The participation of pyridine nucleotides redox state and reactive oxygen in the fatty acid-induced permeability transition in rat liver mitochondria

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Abstract The ability of low concentrations (5–15 μM) of long-chain fatty acids to open the permeability transition pore (PTP) in Ca^{2+}-loaded mitochondria has been ascribed to their protonophoric effect mediated by mitochondrial anion carriers, as well as to a direct interaction with the pore assembly [M.R. Wieckowski and L. Wojtczak, FEBS Lett. 423 (1998) 339–342]. Here, we have compared the PTP opening ability of arachidonic acid (AA) with that of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) at concentrations that cause similar quantitative dissipation of the membrane potential ($\Delta\Psi$) in Ca^{2+}-loaded rat liver mitochondria respiring on succinate. The initial protonophoric effects of AA and FCCP were only slightly modified by carboxyatractyloside and were followed by PTP opening, as indicated by a second phase of $\Delta\Psi$ disruption sensitive to EGTA, ADP, dithiothreitol and cyclosporin A. This second phase of $\Delta\Psi$ dissipation could also be prevented by rotenone or NAD(P)H-linked substrates which decrease the pyridine nucleotide (PN) oxidation that follows the stimulation of oxygen consumption induced by AA or FCCP. These results suggest that, under the experimental conditions used here, the PTP opening induced by AA or FCCP was a consequence of PN oxidation. Exogenous catalase also inhibited both AA- and FCCP-induced PTP opening. These results indicate that a condition of oxidative stress associated with the oxidized state of PN underlies membrane protein thiol oxidation and PTP opening.

Key words: Arachidonic acid; Mitochondrion; Membrane permeability transition; NAD(P)H; Calcium ion

1. Introduction

Although the ability of non-esterified long-chain fatty acids (FA) to uncouple oxidative phosphorylation has been known for a long time [1], only recently was this mechanism clarified by the concept of the fatty acid cycle put forward by Skulachev [2]. According to this model, which has been confirmed by several independent lines of evidence (for reviews, see [3,4]), protonated FA movement to the mitochondrial matrix is passively driven by the ΔpH across the phospholipid bilayer of the inner membrane (flip-flop mechanism). The deprotonated FA anion is subsequently exported electrophoretically by mitochondrial anion carriers such as the ATP/ADP antiporter (ANT) [2], the aspartate/glutamate antiporter [5], the dicarboxylate carrier [6] or uncoupling proteins [2,3]. In each round of the FA cycle, one proton is left behind in the matrix. In addition to their ability to specifically uncouple oxidative phosphorylation, FA are also able to open the permeability transition pore (PTP) [7,8] in Ca^{2+}-loaded mitochondria. Since opening of the PTP is facilitated at lower transmembrane potentials ($\Delta\Psi$) [9–11], the ability of FA, at low concentrations, to open the PTP has been ascribed to their protonophoric action and to their direct interaction with the ANT [7], a putative component of the pore assembly [11,12]. Another characteristic of the PTP is its ability to open under conditions of oxidative stress (for review, see [13]), a condition that may oxidize thiol groups in the ANT [11,14]. Vercesi [9] proposed a link between the mitochondrial membrane potential and PTP opening through the energy-linked NAD(P)H transhydrogenase which uses the $\Delta\Psi$ to displace the redox potential of NADP to a more reduced state [15]. This coenzyme is the source of mitochondrial reducing equivalents that allows glutathione reductase/glutathione peroxidase to scavenge most of the reactive oxygen species (ROS) produced by mitochondria [16], thus protecting the organelle against PTP opening [14] and lipid peroxidation [17]. At low membrane potential, this antioxidant process is impaired and a condition of oxidative stress may arise [16]. In addition, earlier data [18] provided evidence that ANT thiols may be oxidized when the level of NAD(P)H is low, a condition that favors PTP opening [18].

Since both FA and the classical protonophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) stimulate oxygen consumption and shift the electron transfer components of the respiratory chain to a more oxidized state, we have examined the possible participation of the NAD(P) redox state in the mechanism of FA- and FCCP-induced PTP opening. Given the possible participation of the PTP in both necrotic and apoptotic cell death [12] and the fact that arachidonic acid (AA) may participate as a secondary messenger in both modes of cell death [19], we have used this FA to assess the mechanism of PTP opening.

