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Synergetic Inhibition of the Brain Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I) by Fatty Acids and Ca^{2+}

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Abstract—The NADH:ubiquinone oxidoreductase (respiratory complex I) activity of inside-out pig brain submitochondrial particles is inhibited by endogenous or externally added free fatty acids in time-dependent fashion. The rate and degree of the inhibition is dramatically increased by Ca^{2+}. The Ca^{2+}-promoted, fatty acid-induced inhibition is pH dependent, this being particularly evident at pH > 8.0. The inhibition is completely reversed by either EGTA or by bovine serum albumin (BSA). BSA prevents previously described (Kotlyar, A. B., Sled, V. D., and Vinogradov, A. D. (1992) *Biochim. Biophys. Acta*, **1098**, 144-150) inhibitory effect of Ca^{2+} and alkaline pH on the de-active-to-active form transition of complex I. A possible mechanism of synergetic inhibition on complex I by Ca^{2+} and fatty acids is discussed.

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Key words: NADH:ubiquinone oxidoreductase, complex I, fatty acids, divalent cations, brain mitochondria

The final step of the energy-producing nutrient decomposition in mammalian cells proceeds in mitochondria, where NAD^+ required for oxidation is provided by operation of NADH:ubiquinone oxidoreductase (respiratory complex I). The mitochondrial enzyme or its prokaryotic homologs (NDH-1) catalyze oxidation of intramitochondrial or cytoplasmic NADH by ubiquinone coupled with vectorial translocation of protons across the inner mitochondrial (cytoplasmic) membrane. Mammalian complex I is the largest component of the mitochondrial respiratory chain composed of 45 different subunits [1] harboring at least nine distinct redox components [2]. Bacterial operons encoding NDH-1 contain only 13-14 genes [3-5], and their transcription products are highly homologous to 14 core subunits of the mammalian complex I [6]. As the catalytic properties of the mitochondrial enzyme and its prokaryotic homologs are very similar [7], it is safe to assume that only 14 out of 45 subunits of the mammalian enzyme are required for the catalytic capacity of complex I. The functions of the more than 30 accessory subunits remain obscure. The homologous enzymes in lower eukaryotes (*Neurospora crassa* [8], *Yarrowia lipolytica* [9]) and plants [10] are also very complex, being composed of more than 30 individual subunits.

Very recently the three dimensional structures of *E. coli* NDH-1 [11] and *Y. lipolytica* complex I [12] have been determined at 3.9 and 6.3 Å resolution, respectively. The overall L-shaped protein structure consists of two distinct parts: the hydrophilic one is situated almost perpendicular to the membrane plane and protrudes into the mitochondrial matrix, and the hydrophobic part is positioned within the membrane plane. The redox components, FMN and iron–sulfur centers, are located in the hydrophilic part as a chain ended by the iron–sulfur cluster N-2, the site where hydrophobic quinone is presumably reduced. The molecular events at N-2–the quinone junction site where one-electron donor (N-2) reduces two-electron acceptor (quinone) remain obscure, although all the data accumulated so far suggest that they are crucial for electrochemical energy accumulation.

A peculiar property of bovine heart [13] and pig brain complex I [14] as well as those of the homologous enzymes in a number of other eukaryotic species [15, 16] is that they exist in two slowly interconvertible forms: the A-form which is capable of full catalytic activity and the catalytically inactive D-form. The slow A-to-D-form transition occurs when the enzyme resides under condi-

Abbreviations: SMP, submitochondrial particles.

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tions where catalytic turnover is not permitted, i.e. in the absence of NADH and/or oxidized ubiquinone. The slow D-to-A-form transition takes place when the D-form is exposed to permissive conditions that allow turnover. The A-to-D-form transition is highly temperature-dependent, although significantly different activation barriers have been estimated for the enzyme from different species [13, 15, 16]. Thus, pronounced de-activation of the mammalian complex I is seen only at elevated temperature (above 30°C).

The D-form is irreversibly modified by a number of the SH-reagents [17-19], which prevent the turnover-induced activation, whereas the A-form is insensitive to the SH-reagents. The A/D-sensitive SH-group located in ND3 subunit of the bovine heart complex I [20] is exposed to the hydrophilic matrix environment. The D-to-A-form transition is retarded at alkaline pH and in the presence of divalent cations [17].

It has been shown over the years by several independent groups that free fatty acids inhibit electron transfer activities, and their inhibitory efficiency is substantially higher for NADH oxidase activity than that for respiration with succinate or for cytochrome *c* oxidase [21-30]. The phenomenology of inhibitory effects of free fatty acids (particularly palmitate) on complex I activities in inside-out bovine heart submitochondrial particles (SMP) as related to the A/D transition have been described [30]. We have shown that palmitate inhibits the NADH oxidase and NADH:quinone reductase activities and also decreases the rate of the D-to-A-form transition.

