Carboxyatractyloside increases the effect of oleate on mitochondrial permeability transition

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Abstract Addition of a low concentration of carboxyatractyloside (0.075 μM) renders mitochondria susceptible to the opening of the non-specific pore by 5 μM oleate, in a cyclosporin A-sensitive fashion. Matrix Ca^{2+} efflux as well as collapse of the transmembrane potential reveal permeability transition. The effect of oleate is reached after the titration, by carboxyatractyloside, of 38 pmol of adenine nucleotide translocase per mg mitochondrial protein. We propose that permeability transition may result from an additive action of carboxyatractyloside plus oleate on the ADP/ATP carrier.

Key words: Permeability transition; Fatty acid; Carboxyatractyloside; Mitochondrion; ADP/ATP carrier; Calcium efflux

1. Introduction

As established by Skulachev's group [1,2], Schönfeld et al. [3] and Wojtczak et al. [4], long-chain fatty acids, unlike the classic protonophores, uncouple oxidative phosphorylation by passing their dissociated form to the cytosol side through the adenine nucleotide translocase (ANT). Several findings add support to this statement, e.g. Schönfeld et al. [5] have shown that mitochondria from hypothyroid rats are less sensitive to the uncoupling action of fatty acids; such a failure coincides with a diminished expression of ANT. However, the main support is provided by the fact that inhibitors of ANT, carboxyatractyloside (CAT) among them, arrest the uncoupling effect of fatty acids (see [6] for review, also the experiments by Brustovetsky and Klingenberg [7] indicating that the reconstituted ANT mediates H^+ transport by fatty acids). In addition to their effects as uncouplers, fatty acids promote membrane permeability transition [8–10]. Schönfeld and Bohnensack [9] propose that fatty acids open the non-specific pore through a mechanism in which the stabilization of ANT in the cytosolic side appears to be involved. This communication presents experimental results indicating that in kidney mitochondria, CAT at the low concentration of 0.075 μM, increases rather than inhibits the effect of 5 μM oleate on membrane permeability transition. Cyclosporin A (CSA) and *N*-ethylmaleimide (NEM) inhibit the reaction.

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Abbreviations: CAT, carboxyatractyloside; CSA, cyclosporin A; ΔΨ, transmembrane potential; ANT, adenine nucleotide translocase; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone; NEM, *N*-ethylmaleimide

2. Materials and methods

Mitochondria were isolated by the conventional method of differential centrifugation after homogenization of the kidney cortex tissue in 250 mM sucrose-1 mM EDTA pH 7.3. Ca^{2+} movement was followed in a double beam spectrophotometer at 675–685 nm by incubating 2 mg protein from mitochondria in a medium containing 125 mM KCl, 10 mM succinate, 2 mM phosphate, 10 mM HEPES, 50 μM $CaCl_2$, 200 μM ADP, 5 μg rotenone, 5 μg oligomycin, and 50 μM arsenazo III [11]; the medium was adjusted to pH 7.3 with KOH. Transmembrane potential (ΔΨ) was estimated spectrophotometrically at 524–554 nm in a similar medium to that described above, except that 10 μM safranine [12] was used instead of arsenazo III. Oxygen consumption was measured polarographically using a Clark type electrode, by incubating 1.3 mg mitochondrial protein in 2 ml of a similar medium to the one described above. ADP exchange reaction was estimated by adding 1 mg mitochondrial protein to 1 ml of medium, maintained at 4°C, containing 125 mM KCl; 10 mM HEPES pH 7.3, 10 μg oligomycin, 10 μg rotenone, and 20 μM [^{3}H]ADP (specific activity 1000 cpm/nmol), after 30 s of incubation an aliquot of 0.2 ml was withdrawn and filtered through a Millipore filter of 0.45 μm. Protein was determined by the method of Lowry et al. [13].

3. Results

The experiments shown in (Fig. 1A,B), indicating that CAT and oleate respectively cause membrane leakage, resemble those previously published [8–10,14,15]. Nevertheless, they were performed with the aim of establishing the concentration at which CAT or oleate does not promote mitochondrial Ca^{2+} release. As shown in Fig. 1A, CAT at the concentration of 0.075 μM failed to induce Ca^{2+} efflux. Similarly, Fig. 1B indicates that 5 μM oleate was inefficient in promoting Ca^{2+} release. It is known that CAT inhibits the mitochondrial effects of long-chain free fatty acids [6]. Unexpectedly, however, we found that the addition of 0.075 μM CAT, together with 5 μM oleate, potentiated rather than inhibited the effect of the fatty acid on Ca^{2+} efflux (Fig. 1C). This finding is in agreement with results of Andreyev et al. [2] indicating that CAT increases the stimulatory effect of palmitate on state 4 respiration.