2. Materials and methods

2.1. Isolation of rat liver mitochondria and standard incubation procedure

Rat liver mitochondria (RLM) were isolated by conventional differ-

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Abbreviations: ANT, adenine nucleotide translocator; AA, arachidonic acid; CyP-D, cyclophilin D; CsA, cyclosporin A; DTT, dithiothreitol; FA, fatty acids; PTP, permeability transition pore; EGTA, ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NAD(P)H, nicotinamide adenine dinucleotide phosphate; PN, pyridine nucleotides; RLM, rat liver mitochondria; ROS, reactive oxygen species

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ential centrifugation from the livers of adult female Wistar rats fasted overnight. The experiments were carried out at 30°C in a standard medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES (2-hydroxyethyl-1-piperazineethanesulfonic acid), pH 7.2, 2 mM KH_2PO_4, 1 mM $MgCl_2$ and 5 mM succinate. The experiments were done in glass cuvettes with magnetic stirring in a final volume of 2.0 ml and a protein concentration of 0.5 mg ml^{-1}. Other additions are indicated in the figure legends. The results shown are representative or averages of at least three experiments.

2.2. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The mitochondrial membrane potential ($\Delta\Psi$) was estimated by measuring the fluorescence changes of safranin O (5 μM) [20], using a model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operated at excitation and emission wavelengths of 495 nm and 586 nm, respectively, and a slit width of 5 nm. A calibration curve was obtained when mitochondria were incubated in K^+-free reaction medium containing 250 mM sucrose, 10 mM Na–HEPES buffer (pH 7.2) and 0.5 mM EGTA. The changes in the fluorescence of safranin induced by mitochondrial energization were similar in the absence or presence of K^+. In all experiments, one fluorescence unit corresponded to 40 mV.

2.3. Determination of Ca^{2+} movement

Variations in the concentration of free Ca^{2+} were followed by measuring the changes in the absorbance spectrum of arsenazo III [21], using an SLM Aminco DW2000 spectrophotometer set at the wavelength pair 675–685 nm.

2.4. Determination of the NAD(P) redox state

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed in a Hitachi F-4010 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 366 and 450 nm, respectively, with a slit width of 5 nm.

2.5. Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, OH, USA) in 1.3 ml of standard reaction medium (30°C), in a sealed glass cuvette equipped with a magnetic stirrer.

2.6. Materials

Safranin O, catalase (C10), cyclosporin A (CsA), HEPES, arsenazo III, rotenone, EGTA, antimycin A, ADP, α-nicotinamide adenine dinucleotide (reduced form, α-NADH), FCCP, dithiothreitol (DTT), succinate, β-hydroxybutyrate, isocitrate and carboxyatractyloside were from Sigma Chemical Company (St. Louis, MO, USA). All other reagents were of the highest grade available.

3. Results

3.1. Membrane potential alterations induced by AA and FCCP in Ca^{2+}-loaded rat liver mitochondria

Fig. 1 shows that when RLM were added to the standard medium containing 30 μM free Ca^{2+} and 5 mM succinate as substrate, there was a fast decrease in the fluorescence of safranin compatible with the formation of a membrane potential. The addition of 10 μM AA (panel A, line a) or 50 nM FCCP (panel B, line a) caused a biphasic decrease in the membrane potential ($\Delta\Psi$). The first phase (about 20 mV) was compatible with a pure protonophoric effect of these compounds and was followed by a larger second phase of membrane depolarization compatible with the mitochondrial permeability transition (MPT). The initial protonophoric effect was partially inhibited, whereas the second phase of the decrease in $\Delta\Psi$ was totally prevented by the permeability transition inhibitors ADP (lines c), catalase (lines d), DTT (lines e), EGTA (lines f) and CsA (lines g). Rotenone (lines h) and the NAD(P)H-linked substrates β-hydroxybutyrate plus isocitrate (lines i) had similar effects. Carboxyatractyloside (1.0 μM, lines b) caused only a very small inhibition at pH 7.2, in agreement with a previous report [5]. Lines j represent control experiments without AA or FCCP. The addition of antimycin A at the end of the experiments caused a total collapse of $\Delta\Psi$.