Recently we succeeded in the large-scale preparation of coupled inside-out SMP from pig brain mitochondria [14]. In light of the growing evidence for the involvement of complex I in a number of pathophysiological states, particularly in neurodegenerative diseases, and the importance of free fatty acids for metabolism under normal and pathological conditions, it seemed worthwhile to get closer insight into the nature of the complex I–free fatty acid interaction. Here we show that the inhibitory effects of free fatty acids and Ca^{2+} on brain complex I are mutually dependent. Preliminary results of this study were reported in abstract form [31].

MATERIALS AND METHODS

Chemicals. NAD(P)H, fatty acids, EGTA, EDTA, Tris, BSA, hexaammineruthenium (III) chloride, alamethicin, gramicidin D, and rotenone were from Sigma-Aldrich (USA). Sucrose was from MP Biomedicals, Inc (USA). Other chemicals of highest purity were obtained from local suppliers. The stock 10 mM solution of palmitic acid was prepared by dissolving the fatty acid in ethanol and stored at 4°C before use.

Pig brain SMP were prepared and stored as described [14].

Respiratory activity was assayed as oxygen consumption measured by a covered Clark-type electrode at 25°C in reaction mixture composed of 0.25 M sucrose, 10 mM KCl, 0.2 mM EDTA, 5 mM potassium phosphate, pH 7.4, and the respiratory substrates, 1 mM NADH or 10 mM potassium succinate. When succinate oxidase activity was assayed, the samples were preincubated 5-10 min in the presence of 5 mM succinate and 5 μM rotenone.

NADH oxidase was measured photometrically as NADH concentration decrease ($\varepsilon_{340} = 6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) in the standard assay mixture composed of 100 μM NADH, 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, and 0.2 μg/ml gramicidin D.

Protein content was determined by the biuret assay.

RESULTS

We have shown previously that the D-to-A-form transformation is inhibited by either increase of pH or by divalent cations, particularly Ca^{2+} [17]. *In vitro* mitochondria are capable of the energy-dependent accumulation of large amounts of Ca^{2+}, although the concentration of free Ca^{2+} in the mitochondrial matrix depends on a number of particular conditions such as the nature of the penetrating anion, pH, or presence of nucleotides. The possibility of regulatory effect of Ca^{2+} accumulation on catalytic activity of complex I have been discussed. Thus, it was of interest to determine whether Ca^{2+} externally added to inside-out brain SMP influences their complex I activity. We will show here that Ca^{2+} inhibits complex I activity, and the inhibition depends on free fatty acids – either endogenous or externally added. Note should be made that the phenomenology described below is not specific for Ca^{2+}, and qualitatively some effects were observed for Mg^{2+}. Since the intramitochondrial Ca^{2+} concentration is believed to vary greatly depending on the physiological state, whereas Mg^{2+} content seems to be relatively constant, our studies were focused on the Ca^{2+}-induced effects only.

Figure 1a shows that the addition of a high concentration of Ca^{2+} at alkaline pH to SMP resulted in time-dependent inhibition of their NADH oxidase activity. The inhibition was completely reversed by an excess of EGTA, also in time-dependent fashion (curve *3*). No inhibition of the succinate oxidase activity of SMP was seen under the conditions described in Fig. 1a (the results are not shown), thus the inhibitory effect of Ca^{2+} was directed to complex I. The artificial electron acceptor (hexaammineruthenium (III) chloride) [32] reductase activity of complex I was insensitive to Ca^{2+}, thus showing that the inhibition was directed to the ubiquinone junction site.

In an attempt to improve NADH oxidase activity of SMP, BSA was added to the assay mixture since free fatty

