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ATP/ADP antiporter is involved in uncoupling of plant mitochondria induced by low concentrations of palmitate

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Abstract

Carboxyatractyloside partially restored the transmembrane electrical potential difference ($\Delta\Psi$) dissipated by low concentrations of palmitate in pea stem mitochondria. This effect was more marked when mitochondria from sunflower were assayed. It is suggested that the ATP/ADP translocator is involved in the free fatty acid-induced uncoupling of oxidative phosphorylation in plant mitochondria, only when its level is sufficiently high and the concentration of the fatty acid is low to collapse only partially $\Delta\Psi$.

Key words: Fatty acid; Mitochondrion; ATP/ADP antiporter; Uncoupling; Plant species

1. Introduction

Although the uncoupling effect of free fatty acids (FFA) on oxidative phosphorylation of animal mitochondria has been demonstrated for many years [1], the mechanism by which it occurs is still controversial [2].

In a previous paper, it was found that FFA stimulate O_2 consumption, collapse the transmembrane electrical potential difference ($\Delta\Psi$) of intact pea mitochondria and dissipate the proton gradient of submitochondrial particles [3]. The effects of FFA are not reversed, neither by carboxyatractyloside (CAtr), nor by ADP. Therefore, it has been suggested that FFA act as classical uncouplers in plant mitochondria [4], rather than through the adenine nucleotide translocator [5]. However, the lack of effect of CAtr and ADP could depend on the low content of ATP/ADP antiporter in pea mitochondria. Indeed, its level, at least in the animal mitochondria, varies from one source to another and is correlated to the ability of CAtr and ADP to inhibit FFA-stimulated O_2 uptake [6].

In the present work, it is shown that in plant mitochondria the ATP/ADP antiporter is involved in FFA-induced uncoupling only in the presence of low concentrations of FFA, and it is suggested that the uncoupling effect is dependent on the content of ATP/ADP translocator.

2. Materials and methods

2.1. Mitochondria preparation

Crude mitochondria from etiolated pea (*Pisum sativum* L., cv. Alaska) stems and sunflower (*Helianthus annuus* L., cv. Odille, Pioneer) hypocotyls were isolated as previously described [3]. The protein content of the mitochondrial stock suspension was determined by the biuret method [7]. Functional integrity was determined by measuring the respiratory control ratio with 150 μM ADP and 1 mM NADH as substrates. Only mitochondria with a respiratory control ratio greater than 2.5–3 were used. The integrity of the external mitochondrial membrane was assayed by the antimycin A-sensitive NADH-cytochrome *c* oxidoreductase activity. In both intact and osmotically burst mitochondria, NADH (1 mM)-dependent cytochrome *c* reduction was followed at 550 nm by a Perkin–Elmer λ 15 spectrophotometer. On the basis of this criterion, the percentage of mitochondrial membrane integrity was ca. 85–90%. Crude sunflower and pea mitochondria were also purified on a discontinuous gradient formed by three layers (bottom to top) of 45, 21 and 13.5% (v/v) of Percoll in 0.5 M sucrose, 0.2% BSA, and 20 mM MOPS-KOH (pH 7.2). The gradient was centrifuged at 20,000 × *g* for 40 min in a swinging bucket rotor (Sorvall HB-4) and the mitochondria collected at the 21/45% interface. This purification did not greatly increase the respiratory control ratio, or the percentage of mitochondrial membrane integrity. Therefore, crude mitochondria were routinely assayed and purified sunflower mitochondria were used only to confirm some results.

2.2. Oxygen uptake assay

Oxygen uptake by mitochondria was detected by a Clark-type oxygen electrode (YSI, model 5331), at 28°C. The incubation medium was 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, 5 mM Na/K phosphate, 0.5 μg/ml oligomycin, 0.01 mg/ml BSA, 1 mM NADH and 100 μl of mitochondria (ca. 1 mg protein) in a final volume of 2 ml. The incubation medium was supplemented either with 5 mM $MgCl_2$ (in experiments with hexokinase, or with 0.5 mM EGTA, 1.5 mM $MgCl_2$, 5 mM glutamate and 5 mM malate (in experiments with palmitic acid).

