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Cyclosporin A-Sensitive Decrease in the Transmembrane Potential across the Inner Membrane of Liver Mitochondria Induced by Low Concentrations of Fatty Acids and Ca^{2+}

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Abstract—At low Ca^{2+} concentrations the pore of the inner mitochondrial membrane can open in substates with lower permeability (Hunter, D. R., and Haworth, R. A. (1979) *Arch. Biochem. Biophys.*, **195**, 468-477). Recently, we showed that Ca^{2+} loading of mitochondria augments the cyclosporin A-dependent decrease in transmembrane potential ($\Delta\Psi$) across the inner mitochondrial membrane caused by 10 μM myristic acid but does not affect the stimulation of respiration by this fatty acid. We have proposed that in our experiments the pore opened in a substate with lower permeability rather than in the "classic" state (Bodrova, M. E., et al. (2000) *IUBMB Life*, **50**, 189-194). Here we show that under conditions lowering the probability of "classic pore" opening in Ca^{2+}-loaded mitochondria myristic acid induces the cyclosporin A-sensitive $\Delta\Psi$ decrease and mitochondrial swelling more effectively than uncoupler SF6847 does, though their protonophoric activities are equal. In the absence of P_i and presence of succinate and rotenone (with or without glutamate) cyclosporin A either reversed or only stopped $\Delta\Psi$ decrease induced by 5 μM myristic acid and 5 μM Ca^{2+}. In the last case nigericin, when added after cyclosporin A, reversed the $\Delta\Psi$ decrease, and the following addition of EGTA produced only a weak (if any) $\Delta\Psi$ increase. In P_i-containing medium (in the presence of glutamate and malate) cyclosporin A reversed the $\Delta\Psi$ decrease. These data show that the cyclosporin A-sensitive decrease in $\Delta\Psi$ by low concentrations of fatty acids and Ca^{2+} cannot be explained by specific uncoupling effect of fatty acid. We propose that: 1) low concentrations of Ca^{2+} and fatty acid induce the pore opening in a substate with a selective cation permeability, and the cyclosporin A-sensitive $\Delta\Psi$ decrease results from a conversion of $\Delta\Psi$ to pH gradient due to the electrogenic cation transport in mitochondria; 2) the ADP/ATP-antiporter is involved in this process; 3) higher efficiency of fatty acid compared to SF6847 in the Ca^{2+}-dependent pore opening seems to be due to its interaction with the nucleotide-binding site of the ADP/ATP-antiporter and higher affinity of fatty acids to cations.

Key words: fatty acid, mitochondrial permeability transition, Ca^{2+}, cyclosporin A, nigericin, uncoupler, ADP/ATP antiporter, liver mitochondria

Mitochondrial $\Delta\Psi$ controls a series of general energy-dependent processes in cells. Recent studies show that significant $\Delta\Psi$ changes play an important role in a variety of physiological and pathological processes, particularly in apoptosis and necrosis. So, it is clearly of interest to study regulatory effects of endogenous effectors, such as long-chain fatty acids, on $\Delta\Psi$.

More than twenty five years ago fatty acids were found to affect mitochondrial energy coupling because they not only produce a specific uncoupling activity (protonophore effect), but also induce Ca^{2+}-dependent nonspecific low-molecular-weight compound permeability of the inner mitochondrial membrane due to Ca^{2+}-dependent pore opening [1]. Some properties described for the pore in early publications of Hunter, Haworth, and Southard [1-4] are given in the table. A brief review of these early studies is given in [5]. About ten years after the first reports of Hunter and Haworth, a specific inhibitory

Abbreviations: $\Delta\Psi$) transmembrane electric potential difference across the inner mitochondrial membrane; DNP) 2,4-dinitrophenol; P_i) inorganic phosphate; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine; SF, SF6847) 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitryl; CCCP) carbonyl cyanide 3-chlorophenylhydrazone.

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Some properties of the Ca^{2+}-dependent pore as revealed in the primary studies of Hunter, Haworth, and Southard

