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Carboxyatractylate inhibits the uncoupling effect of free fatty acids

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The ATP/ADP-antiporter inhibitors and ADP decrease the palmitate-induced stimulation of the mitochondrial respiration in the controlled state. The degree of inhibition decreases in the order: carboxyatractylate > bongkrekic acid, palmitoyl-CoA, ADP > atractylate. GDP is ineffective. The inhibiting concentration of carboxyatractylate coincides with this arresting the state 3 respiration. Carboxyatractylate inhibition decreases when the palmitate concentration increases. Stimulation of controlled respiration by FCCP or gramicidin D at any concentration of these uncouplers is carboxyatractylate-resistant, whereas that by low concentrations of DNP is partially suppressed by carboxyatractylate. These data together with observations that palmitate does not increase H^+ conductance in bilayer phospholipid membranes and in cytochrome oxidase-asolectin proteoliposomes indicate that the ATP/ADP-antiporter is somehow involved in the uncoupling by low concentrations of fatty acids (or DNP), whereas that by FCCP and gramicidin D is due to their effect on the phospholipid bilayer. It is suggested that the antiporter facilitates translocation of palmitate anion across the mitochondrial membrane.

Fatty acid; Uncoupler; Mitochondria; ATP/ADP antiporter; Carboxyatractylate

1. INTRODUCTION

Uncoupling of mitochondrial oxidation and phosphorylation by free fatty acids was described by Pressman and Lardy in 1956 [1]. The possible physiological significance of such an activity was indicated in 1965 by an observation of our group that free fatty acids mediate thermoregulatory uncoupling in skeletal muscles [2]. More recently, it was found that thermogenin, the H^+-conducting protein specialized in the thermoregulatory uncoupling in brown fat, is activated by free fatty acids [3]. One may suggest that not only in brown fat but also in other tissues, protein component(s) of the mitochondrial membrane are involved in the uncoupling effect of fatty acids. Such a suggestion seems probable since fatty acids do not increase H^+-conductance of BLM and cytochrome oxidase proteoliposomes [4,10], in contrast to artificial protonophorous uncouplers [5]. The problem concerns which protein(s) can mediate the fatty acid-induced uncoupling in, say, muscle mitochondria which are also fatty acid-sensitive but contain no thermogenin.

In 1967 Wojtczak and Zaluska [6] reported on the oleate-induced inhibition of adenine nucleotide translocation in mitochondria. Later it was found that bongkrekic acid, a very specific and potent in-

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Abbreviations: BLM, bilayer phospholipid membrane; BSA, bovine serum albumin; CAtr, carboxyatractylate; DNP, 2,4-dinitrophenol; BHT, 2,6-di(*t*-butyl)-*p*-cresol; FCCP, *p*-trifluoromethoxyphenylhydrazonecarbonyl cyanide; Mops, morpholinopropane sulphonate

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hibitor of the mitochondrial ATP/ADP-antiporter, represents a long-chain fatty acid with three carboxylic groups. Essential adjacent carboxylic groups are inherent in another specific antiporter inhibitor, CAtr [7]. Quite recently, Klingenberg and co-workers [8] found that thermogenin is rather similar to the ATP/ADP-antiporter in amino acid sequence and domain composition. For these and other reasons, we suggested that the ATP/ADP-antiporter is somehow involved in the fatty acid-induced uncoupling in tissues other than brown fat [9]. Below, the data confirming this hypothesis will be reported.