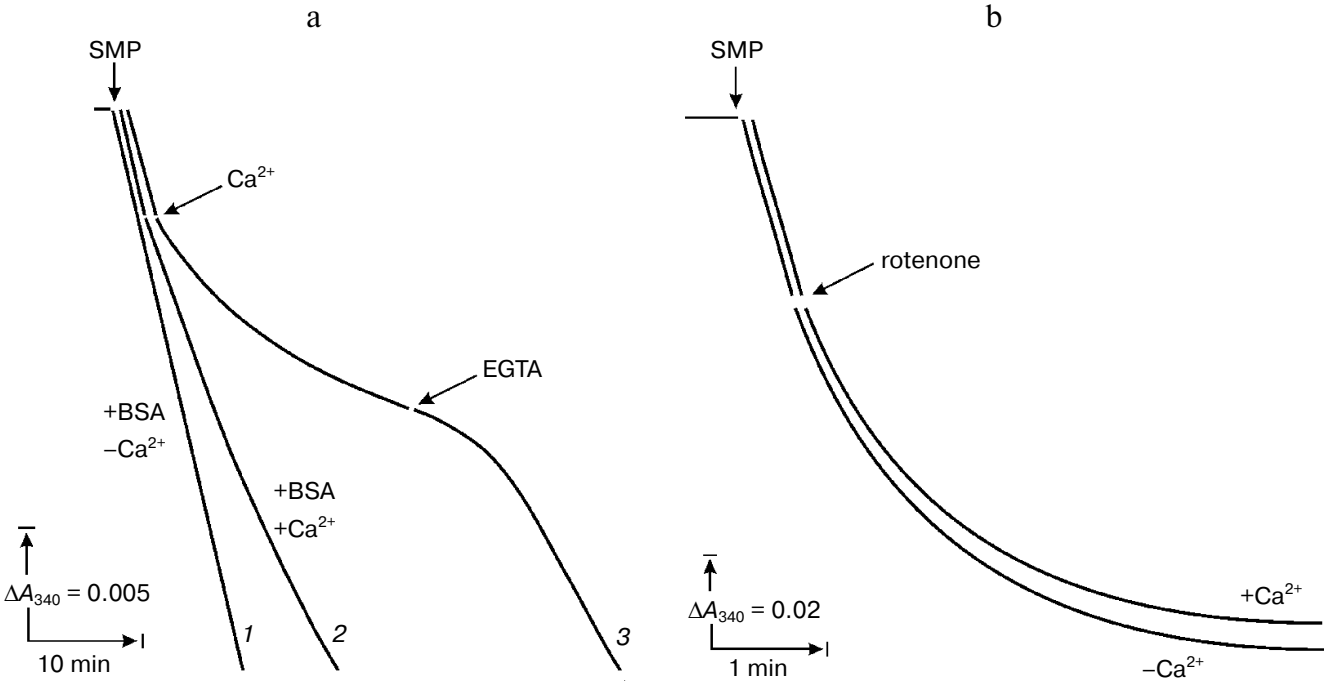

Fig. 1. Effect of Ca^{2+} on NADH oxidase of brain submitochondrial particles: a) 10 mM $CaCl_2$ and 15 mM EGTA were added where indicated to SMP (10 μg of protein per ml) oxidizing 100 μM NADH in the standard reaction mixture (see "Materials and Methods" section) at pH 8.9, 30°C. Curves: *1*) BSA (2 mg/ml) was present; *2*) Ca^{2+} was added to the sample in the presence of BSA; *3*) no BSA was added; b) inhibition of NADH oxidase by rotenone ($5 \cdot 10^{-8}$ M) in the presence or absence of Ca^{2+} (20 μg SMP protein per ml, 10 mM Ca^{2+}, pH 8.7, 30°C).

acids were shown to inhibit complex I [30]. As expected, BSA slightly (about 20%) increased NADH oxidase activity. Unexpectedly, BSA prevented the inhibitory effect of Ca^{2+} (curve *2*). These results suggested that the inhibitory effects of free fatty acids [30] and Ca^{2+} (Fig. 1a) are mutually dependent, and the phenomenon was further investigated. Two explanations of Ca^{2+}-potentiation of the inhibitory effect of fatty acid could be offered. The Ca^{2+} might bind to complex I, thus increasing accessibility (affinity) of the fatty acid-sensitive site to the inhibitor. Alternatively, the effect of Ca^{2+} might be due to Ca^{2+}–fatty acid–membrane phospholipid interaction that resulted in the phenomenon shown in Fig. 1a. If the first possibility were correct, the same or similar effect of Ca^{2+} on inhibition of complex I by other than free fatty acid hydrophobic N-2–quinone junction site-directed inhibitors could be expected. Figure 1b shows that this was not the case: Ca^{2+} affected neither the rate nor the final level of the rotenone-induced inhibition of complex I.

The pH dependence of the inhibitory effect of Ca^{2+} (at some, presumably constant level of endogenous free fatty acids) was evaluated (Fig. 2). Both the rate of Ca^{2+}-induced inhibition and apparent final level of partially inhibited NADH oxidase were strongly pH dependent with an apparent pK_a of about 8.5. It should be noted that because of obvious time-dependency of the inhibitory effect of Ca^{2+}, the final levels of NADH oxidase as depicted in Fig. 2b are arbitrary, and the pH-titration curve should be considered as only provisional.

Since the only plausible explanation for prevention of Ca^{2+}-induced inhibition by BSA seemed the presence of endogenous free fatty acids in SMP, it was of interest to investigate the phenomenon in the presence of externally added fatty acid, which is known to inhibit complex I.