2.3. $\Delta\Psi$ assay

The $\Delta\Psi$ was estimated as fluorescence changes of safranin O [4]. The incubation medium was as in oxygen uptake experiments and was supplemented with 1 mM $MgCl_2$, 0.5 mM EGTA and 5 μM safranin O. The reactions were started by 1 mM NADH (pea mitochondria), or 5 mM glutamate, plus 5 mM malate, plus 1 mM NADH (sunflower mitochondria) and proceeded at 28°C.

2.4. Determination of ATP/ADP translocator level

The amount of ATP/ADP translocator was evaluated from a titration curve obtained by the step-wise addition of CAtr to respiring pea mitochondria [6]. The medium, without oligomycin, was as above (oxygen uptake experiments) plus 10 mM glucose, 1 mM ATP and 9.38 IU/ml hexokinase in a final volume of 2 ml. Oxygen uptake was started by 1 mM NADH and the reaction proceeded at 28°C. Another type of titration was also performed by step-wise addition of CAtr to pea mitochondria in which the NADH-dependent $\Delta\Psi$ was collapsed by the addition of 1 mM glucose, plus 9.38 IU/ml hexokinase and 1 mM ATP.

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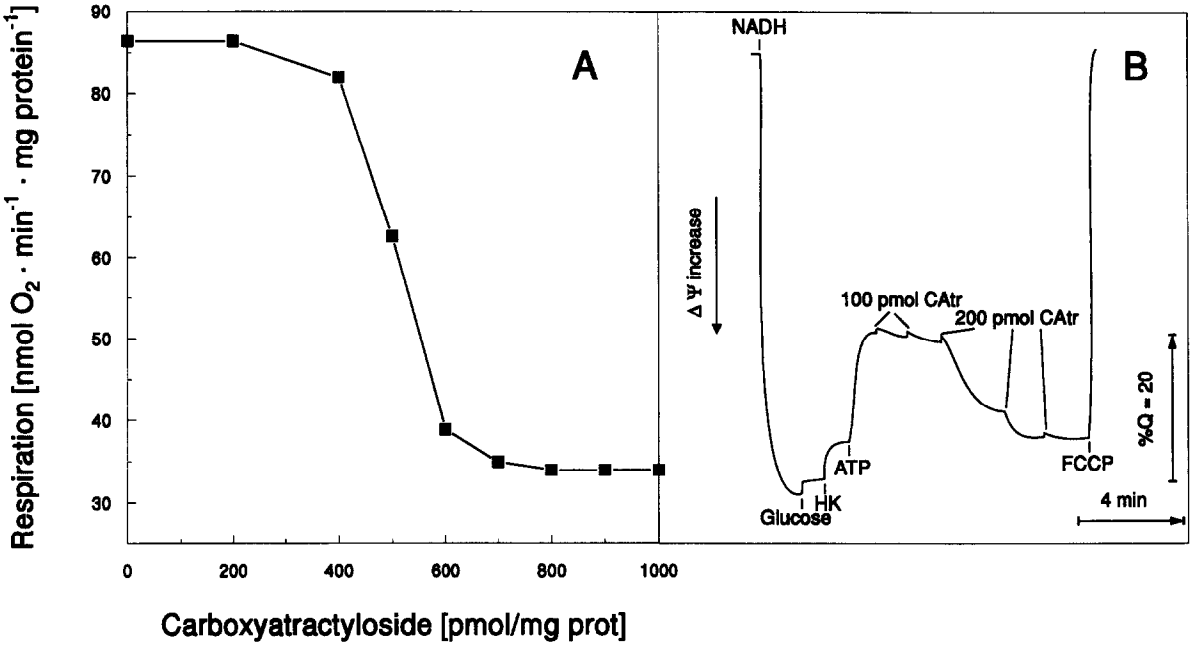

Fig. 1. Evaluation of ATP/ADP antiporter concentration in pea stem mitochondria. Inhibition of NADH-dependent oxygen consumption (Panel A), or restoration of $\Delta\Psi$ monitored by per cent of safranin O fluorescence quenching, %Q (Panel B), as a function of increasing amounts of CAtr. Additions: 1 mM NADH, 2 μM FCCP, 1 mM glucose, 9.38 IU/ml hexokinase (HK), 1 mM ATP.