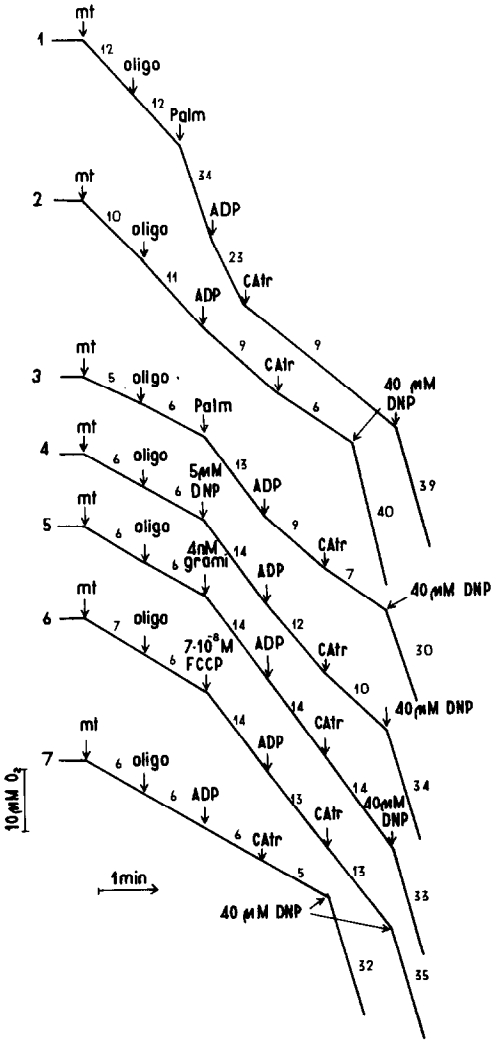

Fig.1. The effect of CAtr and ADP on uncoupler-stimulated respiration of mitochondria from rat skeletal muscle and liver. The incubation mixture (see section 2) was supplemented with BSA (0.2 mg/ml), 0.05 mM nupercaine, 0.05 mM BHT, 4 mM glutamate and 1 mM malate. Additions: oligomycin (oligo) (2 μg/ml); 1×10^{-5} M (curve 1) or 2×10^{-5} M palmitate (Palm) (curve 3); 7×10^{-8} M FCCP; 4×10^{-9} M gramicidin (grami); 2×10^{-4} M ADP; 5×10^{-6} M CAtr. Curves 1 and 2, skeletal muscle mitochondria (mt) (1.2 mg protein/ml); curves 3–7, liver mitochondria (mt) (2 mg protein/ml); t = 37°C. Figures above curves, oxygen consumption (nmol O_2/min per mg protein).

2. MATERIALS AND METHODS

Mitochondria were isolated from 150–250 g white rats. The isolation medium for skeletal muscle mitochondria contained 250 mM sucrose, 50 mM Tris-HCl, and 5 mM EDTA, pH 7.5. The muscle tissue, separated from fat and tendons, was minced in a small stainless steel meat-grinder and homogenized by a teflon pestle in a glass (pyrex) homogenizer for 4 min. After the first centrifugation (800 × g, 10 min), the supernatant was decanted and filtered through 4 layers of gauze. After the precipitation of mitochondria (12000 × g, 10 min), the sediment was washed by the isolation medium supplemented with BSA (3 mg/ml). The final mitochondrial precipitate (12000 × g, 10 min) was suspended in the isolation medium with BSA. From liver, mitochondria were isolated essentially as those from muscle, but the tissue was homogenized for 30 s. The isolation medium contained 250 mM sucrose, 5 mM Mops, 0.2 mM EDTA, pH 7.4.

The incubation mixtures contained 250 mM sucrose, 10 mM Mops, 20 mM Tris, 10 mM KH_2PO_4, BSA (0.2 mg/ml), pH 7.2 (muscle mitochondria) or 250 mM sucrose, 5 mM Mops, 2 mM KH_2PO_4, 0.5 mM EGTA, pH 7.4 (liver mitochondria).

The concentration of mitochondrial protein in a polarographic cell was about 1.2 mg/ml and 2 mg/ml for muscle and liver mitochondria, respectively.

Respiration was measured at 22°C or 37°C with a Clark electrode and an LP-7e polarograph.