	Properties	Experimental data and conclusions	Reference
1	Changes in Mit properties when the pore is opened by Ca^{2+}, P_i, oleate, and arsenate	Mit swelling (configuration changes from aggregated to orthodox), uncoupling, induction of permeability towards sucrose, Mg^{2+}, Ca^{2+}, H^+, etc. Conclusion: Ca^{2+} and other inducers alter permeability rather than Mit configuration	[1]
2	Inhibitors of pore opening	EGTA, RR, La^{3+}, Mg^{2+}, BSA, and increased NADH/NAD^+ ratio	[1, 2]
	Inducers	Ca^{2+} (but not Sr^{2+}), oleate, P_i, and arsenate	
3	Effect of ADP and inhibitors of ADP/ATP antiporter	ADP and BA inhibit pore opening, and atractylate induces pore opening	[2, 3]
4	Existence of pore substates with lower permeability	RR-independent Ca^{2+} release is not accompanied by elevated permeability of the inner Mit membrane towards sucrose at low Ca^{2+} concentration	[4]
5	Effect of energizing	energizing of Mit inhibits pore opening; Mit uncoupling induces pore opening	[2, 4]
6	Pore flickering between opened and closed states	Pore closing kinetics after PEG addition at low Ca^{2+} concentration show evidence for the pore flickering between opened and closed states	[3]
7	Specific Ca^{2+}-binding site (Ca^{2+} trigger site)	Ca^{2+} trigger site resembles in some properties the high-affinity Ca^+-binding site, when Ca^{2+} uptake takes place	[3]
8	Pore size	Pore is permeable for substances with $M_r < 1000$ daltons, and impermeable for substances with $M_r > 1500$ daltons	[3]
9	Inhibition of substrate oxidation	Ca^{2+} entry into Mit inhibits the oxidation of NAD^+-independent substrates even before the pore opening	[1]

Note: Mit, mitochondria; RR, ruthenium red; BA, bongkrekic acid; PEG, polyethylene glycol.

effect of cyclosporin A on the pore was found [6]. And the ability of fatty acids to potentiate Ca^{2+}-dependent pore opening [1] was further confirmed in many studies [7-13].

ADP/ATP antiporter substrates and inhibitors strongly influence the Ca^{2+}-dependent pore; the ability of Ca^{2+} to open the pore depends on the conformation of this anion carrier (table, row 3, [14]). The involvement of ADP/ATP antiporter in the pore opening was shown in experiments on model systems. In particular, an oxidation of liposome-embedded ADP/ATP antiporter SH-groups converts the specific antiporter into a low-selectivity uniporter [15].

In most researchers' opinion, the ADP/ATP antiporter is a part of the pore-forming protein complex, in which it interacts with porin of the outer mitochondrial membrane, cyclophilin D of the matrix, and some other proteins. Both transition of ADP/ATP antiporter to *c*-conformation and oxidation of its SH-groups facilitate the pore opening ([16-18] and references therein).

There is no consensus of opinion on what properties of fatty acids are essential for the opening of the nonspecific Ca^{2+}-dependent pore. Some authors suggest a specific uncoupling effect of fatty acids (their protonophore effect) to play an important role in pore opening [11]. Others belittle the role of this effect [12]. However, the inference from the latter study might be assumed to be only preliminary, because the protonophore effect of CCCP was reported to be lower than that of comparable myristic acid.

A hypothesis was proposed that fatty acids act as negatively charged detergents [10]. However, to test this hypothesis additional experiments are necessary, in which fatty acids and anionic detergents should be compared by stimulation of pore opening and detergent effect.

A prevailing opinion is that ADP/ATP antiporter participates in pore induction by fatty acids in Ca^{2+}-loaded mitochondria [8, 11-13], and fatty acids facilitate ADP/ATP antiporter transition into *c*-conformation [11-

13]. The specific ADP/ATP antiporter inhibitor carboxyatractylate (that turns the antiporter into *c*-conformation) stimulates pore opening [7, 8, 11-13], but inhibits the specific uncoupling activity (protonophore effect) of fatty acids [19].

A "classic" pore opening increases the non-selective permeability of the inner mitochondrial membrane for substances with M_r less than 1500 daltons (table, row 8). However, the pore can open in "pore substates" with lower permeability (table, row 4), when Ca^{2+} concentration is low. The ADP/ATP-antiporter participates in functioning of a K^+-permeable pore, and K^+ is transported via cyclosporin A-sensitive and cyclosporin A-insensitive pathways [16, 21]. In experiments with de-energized mitochondria at gradually increased Ca^{2+} concentration the inner mitochondrial membrane permeability appeared first for K^+, and this effect was prevented by cyclosporin A [22]. Note that even with "classic" pore opening the inner mitochondrial membrane becomes permeable for K^+ and only after several minutes it also becomes permeable for Mg^{2+} and Ca^{2+} [20].

Pro-oxidants are well-studied pore modifiers. Some researchers suppose that pro-oxidants bring the pore to open in a substate in which it is selectively permeable for H^+, and the efflux of Ca^{2+} from mitochondria via Ca^{2+}-uniporter is due to the $\Delta\Psi$ decrease [23]. Others suppose that pro-oxidants can induce cyclosporin A-insensitive cationic channel (impermeable for H^+) that either acts in parallel with the "classic" pore or is the opening step of this pore [24].