3. Results

The ATP/ADP translocator content of pea stem mitochondria was estimated by the inhibitory effect of increasing concentrations of CAtr on NADH-dependent O_2 uptake (Fig. 1, panel A). The mitochondria had an amount of ATP/ADP exchanger (ca. 650 pmol/mg prot.), comparable to that recovered in rat kidney mitochondria [6]. To confirm this finding, its level was also evaluated by another titration assay, using safranin O. As can be seen (Fig. 1, panel B), a comparable value of ATP/ADP translocator (ca. 600 pmol/mg prot.) was determined.

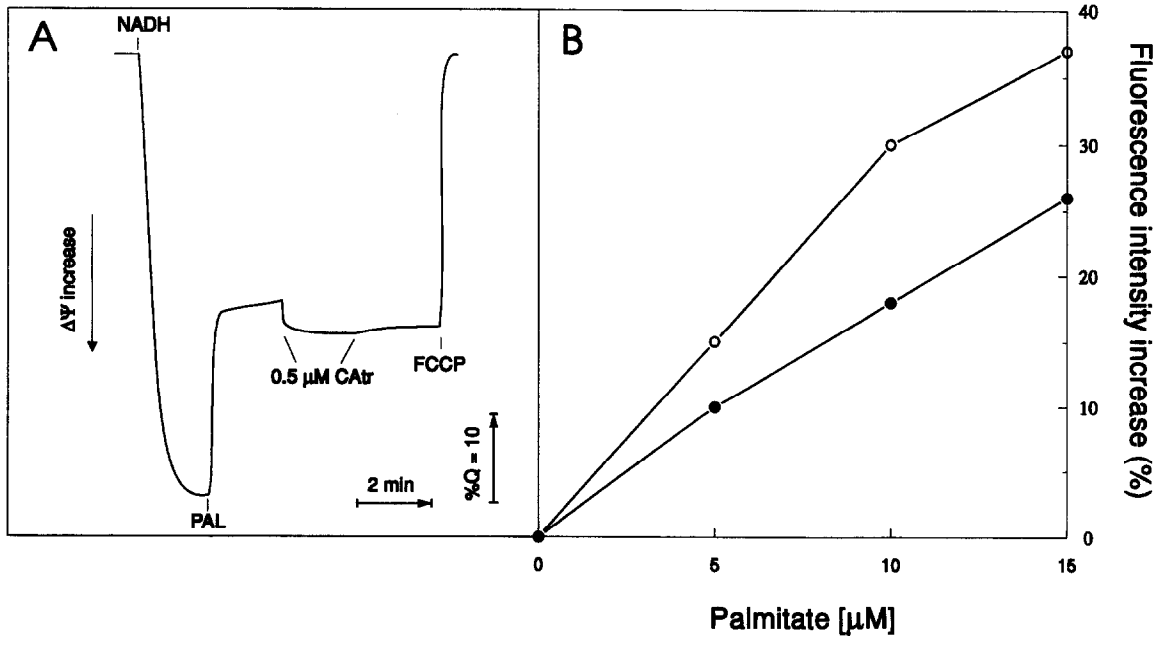

Fig. 2. Effect of CAtr on palmitate-dissipated $\Delta\Psi$ in pea stem mitochondria. Panel A: effect of increasing concentrations of CAtr on palmitate-induced decrease in $\Delta\Psi$ (per cent safranin O fluorescence quenching, %Q). Additions: 10 μM palmitate, 1 mM NADH, 2 μM FCCP. Panel B: relationship between palmitate concentration and dissipation of the $\Delta\Psi$ in the absence (○) or presence (●) of 0.5 μM CAtr.

