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BINDING OF RADIOACTIVELY LABELED CARBOXYATRACTYLOSIDE, ATRACTYLOSIDE AND BONGKREKIC ACID TO THE ADP TRANSLOCATOR OF POTATO MITOCHONDRIA

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SUMMARY

1. The inhibition of the ADP-stimulated respiration of potato mitochondria by carboxyatractyloside is relieved by high concentration of ADP or by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Atractyloside is a much less potent inhibitor than carboxyatractyloside. The inhibition of the ADP-stimulated respiration required about 60-times more atractyloside than carboxyatractyloside.

2. [^{35}S]carboxyatractyloside and [^{3}H]bongkrekic acid bind to potato mitochondria with high affinity (K_d = 10 to 20 nM, n = 0.6–0.7 nmol per mg protein). Added ADP competes with carboxyatractyloside for binding; on the contrary ADP increases the amount of bound bongkrekic acid. [^{3}H]atractyloside binds to potato mitochondria with a much lower affinity (K_d = 0.45 μM) than carboxyatractyloside or bongkrekic acid.

3. Bound [^{3}H]atractyloside is displaced by ADP, carboxyatractyloside and bongkrekic acid. The displacement of bound [^{35}S]carboxyatractyloside by bongkrekic acid and of bound [^{3}H]bongkrekic acid by carboxyatractyloside is markedly increased by ADP.

4. Bongkrekic acid competes with [^{35}S]carboxyatractyloside for binding. Addition of a small concentration of ADP considerably enhances the inhibitory effect of bongkrekic acid on [^{35}S]carboxyatractyloside binding.

5. The adenine nucleotide content of potato mitochondria is of the order of 1 nmol per mg protein. ADP transport in potato mitochondria is inhibited by atractyloside 30- to 40-times less efficiently than by carboxyatractyloside.

INTRODUCTION

Adenine nucleotide translocation has been extensively studied in animal mitochondria by the use of specific inhibitors such as atractyloside, carboxyatractyl-

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

oside called also gummiferin, and bongkrekic acid (cf. ref. 1 for review). In contrast, there are very few data on adenine nucleotide transport in plant mitochondria. Earlier studies by Passam et al. [2–4] had demonstrated that bongkrekic acid inhibits state-3 respiration of Jerusalem artichoke mitochondria in a way similar to that described for rat liver mitochondria, while atractyloside [2–4] and carboxyatractyloside [3] are ineffective at concentrations which are inhibitory for rat liver mitochondria. However, as reported by Jung and Hanson [5], cauliflower and corn mitochondria appear to possess an atractyloside-sensitive adenine nucleotide translocator.

The results presented here demonstrate the occurrence of an ADP/ATP carrier in potato mitochondria and report for the first time binding studies of radioactive [^{3}H]atractyloside, [^{35}S]carboxyatractyloside and [^{3}H]bongkrekic acid to plant mitochondria. A preliminary account of these data has been presented orally [6].

METHODS

Mitochondria from potato tubers were isolated as described previously [7] using a Moulinex mixer 66 (Alençon, France) for tissue disruption. The yield of mitochondria was about 50 mg protein/kg of fresh tissue.

Oxygen uptake was measured at 25 °C with GME oxygraph equipped with a Clark electrode (Yellow Springs, Ohio, U.S.A.). The incubation medium used in respiration experiments contained 0.8 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, pH 7.2, and 1 g/l bovine serum albumin (mannitol medium).

The incubation medium used for binding assays contained 0.12 M KCl, 10 mM morpholinoethane sulfonic acid (MES), pH 6.5, 0.1 mM EDTA (standard binding medium). Binding assays with the labeled inhibitors, [^{3}H]atractyloside [8], [^{35}S]carboxyatractyloside [9] and [^{3}H]bongkrekic acid [10, 11], were carried out in series of tubes containing 5 ml of the standard medium and increasing concentrations of the labeled inhibitor. The incubation was started by addition of mitochondria. After an incubation period of 3 min at 20 °C and then 30 min at 2 °C, the mitochondria were collected by centrifugation and their radioactivity estimated by liquid scintillation following digestion of the pellet by formamide [12]. The incubation conditions were sufficient to allow equilibrium between bound and free ligands.