The fatty acid-free bovine serum albumin (V fraction), Mops, CAtr, atractylate, palmitate and oligomycin were from Sigma; Tris, EDTA, glutamate, EGTA, ADP, ATP, GDP and

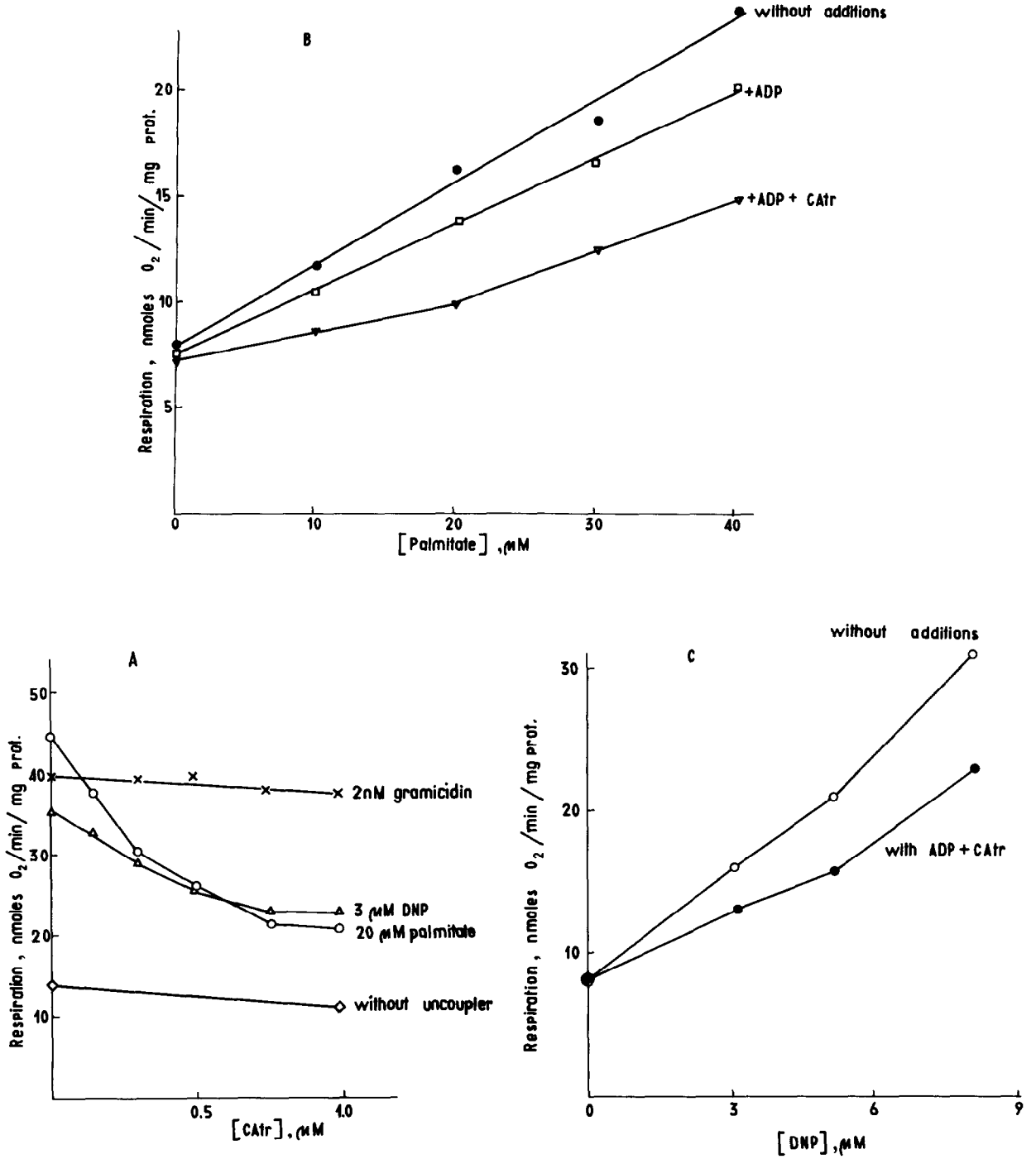

Fig.2. Inhibition of the palmitate- or DNP-stimulated respiration by CAtr. Incubation mixture (see section 2) was supplemented with 5 mM glutamate and 5 mM malate, 5 mM $MgCl_2$, BSA (0.2 mg/ml), oligomycin (2 μg/mg protein) and skeletal muscle mitochondria (1.2 mg protein/ml) (A); or with 4 mM glutamate, 1 mM malate, 1 mM $MgCl_2$, oligomycin (1 μg/mg protein) and liver mitochondria (2 mg protein/ml) (B,C); t = 22°C (A) or 37°C (B,C). Additions in B and C, 0.2 mM ADP and 5 μM CAtr.

gramicidin D from Serva; malate and BHT from Fluka; and FCCP from Boehringer.

Palmitic acid was dissolved in twice distilled ethanol. Sucrose was twice recrystallized from ethanol.

3. RESULTS

Fig.1 shows the effects of various uncouplers, ADP and CAtr upon respiration of mitochondria in the presence of oligomycin. One can see that 10–20 μM palmitate induces 2–3-fold stimulation of the respiratory rate in skeletal muscle mitochondria (curve 1) as well as in liver mitochondria (curve 3). ADP and CAtr strongly inhibit this stimulation. High (40 μM) DNP concentration added after CAtr causes a pronounced increase in the respiration rate. The effect of low (5 μM) DNP inducing the same respiratory stimulation as 20 μM palmitate, is partially inhibited by CAtr (curve 4). On the other hand, the corresponding effects of FCCP and gramicidin D appear to be CAtr-resistant (curves 5 and 6). Respiration of the oligomycin-treated mitochondria without uncouplers is affected by CAtr only slightly (curves 2 and 7).

In fig.2A, the inhibiting effect of CAtr is shown as a function of inhibitor concentration. According to this figure, the CAtr concentration causing half-maximal effect on the respiration stimulated by low palmitate and DNP, is about 2×10^{-7} M. This value is close to the CAtr concentration required to obtain a half-maximal decrease of the state 3 respiration rate (not shown). An excess of CAtr did not cause any additional inhibition of respiration stimulated by high palmitate (not shown).