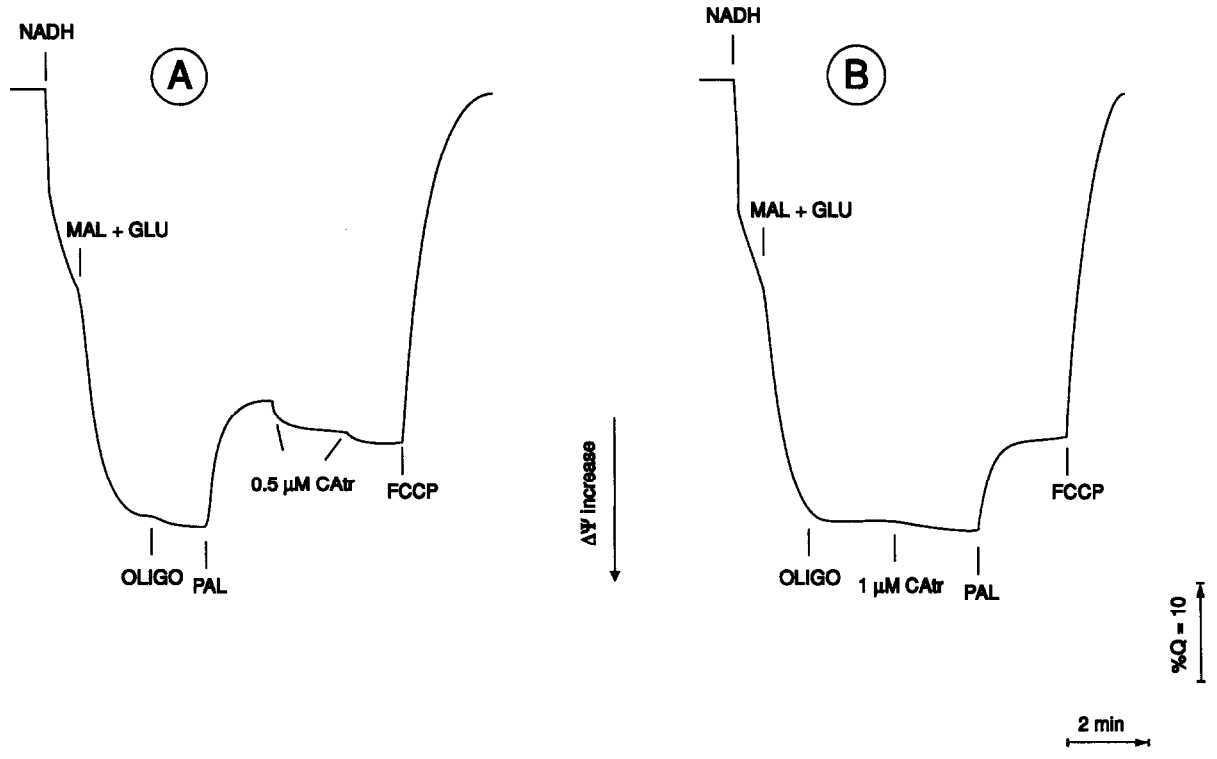

Fig. 3. Effect of CAtr on palmitate-dissipated $\Delta\Psi$ (per cent safranin O fluorescence quenching, %Q) in sunflower hypocotyl mitochondria. Additions: 1 mM NADH, 5 mM malate (MAL), 5 mM glutamate (GLU), 4 μM palmitate (PAL), 2 μg/ml oligomycin (OLIGO), 2 μM FCCP.

When pea stem mitochondria were uncoupled by a very low palmitate concentration (10 μM), a slight stimulation of oxygen consumption (malate plus glutamate served as oxidation substrates), which was inhibited by 1 μM CAtr, was observed (results not shown). A clearer response was obtained assaying the effect of CAtr on palmitate-dissipated $\Delta\Psi$. Fig. 2, panel A, shows that the addition of NADH to pea mitochondria caused the formation of a $\Delta\Psi$ that was partially collapsed by 10 μM palmitate. The subsequent addition of 0.5 μM CAtr induced only a partial restoration of the potential, that did not increase by a further addition of CAtr. The complete relationship between palmitate concentration and the $\Delta\Psi$ decrease is shown in Fig. 2, panel B. The increase in palmitate concentration up to 15 μM, was accompanied by an almost linear increase in the dissipation of the $\Delta\Psi$ that was partially inhibited by CAtr.