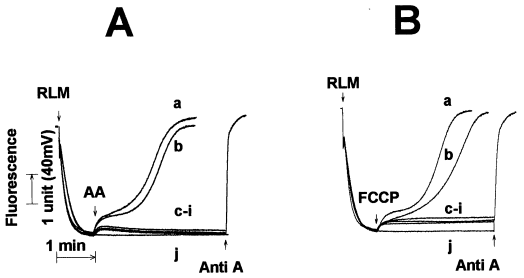

Fig. 1. Mitochondrial membrane potential collapse induced by AA and FCCP: the effects of ADP, catalase, carboxyatractyloside, DTT, EGTA, CsA and rotenone. RLM (0.5 mg ml^{-1}) were incubated in medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM sodium succinate, 30 μM $CaCl_2$ and 5 μM safranin, at 30°C. As indicated, 10 μM AA (panel A, line a) or 50 nM FCCP (panel B, line a) was added in the presence of 1 μM carboxyatractyloside (lines b), 200 nmol ADP and 2 μg oligomycin ml^{-1} (lines c), 2.0 μM catalase (lines d), 2 mM DTT (lines e), 1 mM EGTA (lines f), 1.0 μM CsA (lines g), 4 μM rotenone (lines h) or 5 mM β-hydroxybutyrate plus 0.1 mM isocitrate (lines i). Lines j represent control experiments without additions. Antimycin A (1 μg ml^{-1}) was added at the end of the experiments.

3.2. Stimulation of respiration and mitochondrial NAD(P)H oxidation induced by AA and FCCP

Mitochondrial pyridine nucleotides are a source of reducing equivalents for the antioxidant enzyme system glutathione reductase/glutathione peroxidase, which converts H_2O_2 into H_2O, thereby preventing the formation of hydroxyl radicals ($HO^{\bullet}$) that attack protein thiols leading to MPT [18]. Fig. 2 compares the changes in NAD(P)H fluorescence when the rate of respiration is stimulated by AA or FCCP in mitochondria oxidizing succinate in the absence of rotenone (Fig. 3). The fluorescence measurements were done in the presence of 1.0 μM CsA to prevent any changes in light scattering caused by the mitochondrial swelling that follows NAD(P)H oxidation in Ca^{2+}-loaded mitochondria [18]. The addition of 10 μM AA (panel A, line a) or 50 nM FCCP (panel B, line a) caused a large, fast decrease in fluorescence, compatible with the oxidation of almost 100% of the mitochondrial NAD(P)H [9]. When RLM were incubated with 4 μM rotenone (lines h) or 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines g), the addition of AA or FCCP caused much less NAD(P)H oxidation. Only very low inhibition of NAD(P)H oxidation occurred in the presence of 1.0 μM carboxyatractyloside (lines b), 200 nmol ADP (lines c), 2.0 μM catalase (lines d), 2 mM DTT (lines e) or 1 mM EGTA (lines f). Lines i represent control experiments without AA or FCCP. The rate of respiration (Fig. 3) was slightly faster in the presence of FCCP (151.96 ± 1.45 nmol O_2 min^{-1} mg $protein^{-1}$) than in the presence of AA (128.18 ± 2.65 nmol O_2 min^{-1} mg $protein^{-1}$). The rate of state 4 respiration in the control experiments (line a) was 22.94 ± 0.35 nmol O_2 min^{-1} mg $protein^{-1}$.