The incubation medium for measuring ADP transport was the same as that used for binding assays. [^{14}C]ADP transport was measured by direct exchange [13]; the exchange was stopped by adding 10 μM carboxyatractyloside followed by rapide centrifugation as described previously [12]. The adenine nucleotides contained in the mitochondrial pellet were extracted by perchloric acid and assayed enzymatically [14] in the neutralized perchloric extracts.

RESULTS

Effect of atractyloside, carboxyatractyloside and bongkrekic acid on the ADP-stimulated respiration

Polarographic traces presented in Fig. 1 show that, in mannitol medium, succinate is oxidized with a respiratory control of 5. Carboxyatractyloside at a final concentration of 0.5 μM totally inhibits the stimulation of respiration by 160 μM ADP. Inhibition of respiration is relieved by the uncoupler FCCP (Trace A) or by a

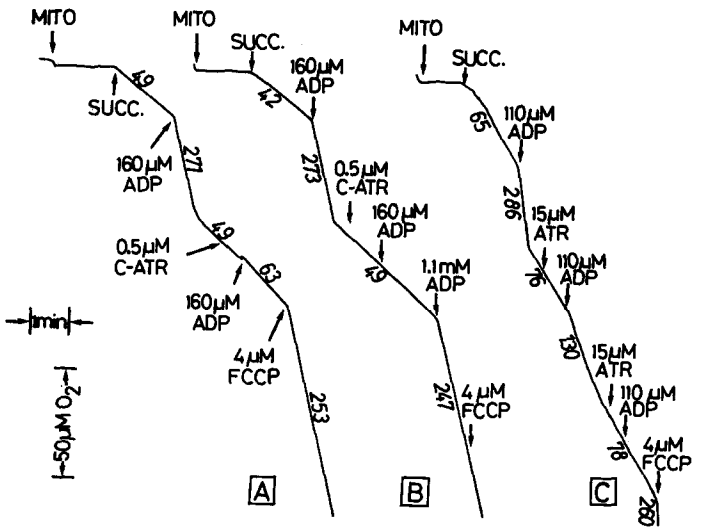

Fig. 1. Effect of atractyloside (ATR) and carboxyatractyloside (C-ATR) on the ADP-stimulated respiration of potato mitochondria. The incubation medium (see Methods) contained 0.15 mM ATP and 10 mM succinate. The volume was 2 ml and the temperature 25 °C. The incubation was started by the addition of mitochondria (0.6 mg protein for A and B and 1.0 mg protein for C). The numbers on the traces correspond to the rates of O_2 uptake in nmol/min per mg protein.

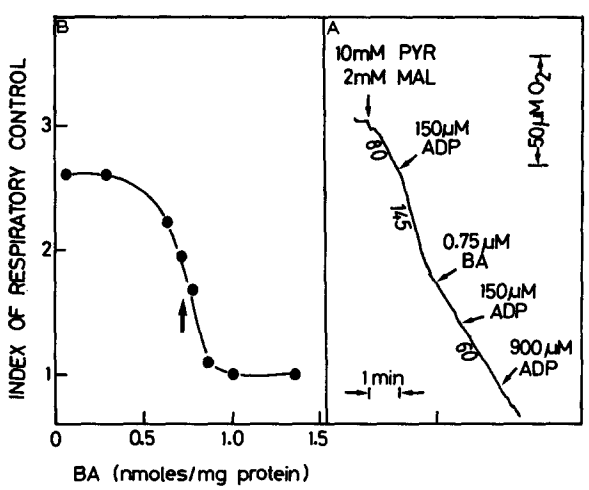

Fig. 2. Effect of bongkrekic acid (BA) on the ADP-stimulated respiration of potato mitochondria. The incubation conditions were the same as those described in Methods except that succinate was replaced by pyruvate (pyr) plus malate (mal), bovine serum albumin was omitted, 150 μM thiamine pyrophosphate was added and the pH of the incubation medium was 6.5. The incubation was started by the addition of mitochondria (1.3 mg protein). (A) Oxygraphic trace of the inhibition of respiration by bongkrekic acid. (B) Titration curve of the effect of bongkrekic acid on the ADP-stimulated respiration.

high concentration of ADP (1.1 mM) (Trace B). Trace C shows that the concentration of atractyloside must be raised to 30 μM to completely inhibit the respiration stimulated by 110 μM ADP, which clearly indicates that the inhibitory effect of atractyloside is much less than that of carboxyatractyloside. The same results were obtained when malate, malate and pyruvate, NADH or glutamate were used as oxidizable substrates.

