0 and 25 °C. Mitochondria (2 mg protein), 33 mM malate, bongkrekic acid (BA, 1 nmole/mg protein, incubated for 2 min prior to ADP addition), 0.83 mM atractyloside, 0.33 μM FCCP and ADP (0.25 μmole per addition) were added where indicated. The broken line shows the rate of O_2 uptake in the absence of bongkrekic acid.

Subsequent addition of 0.33 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) causes a 3-fold increase in the rate of O_2 uptake. On the other hand 0.83 mM atractyloside, a concentration much greater than that which normally inhibits in animal mitochondria, does not prevent the stimulation of O_2 uptake by ADP. The concentration of bongkrekic acid required to bring about complete inhibition of State-3 respiration is dependent on the mitochondrial protein concentration (0.4 nmole/mg protein causing 100% inhibition at pH 6.9 and 25 °C), the pH of the assay medium and the incubation time. Bongkrekic acid also partially inhibits the ATPase activity of artichoke mitochondria but does not inhibit the rate of ATP hydrolysis by submitochondrial particles (see Table I), thus indicating that either the polarity[7] or the integrity of the mitochondrial membrane influences the effect of bongkrekic acid. The rate of ATP hydrolysis by submitochondrial particles was rather low, but this relates to the low pH of the assay medium. Bongkrekic acid inhibits more effectively below pH 7.3, whereas the pH optimum of the ATPase is pH 9–9.3.

TABLE I

THE EFFECT OF BONGKREKIC ACID ON THE ATPase ACTIVITY OF JERUSALEM-ARTICHOKE MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

The ATPase activity was estimated from the decrease in pH during the hydrolysis of ATP[9]. The medium contained, in 1.2 ml, 0.37 M sucrose, 1.8 mM Tris–HCl buffer, 0.83 mM $MgCl_2$, 1.7 mM ATP and mitochondria (4 mg protein) or submitochondrial particles (4 mg protein). The pH at the start of the experiment was approx. 7.0 and the temperature 25 °C. The mitochondria were previously frozen and thawed to enhance the ATPase activity. When included in the assay, bongkrekic acid was first preincubated for 2 min with the particles; 1.7 μM FCCP was added where indicated.

Preparation	*Additions*	*ATPase activity (nmoles ATP hydrolysed/mg protein per min)*
Mitochondria	None	12.0
	FCCP	10.2
	Bongkrekic acid (1.25 nmoles/mg protein)	3.0
Submitochondrial particles	None	29.4
	FCCP	23.4
	Bongkrekic acid (4 nmoles/mg protein)	23.4
	Oligomycin (5 μg)	3.6

The inhibitory effects of bongkrekic acid on the respiration of the artichoke mitochondria closely resemble those earlier described for rat-liver mitochondria[4,5] and suggest therefore a similarity in the mechanism of inhibition. The lack of inhibition of artichoke respiration by atractyloside, however, is in contrast to results reported for animal mitochondria where atractyloside and bongkrekic acid both prevent the stimulation of State-4 respiration by ADP, probably by interaction with the adenine nucleotide translocator[4,6].

In order to see if the inhibition by bongkrekic acid in artichoke mitochondria is related to an inhibition of adenine nucleotide translocation, its effects on the rates of translocation of ^{14}C-labelled adenine nucleotides into these mitochondria were directly measured. Fig. 2 illustrates a typical forward-exchange experiment where artichoke mitochondria were incubated in a medium containing [^{14}C]ATP or [^{14}C]-ADP and the rates of uptake of radioactivity determined. The rates of exchange of ADP or ATP are extremely slow, both in comparison with the rate of translocation described for rat-liver mitochondria and the rate of ATP synthesis in artichoke mitochondria. The exchange reactions are insensitive to both atractyloside and bongkrekic acid. The size of the endogenous nucleotide pool, calculated from the isotopic equilibrium[11], was in the range of 0.5–3.0 nmoles/mg protein, and the translocation velocity was generally less than 3 nmoles/mg protein per min at 25 °C. The nucleotide exchange is not specific for ADP or ATP. As shown in Fig. 3, AMP and GDP also exchange at approximately the same rate as ADP. In the experiment illustrated in Fig. 3 the mitochondria were first preincubated with [^{14}C]ADP for 20 min at 25 °C and, after washing the mitochondria twice with suspending medium, the "backward"