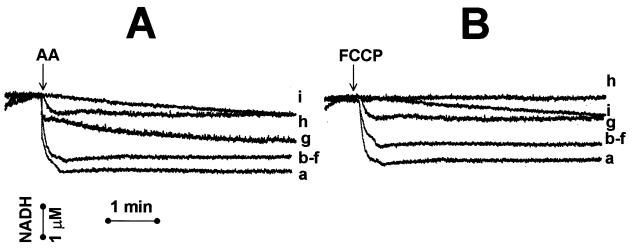

Fig. 2. Mitochondrial NAD(P)H oxidation induced by AA or FCCP. RLM (0.5 mg ml^{-1}) were incubated at 30°C in standard reaction medium containing 1 μM CsA to prevent changes in light scattering due to mitochondrial swelling caused by Ca^{2+}. Panel A, line a, shows the addition of 10 μM AA, and panel B, line a, that of 50 nM FCCP. Other additions were: 1.0 μM carboxyatractyloside (lines b), 200 nmol ADP and 2 μg oligomycin ml^{-1} (lines c), 2.0 μM catalase (lines d), 2 mM DTT (lines e), 1 mM EGTA (lines f), 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines g) and 4 μM rotenone (lines h). Lines i represent control experiments, without additions.

3.3. Re-reduction of NAD(P)$^+$ by rotenone and NAD(P)H-linked substrates prevents PTP opening by AA or FCCP

Although very little is known about the structure and biological role of the PTP, the mechanisms of inhibition of the membrane permeability transition by ADP, DTT, catalase and CsA have been described and are related to direct or indirect interactions with the ANT (by ADP or CsA, respectively) or the antioxidant action of catalase and DTT. The following experiments were designed to ascertain whether re-reduction of $NAD(P)^+$ by rotenone or NAD(P)H-linked substrates correlated with their inhibitory effect against opening of the PTP. The combination of β-hydroxybutyrate and isocitrate promptly re-reduces $NAD(P)^+$ and reverses Ca^{2+} release from rat liver mitochondria treated with prooxidants [9,22]. Fig. 4 shows that β-hydroxybutyrate plus isocitrate (lines b), rotenone (lines c), or rotenone plus β-hydroxybutyrate and isocitrate (lines d), added after AA or FCCP (lines a), caused significant re-reduction of $NAD(P)^+$. As expected, rotenone caused an almost complete reduction of $NAD(P)^+$. This was faster when NAD(P)-linked substrates were added together. To assess whether the second phase of the $\Delta\Psi$ disruption was the consequence of NAD(P)H oxidation, the changes in $\Delta\Psi$ when these $NAD(P)^+$ reductants were added after the first phase of the $\Delta\Psi$ decrease induced by AA or FCCP (lines a) were monitored. Fig. 5 shows that $NAD(P)^+$

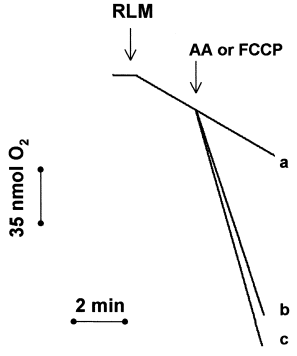

Fig. 3. Release of state 4 respiration by AA or FCCP. RLM (0.5 mg ml^{-1}) were incubated in standard reaction medium at 30°C. Line b shows the addition of 10 μM AA and line c, that of 50 nM FCCP. Line a represents control experiment without additions.

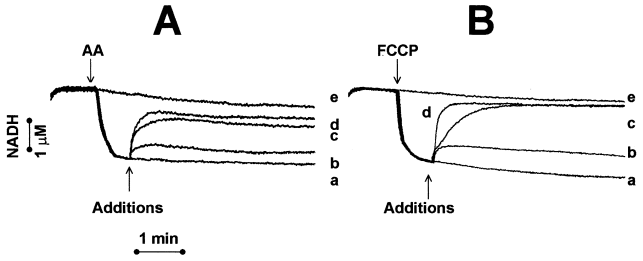

Fig. 4. $NAD(P)^+$ reduction induced by rotenone and NAD(P)H-linked substrates. RLM (0.5 mg ml^{-1}) were incubated in standard reaction medium at 30°C. Panel A, line a, shows the addition of 10 μM AA, and panel B, line a, that of 50 nM FCCP. Where indicated by the arrow, the following were added: 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines b), 4 μM rotenone (lines c) and 4 μM rotenone, 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines d). Lines e represent control experiments without additions.

re-reduction did indeed prevent the second phase of the $\Delta\Psi$ decrease. When the re-reduction was more complete by the simultaneous addition of rotenone and NAD(P)-linked substrates, the $\Delta\Psi$ returned almost to the level of the control experiment in panel A, line d. Lines e represent control experiments without AA or FCCP. The addition of antimycin A caused the collapse of $\Delta\Psi$.