To test the inhibitory effect of bongkrekic acid, a medium free of serum albumin was used in order to avoid the trapping of bongkrekic acid by serum albumin. Data shown in Fig. 2 indicate that bongkrekic acid is also a potent inhibitor of the ADP-stimulated respiration. Half inhibition is obtained with 0.7 nmol of bongkrekic acid per mg protein. Inhibition of the ADP-stimulated respiration by bongkrekic acid is not relieved by increasing concentrations of ADP. The binding studies presented below will show that this is due to an increase of the binding of bongkrekic acid to mitochondria in the presence of ADP.

These data indicate indirectly that carboxyatractyloside and bongkrekic acid, but not atractyloside, are potent inhibitors of ADP transport in potato mitochondria.

Binding of [^3H]atractyloside, [^{35}S]carboxyatractyloside and [^3H]bongkrekic acid

As shown in Fig. 3 and 4, carboxyatractyloside and bongkrekic acid are able to bind to potato mitochondria with high affinity (K_d from 10 to 20 nM). In both cases the total amount of high affinity binding sites is of the order of 0.6–0.7 nmol per mg protein. However the effect of ADP is very different in the two cases. ADP at high concentrations was able to compete with carboxyatractyloside for binding while, on the contrary, it increased the binding of bongkrekic acid to mitochondria. In the

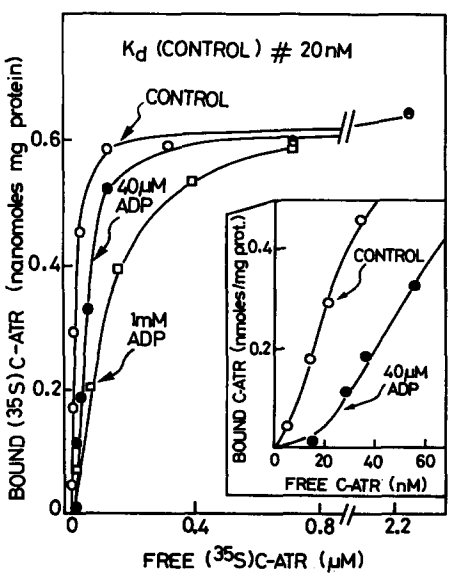

Fig. 3. Effect of ADP on binding of [^{35}S]carboxyatractyloside ((^{35}S)C-ATR) to potato mitochondria. Conditions are as described in Methods. When present, ADP was in the incubation medium with [^{35}S]carboxyatractyloside at the concentrations indicated on the figure. The amount of mitochondrial protein was 1.4 mg.

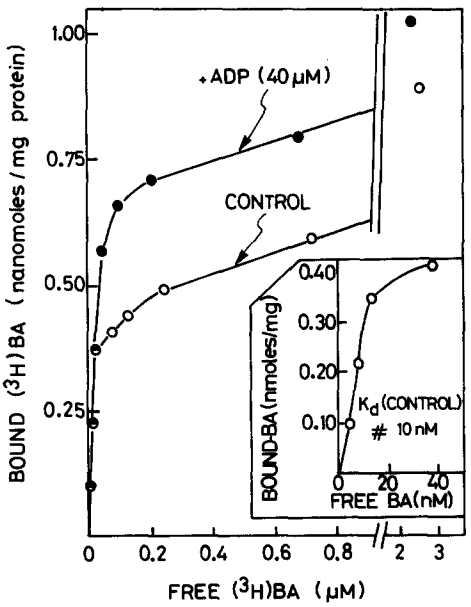

Fig. 4. Effect of ADP on binding of [^{3}H]bongkrekic acid ((^{3}H)BA) to potato mitochondria. Same conditions as in Fig. 3. The amount of mitochondrial protein was 1.4 mg.