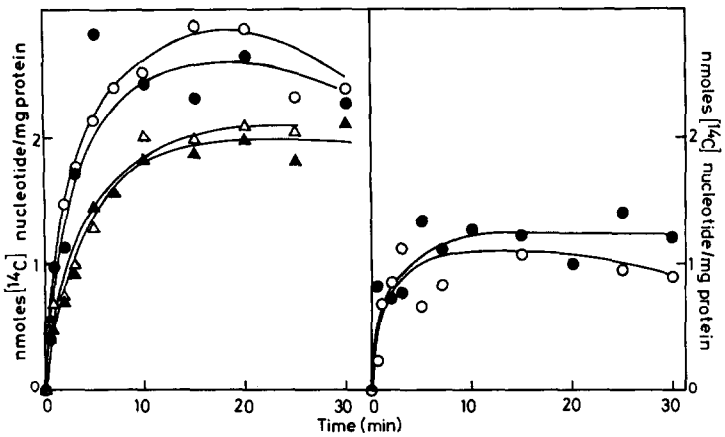

Fig. 2. The effect of atractyloside and bongkrekic acid on the uptake of adenine nucleotides in artichoke mitochondria. The reaction medium contained, in a total volume of 2 ml, 0.24 M sucrose, 0.025 M Tris–HCl buffer, 1.2 mM [^{14}C]ADP or [^{14}C]ATP at pH 7.0 and 25 °C. Other additions were 0.1 mM atractyloside, 7.5 μM bongkrekic acid, mitochondria (5.5 mg protein). Aliquots (0.1 ml) were removed at various intervals after the addition of adenine nucleotide and placed on Millipore filters (pore size 0.45 μm). After washing with 10 ml NaCl (0.9%, w/v), the filters were dried and radioactivity estimated using a scintillation spectrometer. (A) ○—○, ADP; ●—●, ADP+atractyloside; △—△, ATP; ▲—▲, ATP+atractyloside. (B) ○—○, ADP; ●—●, ADP+ bongkrekic acid.

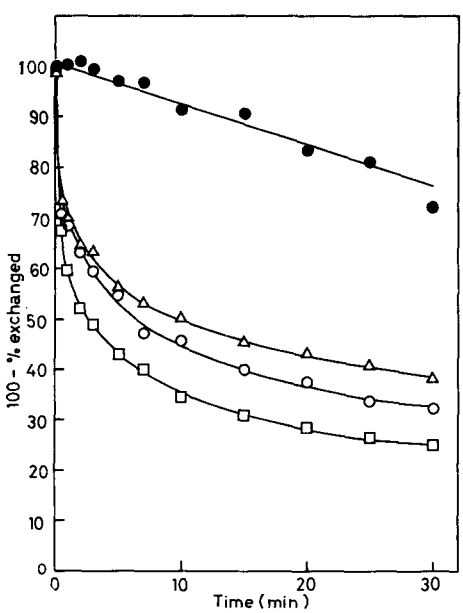

Fig. 3. The specificity of the nucleotide exchange in artichoke mitochondira. Mitochondria (9 mg protein, previously labelled with [^{14}C]ADP) were added to a medium containing, in 2 ml, 0.24 M sucrose and 0.025 M Tris–HCl buffer, at pH 7.0 and 25 °C. Addition of unlabelled nucleotides (1.2 mM) started the assay and aliquots were removed and the radioactivity measured at the times indicated. Unlabelled nucleotides were: □—□, ADP; ○—○, GDP; △—△, AMP. In the control (●—●) no unlabelled nucleotides were added.

exchange of the labelled endogenous nucleotides with unlabelled nucleotides was measured. The backward exchange consists of two phases. The initial fast phase probably corresponds to an actual exchange of bound and free nucleotides, and only occurs when unlabelled nucleotides are added. The slow phase, also occurring in the absence of unlabelled nucleotides, probably represents a gradual leakage of label from the mitochondria.