3.4. Characteristics of mitochondrial Ca^{2+} release induced by AA or FCCP

Fig. 6 shows that when RLM were added to a standard reaction medium containing 5 mM succinate and 30 μM $CaCl_2$, most of the Ca^{2+} present was quickly taken up by mitochondria (lines e). The addition of 10 μM AA (panel A, line a) or 50 nM FCCP (panel B, line a) was followed by the release of accumulated and endogenous Ca^{2+} into the medium. The mitochondrial Ca^{2+} release induced by AA or FCCP was totally inhibited by rotenone (lines b), β-hydroxybutyrate plus isocitrate (lines c) or all of these together (lines d). Similar effects were obtained with ADP with or without oligomycin, DTT, CsA, catalase, but not with car-

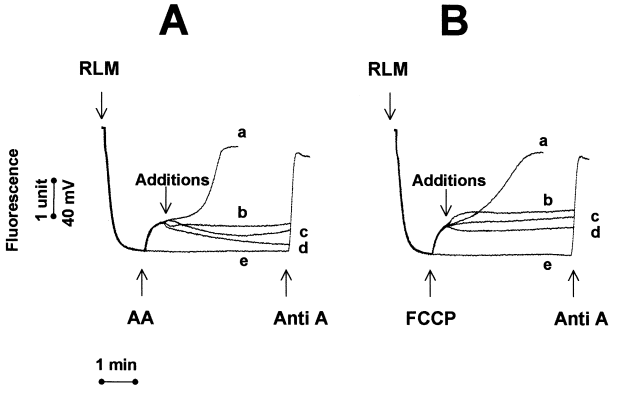

Fig. 5. Rotenone or NAD(P)H-linked substrates added after the initial protonophoric effect of AA or FCCP prevent $\Delta\Psi$ disruption in Ca^{2+}-loaded RLM. RLM (0.5 mg ml^{-1}) were incubated at 30°C in medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM sodium succinate, 30 μM $CaCl_2$ and 5 μM safranin. In all experiments, 10 μM AA (panel A, line a) or 50 nM FCCP (panel B, line a) was added. Where indicated by the arrow, the following were added: 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines b), 4 μM rotenone (lines c) and 4 μM rotenone, 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines d). Lines e represent control experiments.

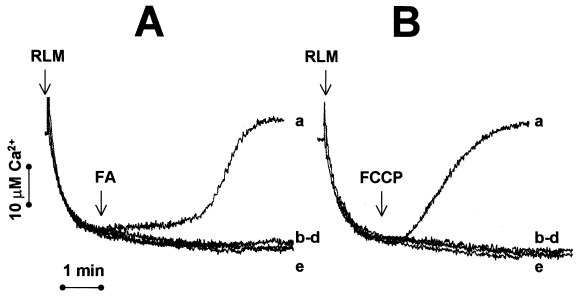

Fig. 6. Mitochondrial Ca^{2+} release induced by AA or FCCP: effects of β-hydroxybutyrate plus isocitrate or rotenone. RLM (0.5 mg ml^{-1}) were incubated in standard reaction medium containing 30 μM $CaCl_2$ and 40 μM arsenazo III. Panel A, line a, shows the addition of 10 μM AA, and panel B, line a, that of 50 nM FCCP, in the presence of 4 μM rotenone (lines b), 5 mM β-hydroxybutyrate plus 0.1 mM isocitrate (lines c) or 4 μM rotenone, 5 mM β-hydroxybutyrate and 0.1 mM isocitrate (lines d). Lines e represent control experiments.

boxyatractyloside (not shown). The amount of Ca^{2+} released by antimycin A was the same in all the experimental conditions used (not shown). This eliminated the possibility that isocitrate could be protecting against PTP by chelating an appreciable amount of the cation.