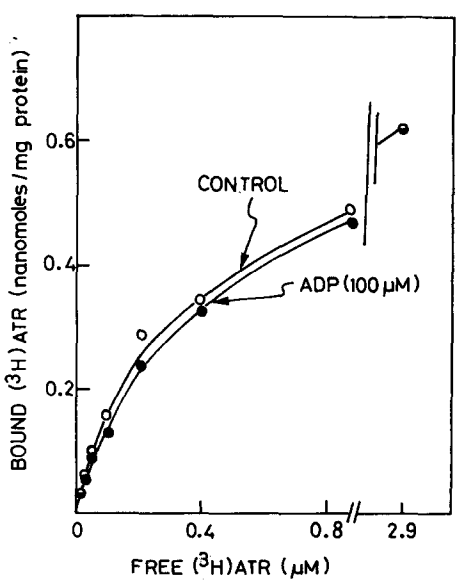

Fig. 5. Effect of ADP on binding of [^{3}H]atractyloside ((^{3}H)ATR) to potato mitochondria. Same conditions as in Fig. 3. The amount of mitochondrial protein was 2 mg.

absence of ADP, the maximal binding capacity for bongkrekic acid is not completely elicited although equilibrium conditions are satisfied. Addition of ADP increases the amount of high affinity sites for bongkrekic acid to a maximal value of 0.6–0.7 nmol/mg protein which is similar to that obtained for carboxyatractyloside binding. This is a characteristic feature of bongkrekic acid binding already observed with heart mitochondria [10, 11].

In contrast to bongkrekic acid and carboxyatractyloside, atractyloside binds to potato mitochondria with low affinity (Fig. 5). Assuming the same number of sites for atractyloside as for carboxyatractyloside [12], i.e. 0.6–0.7 nmol/mg protein, the K_d for atractyloside binding is approx. 0.45 μM, a value 20- to 50-times higher than that found for carboxyatractyloside and bongkrekic acid binding. The K_d values for [^{35}S]carboxyatractyloside and [^{3}H]atractyloside binding (Fig. 3 and 5), determined in the absence of ADP, is much lower than the amount of inhibitor necessary to bring 50 % inhibition of the ADP transport (see below Table III);

TABLE I

RELEASE BY SPECIFIC LIGANDS OF BOUND [^{35}S]CARBOXYATRACTYLOSIDE, [^{3}H]-ATRACTYLOSIDE AND [^{3}H]BONGKREKIC ACID FROM POTATO MITOCHONDRIA

Potato mitochondria were loaded with [^{3}H]atractyloside or [^{35}S]carboxyatractyloside or [^{3}H]-bongkrekic acid as follows: mitochondria (20 mg protein) were incubated in 8 ml of the same medium as that used for binding assays with either [^{3}H]atractyloside (40 nmol) or [^{35}S]carboxyatractyloside (40 nmol) or [^{3}H]bongkrekic acid (40 nmol) for 5 min at 20 °C. These conditions allow complete equilibration between bound and free ligands. Then the mitochondria were sedimented by centrifugation, resuspended in the same standard binding medium and used for the displacement assays. Displacement of bound [^{3}H]atractyloside or[^{35}S]carboxyatractyloside or [^{3}H]bongkrekic acid by ADP, atractyloside, carboxyatractyloside or bongkrekic acid was tested as follows. The mitochondria (0.6 mg protein) loaded with [^{3}H]atractyloside or [^{35}S]carboxylatractyloside or [^{3}H]bongkrekic acid were incubated for 3 min at 20 °C and then 30 min at 0 °C in 5 ml of standard binding medium, pH 6.5, containing either 100 μM ADP, 10 μM atractyloside, 10 μM carboxyatractyloside or 10 μM bongkrekic acid as indicated. The incubation was stopped by centrifugation and the radioactivity of the pellet counted by scintillation.