In animal mitochondria the rate of exchange of adenine nucleotides is so rapid that kinetic measurements are generally performed at 0 °C. The process in rat-liver mitochondria is (a) dependent on the incubation temperature[3], (b) specific for ADP and ATP[12], and (c) inhibited by atractyloside[1,3] and bongkrekic acid[5], all three characteristics probably being related to the properties of the translocator. In artichoke mitochondria attempts to increase the rate of uptake of radioactivity were unsuccessful. Thus, as shown in Table II, variation of the temperature, or inclusion in the reaction medium of oxidizable substrate or KCl (which has been shown to increase the ATPase activity of corn mitochondria[13] and the nucleotide translocation

TABLE II

THE EFFECT OF VARYING THE ASSAY CONDITIONS ON THE RATE OF TRANSLOCATION OF ADENINE NUCLEOTIDES IN JERUSALEM-ARTICHOKE MITOCHONDRIA

The reaction medium contained, in a total volume of 2 ml, 0.2 M sucrose, 0.025 M Tris–HCl buffer, antimycin (3 μg/mg protein), [^{14}C]ADP or [^{14}C]ATP (1.2 mM unless otherwise stated) and mitochondria (9 mg protein) at pH 7.0. Other additions were made as indicated: 50 mM malate, 25 mM glutamate, 5 mM P_i, 3.2 mM atractyloside, 5 μM bongkrekic acid. KCl medium consisted of 0.3 M KCl only. The internal pool and translocation velocity were calculated from the isotopic equilibrium[11].

Expt	*Temp.* (°C)	*Additions*	*Internal pool (nmoles/mg protein)*	*Translocation velocity (nmoles/mg protein per min)*
1	25	ADP	0.88	0.74
		ADP + malate + glutamate + P_i*	0.81	1.71
		ADP + bongkrekic acid	0.98	1.65
		ADP + atractyloside	0.69	1.47
2	0	ADP	0.90	0.34
		ADP + atractyloside	0.77	0.58
		ATP	0.31	0.19
		ATP + atractyloside	0.58	0.35
3	25	KCl medium + ADP	0.77	1.60
		KCl medium + ADP + bongkrekic acid	0.80	1.16
4	25	ADP (0.075 mM)*	0.29	0.43
		ADP (0.3 mM)*	0.53	0.76
		ADP (1.2 mM)*	1.05	1.09

* No antimycin present.

of animal mitochondria[14]) did not substantially increase either the rate of uptake or the size of the exchangeable endogenous pool. The internal pool, calculated from the isotopic equilibrium, did however appear to be related to the exogenous concentration of adenine nucleotides. This contrasts with animal mitochondria, where the size of the inner pool is independent of the concentration of adenine nucleotides outside the mitochondria (Souverijn, J. H. M., unpublished).

DISCUSSION

The absence of inhibition by atractyloside or bongkrekic acid and the extremely slow and non-specific nature of the uptake of nucleotides suggests that the uptake measured probably represents a non-specific binding of nucleotides to the membrane. Atractyloside, even when added at concentrations of 0.8 mM, does not inhibit adenine nucleotide-dependent reactions in artichoke mitochondria, indicating the absence of a translocator system similar to that described for animal mitochondria.

The strong inhibition of State-3 respiration in intact artichoke mitochondria by bongkrekic acid clearly distinguishes its mode of action from that of atractyloside. Its failure to inhibit the ATPase of submitochondrial particles is probably not simply due to a loss of coenzyme A (apparently essential for bongkrekic acid inhibition[15]), since addition of coenzyme A and glutathione to submitochondrial particles in the presence of bongkrekic acid did not cause any inhibition of the ATPase activity.

It is suggested therefore that either: (a) there is no adenine nucleotide translocator in artichoke mitochondria—and hence atractyloside does not inhibit because there is no binding site (in this case bongkrekic acid may inhibit by a reaction elsewhere in the mitochondria), or (b) there is an active exchange of an extremely small endogenous pool of nucleotides that is too low to be detected by the methods used. Such an active exchange may be facilitated by a translocator that is sensitive to bongkrekic acid but not to atractyloside, the difference in sensitivity possibly relating to a difference in the affinity of these inhibitors for the translocator.

In agreement with the present results, and in order to account for the unusual ATPase properties of artichoke mitochondria[16], it is further proposed that either: (a) in the absence of a translocator the membrane is freely permeable to adenine nucleotides, and the specificity is imposed by the ATPase or (b) in the presence of at translocator the active exchange of an immeasurably small endogenous nucleotide pool must be extremely rapid and specific for

$$ADP_{out} \rightarrow ADP_{in}$$

$$ATP_{out} \leftarrow ATP_{in}$$

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