As we have shown previously, loading of mitochondria with Ca^{2+} potentiates the cyclosporin A-dependent decrease in $\Delta\Psi$ by 10 μM myristic acid, but does not affect the stimulation of respiration by this fatty acid. These data suggest that under our experimental conditions the pore opens not in the "classic" state (table, rows 1 and 8), but in a substate with restricted permeability [25].

The study presented is aimed to investigate a mechanism of cyclosporin A-dependent $\Delta\Psi$ decrease by low concentrations (5-10 μM) of fatty acid and Ca^{2+}. Our main concern is thus to find out how sensitive the pore opening in the substate studied is to cyclosporin A, nigericin, and carboxyatractylate, and to clear up the role of specific uncoupling effect of fatty acid.

MATERIALS AND METHODS

Mitochondria were isolated from the liver of white rats (body weight about 180 g) by differential centrifugation. In some experiments (see below) rats were starved 12-15 h before the experiment (water was available in excess). The medium for isolation contained 250 mM sucrose, 1 mM EGTA, and 5 mM MOPS-KOH, pH 7.4. Liver homogenate was centrifuged for 10 min at 700*g*. Mitochondria (from supernatant) were sedimented at 9000*g* for 10 min, resuspended in 1 ml of the isolation medium (without EGTA) containing 3 mg/ml BSA, adjusted to 30 ml with isolation medium (without EGTA and BSA), and sedimented again at 12,000*g* for 10 min. The pellet was suspended in 30-50 μl of the isolation medium (without EGTA and BSA). Protein content in the mitochondrial suspension was 80-120 mg/ml.

To study high-amplitude swelling, the kinetics of light scattering by mitochondrial suspension was monitored by an Aminco DW2000 spectrophotometer in a split-beam mode, scale "Absorption". The oxygen uptake rate in mitochondrial suspension was determined polarographically using an LP9 polarograph equipped with a Clark electrode. Variations in $\Delta\Psi$ were estimated using safranin O dye [26]. The difference in absorption at 555 and 523 nm (ΔA) was determined on the Aminco DW2000 spectrophotometer in double-beam mode. Free Ca^{2+} concentration in isolation and incubation media (without EGTA) was estimated using the metallochromic indicator Arsenazo III [27] on the Aminco DW2000 spectrophotometer in double-beam mode; the difference in absorption at 666 and 692 nm was determined. This concentration was about 10 μM.

All measurements were performed at 26°C; mitochondrial suspension was stirred using a Teflon-coated magnetic stir bar. Incubation medium compositions are given in legends to figures.

Mitochondrial protein was determined by the biuret assay using BSA as a standard. The mitochondrial protein content was about 0.7 mg/ml when $\Delta\Psi$ and swelling of mitochondria were studied, and about 1 mg/ml when oxygen uptake was recorded.

The following chemicals were used: MOPS, myristic acid, oligomycin, carboxyatractylate, nigericin, safranin O, gramicidin D, potassium succinate, fatty acid-free BSA, potassium glutamate, and β-hydroxybutyrate (Sigma, USA); EGTA, rotenone, and calcium chloride (Serva, Germany); 2,4-DNP, cyclosporin A, and TMPD (Fluka, Germany). Sucrose (manufactured in Russia) was recrystallized by precipitation from aqueous solution with previously distilled ethanol.

RESULTS

Comparative studies on kinetics of mitochondrial $\Delta\Psi$ and swelling during pore opening by myristic acid or SF in Ca^{2+}-loaded mitochondria. To prevent $\Delta\Psi$ decrease in samples stored for long periods, in some experiments we added 100 μM P_i to samples just before Ca^{2+} and myristic acid (in the presence of oligomycin).

To compare the effects of myristic acid and SF on pore opening, the concentrations of these substances were chosen so that their protonophore activities were

equal. We estimated the protonophore activities of myristic acid and SF from their effect on mitochondrial respiration and ΔΨ. For these experiments, 1 mM EGTA was added into the medium for washing and suspending of mitochondria (see "Materials and Methods" section), and the incubation medium contained 0.5 mM EGTA. In these experiments 5 μM myristic acid stimulated respiration and decreased ΔΨ approximately to the same extent as 1-2 nM SF.

Under our experimental conditions 2.5 nM SF induces the cyclosporin A-sensitive pore less effectively than 5 μM myristic acid does: in the presence of SF both ΔΨ decrease (Fig. 1, a and c) and the swelling of mitochondria (Fig. 1, b and d) developed slower. Independently of what substance (myristic acid or SF) induced the pore opening, ADP retarded and carboxyatractylate accelerated the ΔΨ decrease (data not shown).