Addition	[^{3}H]Atractyloside		[^{35}S]Carboxy-atractyloside		[^{3}H]Bongkrekic acid	
	Remaining bound (pmol/mg protein)	Released (%)	Remaining bound (pmol/mg protein)	Released (%)	Remaining bound (pmol/mg protein)	Released (%)
None	160	–	620	–	470	–
ADP	40	75	570	8	510*	–
Atractyloside	20	87	550	11	410	13
Carboxy-atractyloside	16	90	300	52	290	38
Bongkrekic acid	40	75	570	8	165	65
Carboxy-atractyloside+ ADP	15	91	150	76	260	45
Bongkrekic acid +ADP	16	90	250	60	160	66

* Increase of binding.

TABLE II

COMPETITION BETWEEN [^{35}S]CARBOXYATRACTYLOSIDE, BONGKREKIC ACID AND ADP FOR BINDING TO POTATO MITOCHONDRIA

Potato mitochondria (2 mg protein) were incubated as described in Methods with 0.8 μM [^{35}S]-carboxyatractyloside and, as indicated, with 1 μM bongkrekic acid or/and 20 μM ADP in 10 ml of standard binding medium, pH 6.5. The incubation was ended by centrifugation and the radioactivity of the pellet counted by scintillation.

Additions	Bound [^{35}S]carboxyatractyloside (pmol/mg protein)
[^{35}S]carboxyatractyloside	660
[^{35}S]carboxyatractyloside plus bongkrekic acid	570
[^{35}S]carboxyatractyloside plus ADP	650
[^{35}S]carboxyatractyloside plus ADP plus bongkrekic acid	70

this is due to the fact that atractyloside and carboxyatractyloside compete with ADP for binding to the ADP transport system.

To demonstrate that atractyloside interacts with other inhibitors for binding to the ADP translocator, we have tested the effect of ADP, atractyloside, carboxyatractyloside and bongkrekic acid on the release of bound [^{3}H]atractyloside, [^{35}S]-carboxyatractyloside and [^{3}H]bongkrekic acid (Table I). The fact that bound [^{3}H]-atractyloside is efficiently removed by ADP, atractyloside, carboxyatractyloside and bongkrekic acid indicates that, in spite of its low affinity, atractyloside binds specifically to the ADP translocator. Bound [^{35}S]carboxyatractyloside and bound [^{3}H]-bongkrekic acid are also displaced from their sites by carboxyatractyloside and bongkrekic acid, which is also consistent with their specific binding to the ADP translocator. Addition of ADP increases the displacement of bound [^{35}S]carboxyatractyloside by bongkrekic acid and of bound [^{3}H]bongkrekic acid by carboxyatractyloside, an effect previously observed for rat liver mitochondria [12]. Atractyloside, at a concentration of 10 μM, displaces between 10 and 15 % of bound [^{35}S]-carboxyatractyloside or bound [^{3}H]bongkrekic acid. A further displacement of less than 5 % was obtained by doubling the concentration of atractyloside (not shown). This is in agreement with the fact that atractyloside binds to potato mitochondria with a much lower affinity than carboxyatractyloside or bongkrekic acid (see above).

As shown in Table II, bongkrekic acid competes with [^{35}S]carboxyatractyloside for binding to potato mitochondria. Addition of a small concentration of ADP (20 μM), (which on its own is hardly effective on carboxyatractyloside binding) together with bongkrekic acid considerably enhances the competitive interaction between bongkrekic acid and [^{35}S]carboxyatractyloside. This synergistic effect of ADP and bongkrekic acid for preventing [^{35}S]carboxyatractyloside fixation has already been observed with rat liver mitochondria [12].