As shown in fig.2B, CAtr inhibition of the palmitate-stimulated respiration is more pronounced at low palmitate concentrations. Under the same conditions, DNP-stimulated respiration is less sensitive to CAtr (fig.2C).

Besides CAtr, some inhibition of the palmitate-stimulated respiration was found to be produced by ADP (figs 1 and 2B), bongkrekic acid, palmitoyl CoA and atractylate (not shown). The maximal degree of inhibition decreased in the order: CAtr > bongkrekic acid, palmitoyl-CoA, ADP > atractylate.

In skeletal muscle mitochondria, ADP concentrations required for 50% inhibitory effect on the palmitate-stimulated respiration (2 μM) proved to be close to the K_m value of the ATP/ADP-antiporter. GDP failed to replace ADP. ATP was inhibitory only at much higher concentrations than ADP. In the presence of Mg^{2+}, higher ADP concentrations were required to obtain inhibition (not shown).

4. DISCUSSION

Inhibition of the palmitate-stimulated respiration by CAtr indicates that in muscle and liver mitochondria, the ATP/ADP-antiporter is involved in the uncoupling effect of low concentrations of fatty acids. Indeed, effective concentrations of CAtr required to inhibit (i) state 3 respiration and (ii) palmitate-stimulated respiration proved to be similar (2×10^{-7} M). It is known that effect (i) is due to specific inhibition of the ATP/ADP-antiporter by CAtr [7]. The inhibitory action of micromolar concentrations of ADP seems to be another piece of evidence for the above concept. The very facts that (i) GDP fails to substitute for ADP, (ii) ATP is effective at much higher concentrations than ADP, and (iii) Mg^{2+} lowers the inhibiting effect of ADP are in agreement with the properties of the ATP/ADP-antiporter which binds neither GDP nor Mg·ADP, and under energized conditions, favours extramitochondrial ADP over ATP [7].