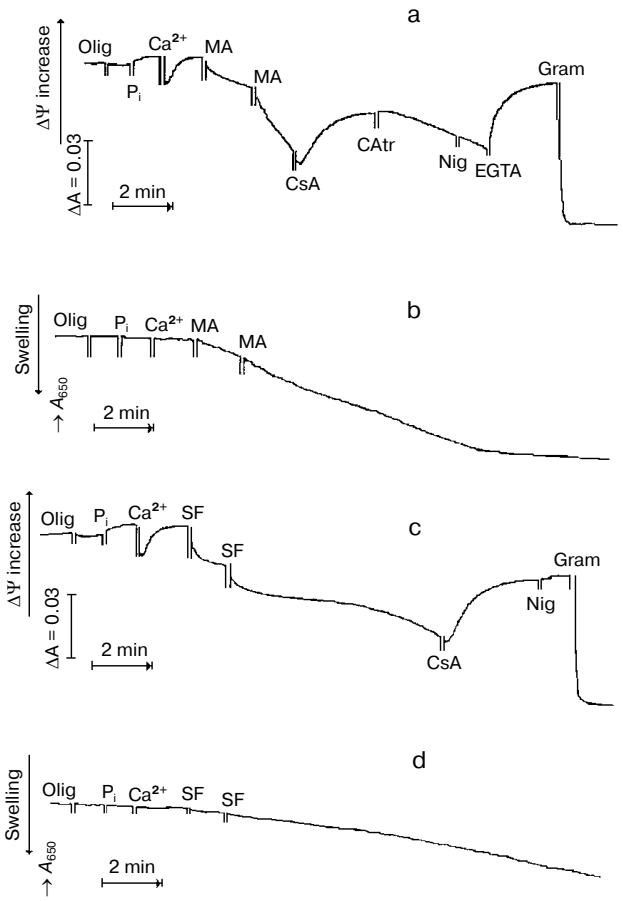

Fig. 1. ΔΨ decrease and mitochondrial swelling caused by Ca^{2+} and either myristic acid or SF. The incubation medium contained 5 mM MOPS-KOH (pH 7.4), 250 mM sucrose, 5 μM EGTA, 5 mM potassium succinate, 3 mM potassium glutamate, 0.1 mg/ml BSA, rotenone (2 nmol/mg protein), 10 μM safranin O, and mitochondria (0.7 mg/ml protein). Additions: Olig, oligomycin (2 μg/mg protein); P_i, 100 μM KH_2PO_4; Ca^{2+}, 5 μM $CaCl_2$; MA, 5 μM and 2.5 μM myristic acid; SF, 2.5 nM and 1.25 nM SF; CsA, cyclosporin A (0.4 nmol/mg protein); CAtr, carboxyatractylate (0.5 nmol/mg protein); Nig, 4 nM nigericin; EGTA, 500 μM EGTA; Gram, gramicidin D (0.4 μg/mg protein).

These data show that similar (on the qualitative level) ΔΨ and mitochondrion swelling kinetics is observed when either myristic acid or protonophore uncoupler SF opens the cyclosporin A-sensitive pore. However, the fatty acid is more potent pore inducer than the uncoupler is, despite their equal protonophore activities (Fig. 1). Similar conclusions were made earlier from studies on the "classic" pore [11].

In incubation medium containing 0.5 mM EGTA an addition of 5-10 μM myristic acid led to a negligible ΔΨ decrease. And in incubation medium containing 5 μM EGTA the addition of 5-10 μM myristic acid following the addition of 5-10 μM $CaCl_2$ led to a considerable ΔΨ decrease ([25] and Fig. 1a). However, in both cases the respiration levels in the presence of fatty acid as well as the levels of respiratory stimulation by fatty acid were equal. The respiration rates before and after 0.5 mM EGTA was added were, respectively, 9.7 ± 0.07 and 9.1 ± 0.07 nmol O_2/min per mg protein before and 14.1 ± 0.09 and 13.8 ± 0.1 nmol O_2/min per mg protein after myristic acid was added (the mean values of five experiments are given). Earlier we obtained similar data [25].

The increase in respiration rate under low uncoupling may occur without ΔΨ decrease (when the respiratory activity is high enough, that is at high rate of ΔΨ generation). However, in our experiments we observed an opposite effect: ΔΨ decrease with no respiratory stimulation. Such kind of effect is characteristic of ΔΨ to ΔpH conversion observed particularly upon electrogenic cation transport into the matrix.

Thus, under our experimental conditions ΔΨ decrease under the influence of low (5-10 μM) concentrations of Ca^{2+} and fatty acid cannot be explained from a specific uncoupling effect of fatty acids.