Certainly, the described effect of CAT-oleate on Ca^{2+} release must be due to the opening of the non-specific pore. Starkov et al. [8] reported that CSA inhibits fatty acid-induced membrane permeabilization. In agreement, Fig. 2A shows that the immunosuppressant effectively inhibited Ca^{2+} efflux as promoted by CAT-oleate. In an early work Aquila et al. [16] demonstrated that NEM antagonizes the binding of CAT to the adenine nucleotide translocase (ANT). Accordingly, Fig. 2B illustrates that the addition of 15 μM NEM suffices to inhibit the effect of the pair CAT-oleate on permeability transition, presumably by blocking the binding of CAT.

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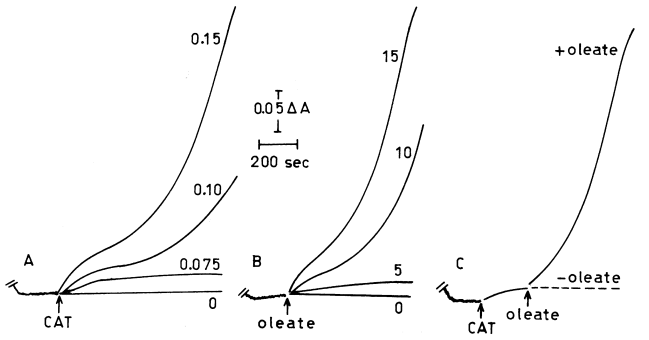

Fig. 1. Effect of CAT and oleate on mitochondrial Ca^{2+} efflux. Experimental conditions were as described in Section 2. In A and B the numbers at the side of the traces signify μM added concentrations of CAT or oleate, respectively. Where indicated in C, 0.075 μM CAT and 5 μM oleate were added. Final volume 3 ml. Incubation temperature 30°C.

Considering that oleate plus CAT induce pore opening it can be expected that they also collapse ΔΨ, which is a characteristic of membrane permeability transition [17]. Fig. 3A shows that CAT or oleate when added separately did not induce a drop in transmembrane potential. In contrast, in Fig. 3B it is shown that 5 μM oleate discharges ΔΨ if added after 0.075 μM CAT. This result is in agreement with the findings of Amerkhanov et al. [18] showing that in liver mitochondria from arousing ground squirrels CAT collapses ΔΨ. According to Brustovetsky et al. [19], the drop in ΔΨ by CAT-oleate is inhibited by 0.5 μM cyclosporin A (not shown).

A distinctive effect of long-chain fatty acids is to increase respiratory rate, which in turn is inhibited by CAT [2]. The experiment in Fig. 4A was carried out with the purpose of exploring whether or not CAT, at the low concentrations used, is able to inhibit oleate-stimulated respiration. It is seen that indeed 0.075 μM CAT arrests a 17% respiratory rate stimulated by 5 μM oleate. Interestingly, CSA did not inhibit oleate-dependent increased respiration. Fig. 4B shows that in agreement with previous reports [2,20], 2.5 μM CAT suppressed the increase in oxygen consumption mediated by 20 μM oleate.

At this stage of the experimental results two questions arose: (i) how much inhibition, if any, of ANT function resulted with the used concentrations of CAT (0.075 μM); (ii) how many nmol adenine nucleotide translocase are titrated by

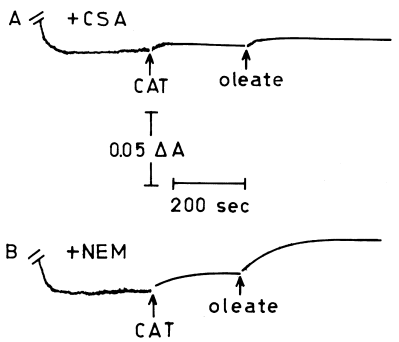

Fig. 2. Protective effect of CSA and NEM on Ca^{2+} release as induced by CAT-oleate. Experimental conditions were as described in Section 2. Where indicated 0.5 μM CSA, 15 μM NEM, 0.075 μM CAT, and 5 μM oleate were added. Final volume 3 ml. Incubation temperature 30°C.

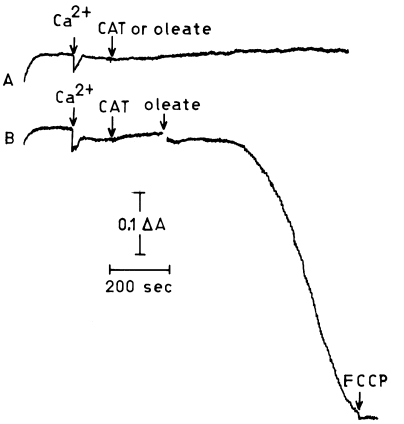

Fig. 3. Effect of CAT and oleate on the transmembrane electric gradient. Kidney mitochondria were incubated under the conditions described in Section 2. Where indicated, in traces A and B, 50 μM $CaCl_2$, 0.075 μM CAT, 5 μM oleate, and 1 μM *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP). Final volume 3 ml. Incubation temperature 30°C.