Involvement of protein(s) in fatty-acid mediated uncoupling explains why palmitate fails to increase H^+ conductance in BLM and the cytochrome oxidase proteoliposomes [4,10]. This means that fatty-acid mediated uncoupling is not a common property of any protein-containing membrane. Rather, a specific protein should be present.

According to the data of Tsofina and Vygodina [4], palmitate increases $\Delta\psi$ across the membrane of cytochrome oxidase-asolectin proteoliposomes. Monensin substitutes for palmitate. This effect of palmitate may be explained by diffusion of the protonated form of fatty acid (RCOOH) across the proteoliposomal membrane, resulting in a ΔpH decrease and corresponding $\Delta\psi$ increase. Thus, it is the transport of the deprotonated form ($RCOO^-$), rather than RCOOH, that seems to be a rate-limiting step in the circulation of fatty acids in the membrane systems.

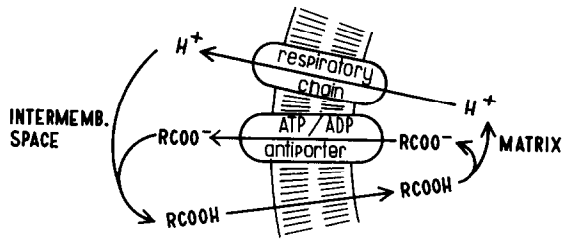

Fig.3. A scheme illustrating possible mechanism of uncoupling by low concentrations of free fatty acid. RCOOH and $RCOO^-$, protonated and deprotonated forms of fatty acid, respectively.

Within the framework of such a concept one may understand how the ATP/ADP-antiporter facilitates the uncoupling effect of fatty acids. Most probably, this protein, exchanging anions of ATP^{4-}_{in} for anions of ADP^{3-}_{out}, increases also in some way the membrane permeability for anion $RCOO^-$ (fig.3).

One may assume that the ATP/ADP-antiporter is composed of (i) very specific gates discriminating, e.g., ADP from GDP and (ii) a rather non-specific mechanism of anion translocation ('rotor', channel or relay of fixed cationic groups crossing the hydrophobic barrier of the membrane). Fatty acid anions $RCOO^-$ might reach, say, cationic groups of the rotor with no gate involved, or, alternatively, there is another gate specific for $RCOO^-$. The former possibility seems more probable since not only palmitate but also low (micromolar) concentrations of DNP were shown to stimulate respiration in a CAtr-sensitive fashion. One may speculate that it is DNP^- that is transported by the ATP/ADP-antiporter whereas DNPH, like RCOOH, crosses the phospholipid bilayer without the assistance of any protein. As found by Hanstein and Hatefi [11], 2-azido-4-nitrophenol combines with mitochondrial protein(s) of apparent molecular mass 20–30 kDa which roughly corresponds to that of the ATP/ADP-antiporter. According to Cyboron and Dryer [12], palmitate as well as FCCP completely inhibits binding of 2-azido-4-nitrophenol in liver and brown fat mitochondria. Since FCCP effectively decreases BLM resistance, we may conclude that FCCP can cross the phospholipid bilayer not only in the protonated but also in the anionic form. Therefore, assistance of the ATP/ADP-antiporter seems to be unnecessary. Similarly, the antiporter is not involved in the action of gramicidin operating as a channel permeable for H^+, Na^+ and K^+.

Apparently, two adjacent COO^- groups are essential for CAtr inhibition of the palmitate effect. The inhibitory effect of atractylate differing from CAtr by the absence of one of these groups, proved to be much weaker.

Maybe, combination of the gate part of the antiporter with CAtr suppresses operation of the rotor part stronger than that with atractylate.

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