Effect of nigericin on ΔΨ decreased by Ca^{2+} and myristic acid. When mitochondria were incubated in the presence of succinate (and rotenone in either presence or absence of glutamate), cyclosporin A blocked the Ca^{2+}- and myristic acid-induced ΔΨ decrease. In incubation media without P_i cyclosporin A either reversed this effect of Ca^{2+} and myristic acid or only stopped ΔΨ decrease depending on the animals' living conditions before the experiment. When the animal starved before the experiment (see "Materials and Methods" section), cyclosporin A reversed the myristic acid-induced ΔΨ decrease in most experiments, as we described previously [25]. When food was not withheld beforehand, then, as a rule, cyclosporin A in the absence of P_i did not reverse, but only stopped the myristate- and Ca^{2+}-induced ΔΨ decrease (Figs. 2 (b and c) and 3a). The typical for such mitochondria effects of cyclosporin A, nigericin, and carboxyatractylate on ΔΨ

upon its decrease by Ca^{2+} and myristic acid are shown in Figs. 2 and 3. Taken separately, 5 μM Ca^{2+} and 5 μM myristic acid decreased ΔΨ in significantly less than when added simultaneously (Fig. 2, b and c). We observed a sharp drop of ΔΨ under the action of myristic acid and Ca^{2+} taken together also in the presence of cyclosporin A as well (Fig. 2a).

In the absence of P_i, Ca^{2+} influx into mitochondria is accompanied by a considerable alkalosis of the matrix [28] that leads to ΔΨ decrease also in the presence of cyclosporin A. As it takes place, Ca^{2+} concentration in the matrix decreases below the level necessary not only for the "classic" pore opening (table, row 7), but presumably for the pore opening in a substate characterized by the fast cyclosporin A-sensitive ΔΨ decrease [25]. Note that fatty acids can transport cations via a cyclosporin A-independent pathway through the inner mitochondrial membrane [29, 30] and through a liposome membrane [31].

The fast drop of ΔΨ was followed by its slow decrease with a constant rate (Fig. 2d). The latter was sensitive to cyclosporin A that stopped this ΔΨ decrease (Fig. 2, b and c). Ethanol taken in equal volume had no effect on the rate of ΔΨ decrease (Fig. 3, a and b).

Nigericin added after (Figs. 2b and 3a) or instead of cyclosporin A (Fig. 3b) almost completely restored the ΔΨ level. Subsequent addition of EGTA did not alter or only slightly increased ΔΨ.

Note that in samples without cyclosporin A carboxyatractylate added before can reduce or cancel the effect of nigericin (Fig. 2d). One can suppose that carboxyatractylate expresses this activity at relatively higher intramitochondrial Ca^{2+} concentration. As it took place, carboxyatractylate (which favors ADP/ATP antiporter transition into *c*-conformation) could accelerate the cation influx to the point where it became comparable to the rate of K^+/H^+ exchange caused by nigericin. In this case nigericin was unable to increase ΔΨ, and the latter increased after the following EGTA addition (Fig. 2d).

Cyclosporin A displayed its effect either in medium containing both succinate and rotenone (Fig. 2) or in the same medium supplied with glutamate (Fig. 3a). Effects of nigericin and cyclosporin A also persisted when 3 mM β-hydroxybutyrate was added to samples along with succinate and rotenone (data not shown).

During the slow cyclosporin A-dependent ΔΨ decrease a slight swelling of mitochondria was observed (Fig. 3c). The swelling accelerated after 0.1 mM P_i was added; further swelling was stimulated by 3 mM P_i. It is well-known that Ca^{2+}-induced swelling increases after P_i addition; this is explained by faster and more complete influx of Ca^{2+} into the matrix [14, 28] resulting in larger portion of mitochondria whose pores are opened.

The mitochondrial swelling can result from the prevailing cation influx into the matrix (via uniporter) over its efflux in exchange for protons (via antiporter). If both the processes are comparable in rate, the swelling is weak,