0.075 μM CAT. To answer such questions a titration of ANT with different concentrations of CAT was performed. Fig. 5 shows the inhibition of ADP exchange by increasing concentrations of CAT. As shown, the addition of 0.075 μM CAT, inhibited ADP exchange reaction by 75%, i.e. it was diminished from 0.95 to 0.3 nmol/mg. By following the treatment described by Gellerich et al. [21] and García et al. [22], with the values obtained from the curve, it was estimated that 38 pmol ADP/ATP carrier/mg were titrated when 0.075 μM CAT was added.

4. Discussion

In contrast to the reported inhibitory role of CAT on the effects of fatty acids [2,4,7,20], we have shown that CAT promotes it. As demonstrated, 0.075 μM CAT enhanced the effect of 5 μM oleate on CA^{2+} release, as well as on the drop of ΔΨ. The described effects of the pair CAT-oleate are consistently similar to those shown when mitochondria undergo

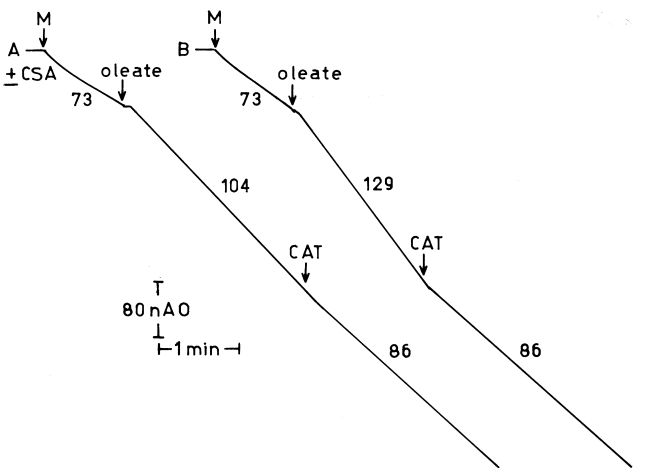

Fig. 4. The inhibitory effect of CAT on oleate-dependent respiratory rate. Mitochondria were incubated under the conditions described in Section 2. In trace A the additions were: 5 μM oleate and 0.075 μM CAT, and 0.5 μM CSA. In trace B the additions were as follows: 20 μM oleate and 2.5 μM CAT. Temperature 30°C.

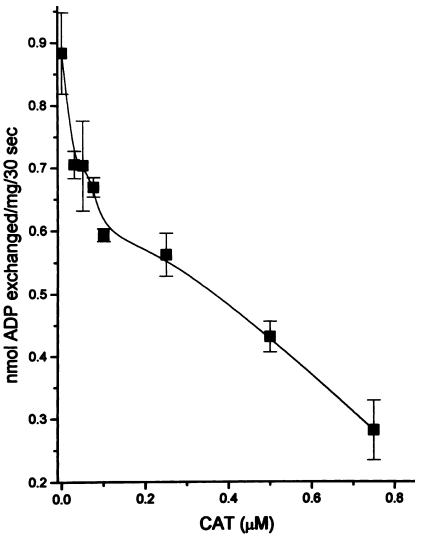

Fig. 5. The inhibitory effect of increasing concentrations of CAT on ADP exchange reaction. Experimental conditions were as described in Section 2. The data represent mean values ± S.D.

membrane permeability transition. Certainly it is well recognized that separately, CAT and fatty acids are inducers of permeability transition, although at a higher concentration than the one used in this work [8–10,14,15]. The assumption that CAT-oleate opens the non-specific pore is mainly based on the finding that CSA restrains membrane leakage. Interestingly, CSA does not inhibit oleate-dependent oxygen consumption. This opens up the possibility that the protonophoric action of long-chain fatty acids is not necessarily involved in the induction of permeability transition. A growing body of evidence points to adenine nucleotide translocase as the non-specific pore [23,24]. The fact that in this work CAT was required to induce oleate-dependent pore opening favors such an assumption. In addition we must consider that NEM blocks the CA^{2+} releasing effect of CAT-oleate. Certainly, it is recognized that NEM inhibits the binding of CAT to adenine nucleotide carrier [16]; however, it must be also recognized that NEM fixes the carrier in the matrix side [16], a condition that inhibits membrane permeability transition (see [23] for review). Furthermore, the participation of ANT can be reinforced by the fact that glutamate does not inhibit the effect of CAT-oleate on matrix Ca^{2+} efflux (not shown); in this regard it should be noted that Wieckowski and Wojtczak [24] and Samartsev et al. [25] reported that the dicarboxylate carrier is also involved in the uncoupling action of fatty acids. Concerning the mechanism by which the pair CAT-oleate induces pore opening, we would like to consider the scheme of Majima et al. [26] postulating that the binding of CAT to ANT induces conformational changes. It may be provocative to speculate that after the titration of 38 nmol ANT, such a change in configuration would expose more positive charges, thus facilitating the passing of the dissociated molecule of oleate. However, to put the action of CAT-oleate on firmer ground it is worthwhile taking into account the proposal by Schönfeld and Bohnensack [9] who argue that oleate fixes ANT on the cytosol side just as CAT does. Thus, permeability transition would result from a synergistic effect of CAT and oleate.

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