4. Discussion

It is now widely accepted that the ANT is a component of the PTP assembly [12] and that the redox state of two of its thiol groups plays a crucial role in the mechanism of PTP opening [11]. Oxidation of these thiols by thiol reagents, reactive oxygen or via a redox link with the oxidized state of pyridine nucleotides (PN) facilitates PTP opening [13]. An earlier report [11] provided evidence that both cyclophilin D (CyP-D) binding and ADP displacement from distinct sites on the ANT increased the affinity of Ca^{2+} binding to the protein site(s) which trigger(s) pore opening. Both mechanisms appear to be stimulated by oxidation of the thiol groups on the ANT [11], a process that may also mediate the DTT-sensitive PTP opening induced by both AA and FCCP in the present work. Linolenic acid, which, together with AA, accounts for the bulk of mitochondrial phospholipids (see [24]), has an effect similar to that produced by AA (not shown).

The data presented here demonstrate that NAD(P)H and protein thiol oxidation associated with the stimulation of oxygen consumption induced by mild uncoupling by either FCCP or AA participate in the mechanism of PTP opening. This is illustrated by the inhibition of pore opening by either rotenone or NAD-linked substrates which significantly decrease NAD(P)H oxidation in RLM oxidizing succinate. If $NAD(P)^+$ is re-reduced by rotenone or NAD(P)H-linked substrates, opening of the PTP is also prevented. Therefore, a link between the thiol redox state of the translocase and that of NAD(P)H seems to underlie the mechanism of PTP opening by both AA and FCCP. In this regard, a decrease in the number of inner membrane protein sulfhydryl groups and ANT conformation is reportedly associated with the oxidation of PN in Ca^{2+}-loaded rat liver mitochondria [18]. PTP opening occurred under these conditions. The inhibition of pore opening by DTT (Fig. 1), a dithiol reductant, is consistent with this mechanism. On the other hand, ADP and CsA, although unable to prevent NAD(P)H and, probably, ANT thiol oxidations, did inhibit PTP opening by a direct interaction with the inhibitory site on the ANT or by removing bound CyP-D, respectively. In both cases, the oxidation of ANT thiols was overcome by the effects of CsA and ADP [11]. Based on the present results, we propose that the ability of FA to open the PTP is, as in the case of FCCP, a consequence of NAD(P)H oxidation that occurs through an increase in the rate of respiration mediated by the protonophoric action of these compounds. In both situations, a condition of oxidative stress generated by the lack of reducing equivalents from NAD(P)H to the antioxidant system glutathione reductase/peroxidase favors PTP opening. This notion is strongly supported by the experiment showing that removal of H_2O_2 by exogenous catalase prevented the mitochondrial permeability transition. It is assumed that AA-induced uncoupling was mediated by ANT and the aspartate/glutamate antiporter as proposed by Samartsev et al. [5], since liver mitochondria do not contain a sufficient amount of uncoupling protein-2 [23]. This assumption was confirmed by the lack of the inhibitory effect of GDP on the first uncoupling phase (not shown).

The present results should also be discussed in view of the findings of Korshunov et al. [24] and Cocco et al. [25]. The first group presented evidence that micromolar concentrations of FA cause mild uncoupling and arrest ROS formation by rat heart mitochondria respiring on succinate in Ca^{2+}-free medium. The second group reported that AA and other FA interactions with intact bovine heart mitochondria, while causing uncoupling, also markedly inhibit uncoupled respiration at the level of respiratory complexes I and III, with a consequent stimulation of ROS production. Our results are not contrary to these studies since our experiments were carried out with Ca^{2+}-loaded rat liver mitochondria under experimental conditions in which there was only a slight difference in the rate of AA- and FCCP-induced uncoupling (Fig. 3). The accumulation of ROS is certainly a consequence of increased production resulting from the membrane perturbation caused by Ca^{2+} binding to negative phospholipids on the internal face of the inner mitochondrial membrane [26]. As previously proposed [27], this Ca^{2+} effect is potentiated when associated with the lack of reducing equivalents from NAD(P)H to feed the mitochondrial antioxidant systems.

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