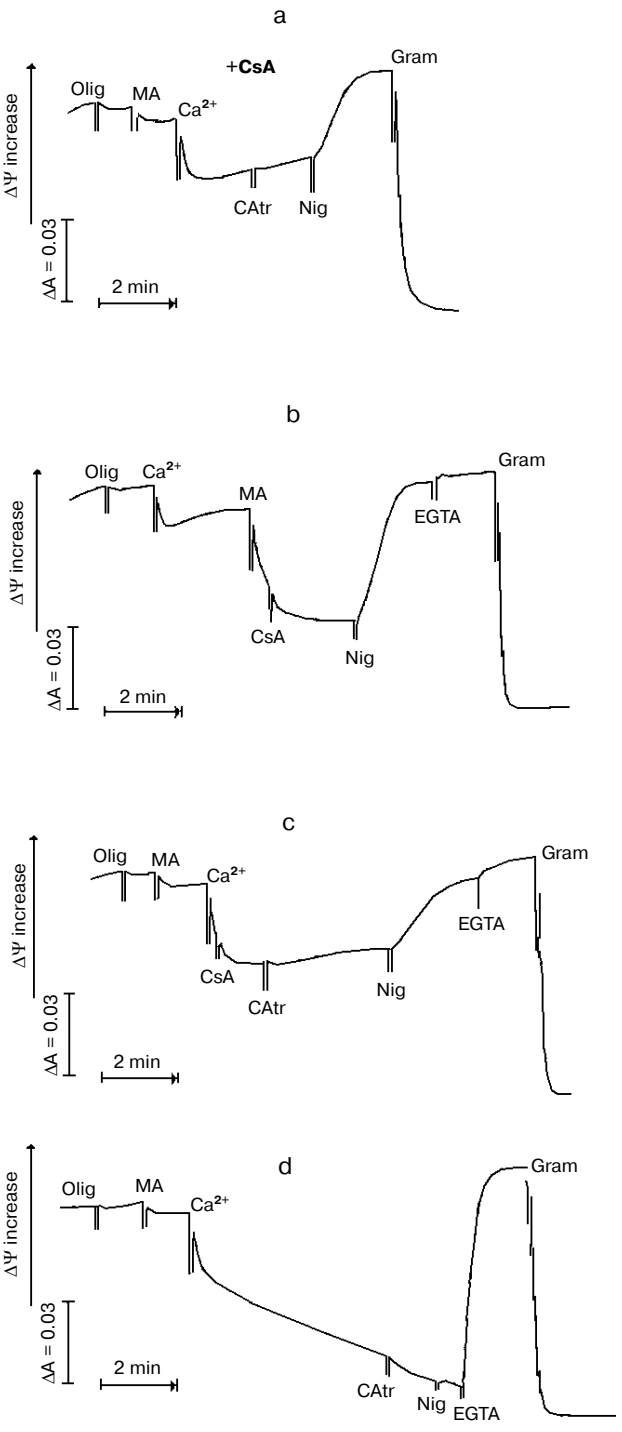

Fig. 2. Effects of cyclosporin A and nigericin on the kinetics of ΔΨ when the pore is opened by myristic acid and Ca^{2+} in the presence of succinate. The incubation medium contained 250 mM sucrose, 5 mM MOPS-KOH, pH 7.4, 5 μM EGTA, 5 mM potassium succinate, rotenone (2 nmol/mg protein), 0.1 mg/ml BSA, 10 μM safranin O, and mitochondria (0.7 mg/ml protein). Additions: MA, 5 μM (curves (a), (c), (d)) or 7.5 μM (curve (b)) myristic acid; in the variant (a) cyclosporin A (0.4 nmol/mg protein) was added into the medium simultaneously with mitochondria. Other additions and their notations are the same as in Fig. 1.