A similar picture was obtained by using sunflower mitochondria (Fig. 3). The addition of substrate (malate plus glutamate plus NADH) induced the formation of an $\Delta\Psi$ that was decreased by 4 μM palmitate. Carboxyatractyloside (0.5 μM) partially restored the $\Delta\Psi$ and, conversely from pea mitochondria, subsequent additions of CAtr further induced increases in the $\Delta\Psi$ (trace A). CAtr (1 μM), added before palmitate, lowered the dissipation of $\Delta\Psi$ caused by this fatty acid (trace B). This suggests that sunflower mitochondria could contain a higher level of ATP/ADP translocator than pea mitochondria. However, in these mitochondria the evaluation of the content of ATP/ADP translocator was hindered, because of the impossibility to use both titration methods.

4. Discussion

Fatty acids are known to differ from classical uncouplers. A new concept of uncoupling, termed 'decoupling', has been proposed to rationalize oleate and palmitate-induced uncoupling in rat liver mitochondria, without a significant decrease in proton electrochemical gradient [8]. The oleate-induced uncoupling of animal mitochondria may also depend on the ability of FFA to act only as protonophores [9] or, by a mixed mechanism, as protonophores as well as intrinsic uncouplers of redox proton pumps [10,11]. Finally, the involvement of proteins of the inner mitochondrial membrane to induce uncoupling has been suggested [5]. These are the ATP/ADP antiporter and thermogenin in brown fat, which have very similar aminoacid sequences and domain structures [12]. Part of the controversy has been resolved in the light that a threshold membrane potential of about 125 mV is necessary for fatty acid-induced permeability [13].

In agreement to what was found with animal mitochondria [14,15], the results presented in this paper demonstrate that also in the plant ones the adenine nucleotide translocator may be involved in FFA-induced uncoupling, when the concentration of FFA is suffi-

ciently low and FFA collapse the $\Delta\Psi$ only partially. In these circumstances, hence, palmitate-induced uncoupling of oxidative phosphorylation may in part be explained by the involvement of ATP/ADP antiporter in FFA-induced uncoupling [5]. The lack of effect of CAtr on the palmitate-dissipated $\Delta\Psi$ and on the palmitate-stimulated oxygen uptake by pea stem mitochondria [3,4], can now be explained by both the low content of ATP/ADP translocator present in these mitochondria and by the higher concentration of palmitate used which completely collapsed $\Delta\Psi$.

In animal mitochondria CAtr is a noncompetitive inhibitor [16], and this renders it useful to estimate the ATP/ADP translocator by titration experiments [6]. Conversely, in plant mitochondria CAtr may act as a competitive inhibitor [17]. Nevertheless, it may be used to approximately evaluate the amount of ATP/ADP antiporter in pea mitochondria, when control experiments in the presence of different ADP concentrations are performed.

Heat production in plants is commonly linked to cyanide-insensitive respiration (alternative pathway), which does not result in ATP synthesis [18], and can represent a physiological response to low temperatures [19,20]. Practically all the plant mitochondria isolated so far show a residual cyanide-insensitive respiration [18], although there are plant cultivars lacking the alternative path, thus suggesting that it is not essential for metabolism [21]. Sunflower hypocotyl mitochondria are almost completely cyanide-sensitive. Nevertheless, this plant tolerates cold during germination more than pea. Therefore, it is suggested that sunflower hypocotyl mitochondria have a high level of ATP/ADP carrier to allow a transient uncoupling of mitochondria for heat generation. This would facilitate seed germination and a rapid hypocotyl development at low environmental temperatures. Free fatty acids, released from cotyledon triacylglycerols by lipases [22], may be used as substrates, but they can also uncouple mitochondria. This mechanism could, hence, be similar to cold-induced urgent heat production in warm-blooded animals [2].

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