Effect of atractyloside and carboxyatractyloside on ADP transport

The adenine nucleotide content of potato mitochondria is between 1 and 2 nmol/mg protein, 50 per cent being AMP and the remaining being ADP plus ATP. A small pool of adenine nucleotides of about the same size was also found in Jerusalem artichoke mitochondria [2, 3] in contrast with the much higher values reported for

TABLE III

EFFECT OF ATRACTYLOSIDE AND CARBOXYATRACTYLOSIDE ON THE RATE OF ADP TRANSPORT IN POTATO MITOCHONDRIA

Potato mitochondria (0.9 mg protein) were preincubated in 5 ml of the mannitol medium, pH 7.2, in the absence or presence of atractyloside, as indicated, 3 min at 20 °C and 7 min at 0 °C. Then [^{14}C]ADP in 100 μl was added to a final concentration of 10 μM. The incubation with [^{14}C]ADP lasted for 10 s at 0 °C and was stopped by addition of 10 μM carboxyatractyloside followed by rapid centrifugation. The amount of [^{14}C]ADP incorporated was calculated from the amount of [^{14}C]-ADP present in the pellet after correction of the [^{14}C]ADP in the sucrose space, as described in ref. 12. The exchange refers to internal ADP+ATP.

Inhibitor	Exchange (percent of maximum)	Inhibition (percent)
None	24	–
Atractyloside (1 μM)	23	4
Atractyloside (6 μM)	16	33
Atractyloside (12 μM)	12	50
Atractyloside (30 μM)	2	91
Carboxyatractyloside (0.2 μM)	14	42
Carboxyatractyloside (0.6 μM)	4	83

mammalian mitochondria [13]. Although the kinetics of transport was not easy to resolve, due to the very low amount of internal ADP plus ATP, data in Table III show that the inhibitory effect of atractyloside on ADP transport is much less than that of carboxyatractyloside. The ratio of efficiency between the two inhibitors was between 30 and 40, a value close to the ratio of the K_d values calculated from binding experiments (see above). The inhibitory effect of bongkrekic acid on ADP transport in potato mitochondria was difficult to assess, because bongkrekic acid addition results in an extra binding of external [^{14}C]ADP to carrier sites. The extra binding of ADP, which is dependent on bongkrekic acid, has already been reported for heart mitochondria [15, 16].

DISCUSSION

The adenine nucleotide transport system in plant mitochondria differs from that of mammalian mitochondria by several features which include: (1) the small size of the internal pool of adenine nucleotides in plant mitochondria, which is 5- to 10-times less than that of mammalian mitochondria, (2) the low sensitivity to atractyloside, as compared to the high sensitivity of mammalian mitochondria to atractyloside, (3) the competitive inhibitory effect of carboxyatractyloside on the ADP transport as compared to the apparent non-competitive effect of carboxyatractyloside in mammalian mitochondria, in spite of a similar K_d value for the binding of [^{35}S]-carboxyatractyloside to both types of mitochondria.

Although contradictory results concerning the inhibitory effects of atractyloside on ADP transport in plant mitochondria have appeared in the literature, a careful scrutiny of previously published results reveals that contradictions are only apparent and may arise from the material and the assays used. In particular it must be kept in mind that any alteration of the mitochondrial membrane may result in a

loss of the binding properties of the ADP translocator.

A quantitative assessment of the difference of efficiency between atractyloside, carboxyatractyloside and bongkrekic acid on the ADP transport system of potato mitochondria is presented in this paper. Binding data obtained with labeled inhibitors are in good agreement with data on the effects of these inhibitors on the rate of ADP transport. In particular, the fact that the inhibitory efficiency of atractyloside on ADP transport is 30- to 40-times less than that of carboxyatractyloside is consistent with the fact that the binding affinity of atractyloside is 30- to 50-times less than that of carboxyatractyloside.

In short, the adenine nucleotide translocator in potato mitochondria is highly sensitive to bongkrekic acid and to carboxyatractyloside, but much less sensitive to atractyloside. Furthermore, whereas the inhibition of ADP transport by carboxyatractyloside is apparently not relieved by adding ADP in mammalian mitochondria [12], it is easily relieved by adding ADP in potato mitochondria. The additional binding of bongkrekic acid to potato mitochondria induced by the presence of ADP has also been observed in heart mitochondria [10, 11, 15] and yeast mitochondria (unpublished results), and is ascribed to an increased affinity of bongkrekic acid in the presence of ADP [11, 15]. It is concluded that the binding properties of the adenine-nucleotide translocator in plant and mammalian mitochondria are similar with respect to bongkrekic acid but somewhat different with respect to atractyloside and carboxyatractyloside.

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