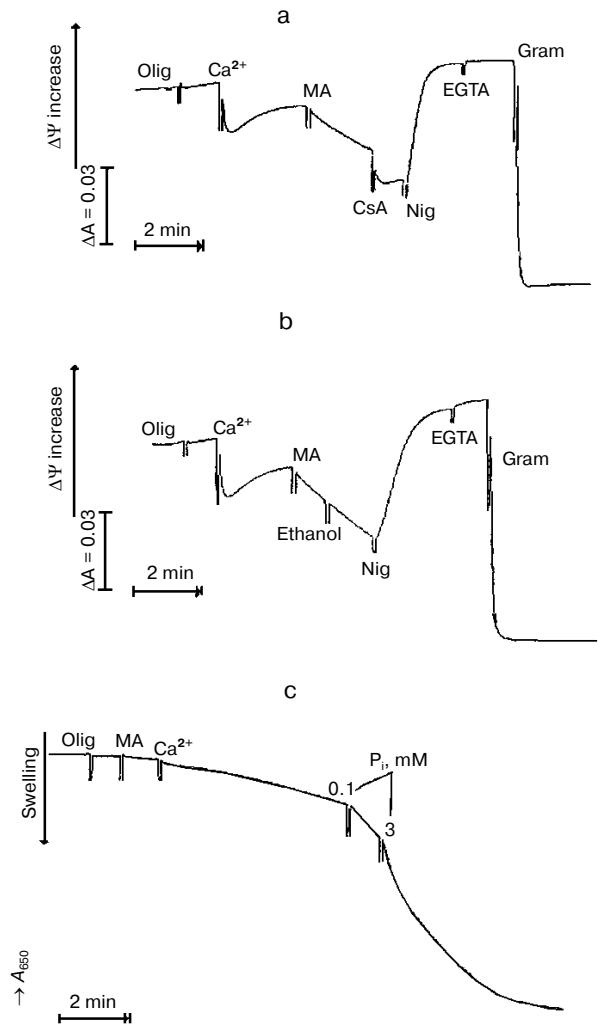

Fig. 3. Nigericin-induced reverse of ΔΨ decreased by Ca^{2+} and myristic acid. The incubation medium contained 250 mM sucrose, 5 mM MOPS-KOH, pH 7.4, 5 μM EGTA, 3 mM potassium glutamate, 5 mM potassium succinate, rotenone (2 nmol/mg protein), 0.1 mg/ml BSA, 10 μM safranin O, and mitochondria (0.7 mg/ml protein). Additions: MA, 5 μM myristic acid; Ethanol, 2.5 μl ethanol in 2 ml incubation medium. Other additions and their notations are the same as in Fig. 1.

but respiration is activated. In our experiments the respiration rate was unchanged when ΔΨ decreased and the swelling developed under the action of Ca^{2+} and myristic acid in the presence of 100 μM P_i. This indicates that cyclic cation transport is either absent or considerably slower than electrogenic cation influx into the matrix of mitochondria.

Glutamate and malate as oxidative substrates. NAD^+-dependent substrates are not commonly used in studies on the "classic" pore, because of the respiratory inhibition occurring from before the pore opening (table, row 9). Under our experimental conditions the pore opening and closing kinetics were also observed for at least 10-min incubation when glutamate and malate were used as oxidative substrates.

Cyclosporin A almost completely reversed the myristic acid-induced ΔΨ decrease (Fig. 4a). This fact means that in our experiments the pore opened in a substate in which a strong inhibition of NAD^+-dependent substrate oxidation is not yet achieved. The following addition of carboxyatractylate results in ΔΨ increase (Fig. 4a), thus providing additional evidence for complete closing of the myristic acid-induced pore by cyclosporin A under those conditions. This effect of carboxyatractylate is characteristic of the specific uncoupling effect of fatty acids [19], whereas in the case of Ca^{2+}-dependent uncoupling effect of fatty acids the carboxyatractylate addition might result in the "classic" pore opening accompanied by respiratory stimulation [7, 19] and ΔΨ decrease [8, 12, 13, 25].

The observed decrease in ΔΨ level was accompanied by the swelling of mitochondria (Fig. 4b).

In the presence of glutamate and malate DNP increased the respiration rate (in the presence of oligomycin) by more than sixfold.

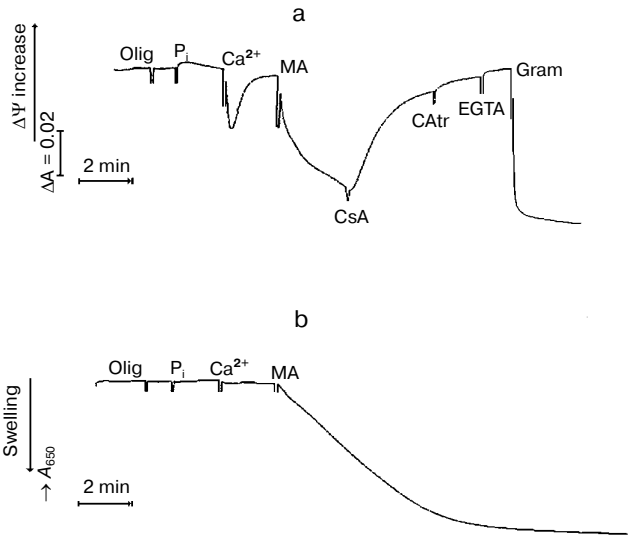

Fig. 4. Pore opening upon NAD^+-dependent substrate oxidation. The incubation medium contained 250 mM sucrose, 5 mM MOPS-KOH, pH 7.4, 5 μM EGTA, 4 mM potassium glutamate, 1 mM malate, 10 μM safranin O, and mitochondria (0.7 mg/ml protein). Additions: Ca^{2+}, 10 μM $CaCl_2$; MA, 5 μM myristic acid. Other additions and their notations are the same as in Fig. 1.

DISCUSSION

To examine the pore substates it is necessary to provide stable low intramitochondrial Ca^{2+} concentration for a long time. As a rule, de-energized mitochondria are used for this purpose. We could not use this approach for studies on the mechanism of $\Delta\Psi$ decrease caused by Ca^{2+} and fatty acids.

To extend the lifetime of the pore substate studied we performed our experiments under conditions when the "classic" pore opening probability was small. To accomplish this, we increased both NADH/NAD^+ ratio (table, row 2, [32]) and endogenous ADP concentration (table, row 3) by the addition of rotenone and oligomycin A into the succinate-containing medium for incubation of mitochondria. In most experiments we did not add P_i into the medium to decrease Ca^{2+} uptake by mitochondria.

The data we gained to suggest that under our experimental conditions $\Delta\Psi$ decrease caused by low concentrations of Ca^{2+} and fatty acid results from the pore opening in a substate with selective permeability for cations except H^+ (as suggested for prooxidants [24]); and cyclosporin A-sensitive $\Delta\Psi$ decrease results from $\Delta\Psi$ to ΔpH conversion due to the electrogenic cation transport into mitochondria. Effects of carboxyatractylate are also indicative of ADP/ATP antiporter involvement in the $\Delta\Psi$ decrease upon the pore opening in the substate studied (Fig. 2d; see also [25]).

It is well-known that $\Delta\Psi$ increase leads to dramatic decrease in the pore opening probability [33], and this fact explains the inhibitory effect of nigericin on the pore opening. The ability of nigericin to increase $\Delta\Psi$ level decreased by Ca^{2+} and myristic acid (Figs. 2 and 3) is thus evidence that this decrease in $\Delta\Psi$ was due to its conversion to pH gradient.

Both low rate of cyclosporin A-dependent $\Delta\Psi$ decrease (Fig. 2d) and stable $\Delta\Psi$ increase by saturating nigericin concentration in the absence of cyclosporin A (Fig. 3b) are indicative of either very slow cation influx or short-term cationic conductivity due to the pore flickering between the opened and closed states (table, row 6; see also review [18]). If cation influx into mitochondria was not significantly slower than K^+/H^+ exchange by nigericin (that is, the rates of both processes were similar) then nigericin would induce the uncoupling effect accompanied by both stimulation of respiration and $\Delta\Psi$ decrease rather than $\Delta\Psi$ increase.

When comparing the effects of myristic acid and SF, note that a fatty acid is characterized by its interaction with the nucleotide-binding center of ADP/ATP antiporter and by high affinity to Ca^{2+}.

Due to the complex formation with Ca^{2+}, fatty acids can transport Ca^{2+} through hydrophobic membrane parts [31]. Myristic acid and Ca^{2+} taken in combination decrease $\Delta\Psi$ to a far greater extent than each of them taken separately (Fig. 2, b and c). It has long been known that fatty acids potentiate Ca^{2+}-dependent stimulation of both swelling and respiration. This is because of increased phospholipase A_2 activity ([34] and references therein).

It remains unclear how phospholipase A_2 is involved in the pore induction [10]. The pore opening is probably accompanied by some alterations in phospholipid composition at least in the pore-forming protein location. Note that protonophore uncouplers, such as DNP, at low concentration can increase phospholipase A_2 activity [35].

Many researchers think that fatty acids potentiate the pore opening by facilitating the ADP/ATP antiporter transition into *c*-conformation [11-13]. According to the hypothesis of Skulachev, in the course of uncoupling fatty acid anions are transported via ADP/ATP antiporter across the hydrophobic barrier of the inner mitochondrial membrane; in this process they interact with the nucleotide-binding site of this anion carrier [36]. This would shorten the period of ADP/ATP antiporter interaction with ADP and thereby elevate the probability of antiporter transition into *c*-conformation and, hence, the probability of the pore opening. SF would not be able to do so, because the ADP/ATP antiporter is involved in the specific uncoupling effect of fatty acids [19], but not of SF [37].

It is of interest to find out whether fatty acids under physiological conditions cause the pore opening in the substate examined and/or whether this substate is the initial step of the pore opening by fatty acids. Note that the cytoplasmic P_i concentration is significantly higher, whereas cytoplasmic Ca^{2+} concentration is significantly lower than those in our experiments. It was previously found that 5-10 μM palmitic acid added to the liver mitochondria stimulated the oxidation of succinate with kinetics characteristic of the pore opening in almost equal extents independently of whether 100 μM $CaCl_2$ (in the presence of endogenous P_i) or 1 mM P_i (in the presence of endogenous $CaCl_2$) was present [7]. This problem requires further studies.

We propose that partial conversion of $\Delta\Psi$ into ΔpH caused by Ca^{2+} and fatty acids is efficient when it is necessary to enhance the capacity of mitochondrial energy buffer [38] or to accelerate the transport of oxidative substrates or other substances into mitochondria along the pH gradient. Note that a moderate $\Delta\Psi$ decrease virtually did not affect the Ca^{2+} influx into mitochondria [18] though it might impair other $\Delta\Psi$-dependent functions.

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