Figure 3 shows that 10 mM Ca^{2+} only slightly inhibited NADH oxidase (curve *1*). Palmitate caused slowly (within the time scale used) developing inhibition (curve *2*). When the same concentration of Ca^{2+} was added after SMP had been respiring for about 1 min in the presence of palmitate, almost instant inhibition was evident (curve *3*). If palmitate was added after Ca^{2+}, no significant inhibition occurred, and the activity was the same as observed in the presence of Ca^{2+} alone (curves *4* and *1*, respectively). The data shown in Fig. 3 suggest that Ca^{2+} strongly promotes the inhibitory effect of the membrane-bound fatty acid; when fatty acid comes across the solution containing Ca^{2+}, insoluble salt precipitates and the inhibitory specie (free fatty acid) disappears.

The Ca^{2+}-promoted inhibitory effects of four other fatty acids – lauric, myristic, stearic, and palmitoleic – were tested, and some specificity was observed. Palmitate was the most effective (Fig. 4).

As it was briefly described in the introduction, complex I exists in two slowly interconvertible forms, and palmitate inhibits the D-to-A-form transition [30]. It

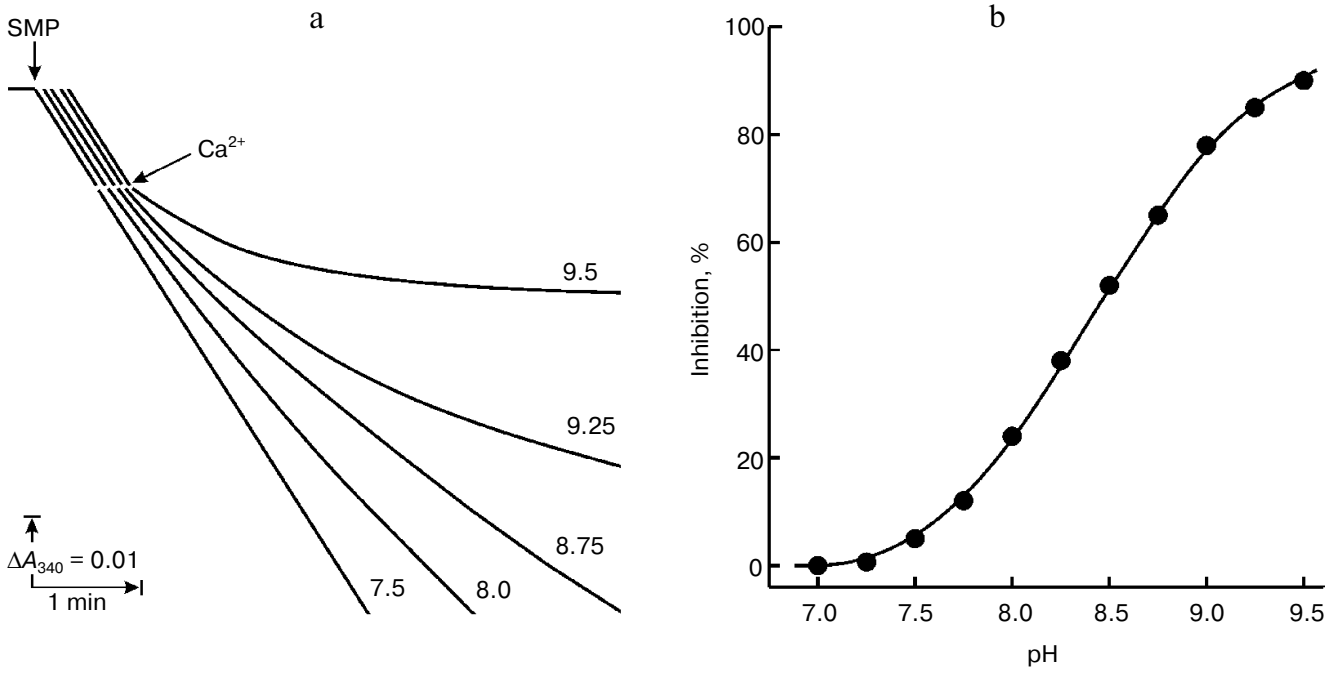

Fig. 2. The pH profile of the albumin-sensitive inhibitory effect of Ca^{2+} on NADH oxidase: a) actual tracings of NADH (100 μM) oxidation after the addition of Ca^{2+} (10 mM) at different pH values (indicated on the curves). The experimental conditions were as in Fig. 1a; b) inhibitory effect of Ca^{2+} expressed as the degree of inhibition (percent) developed 10 min after the addition of Ca^{2+}.

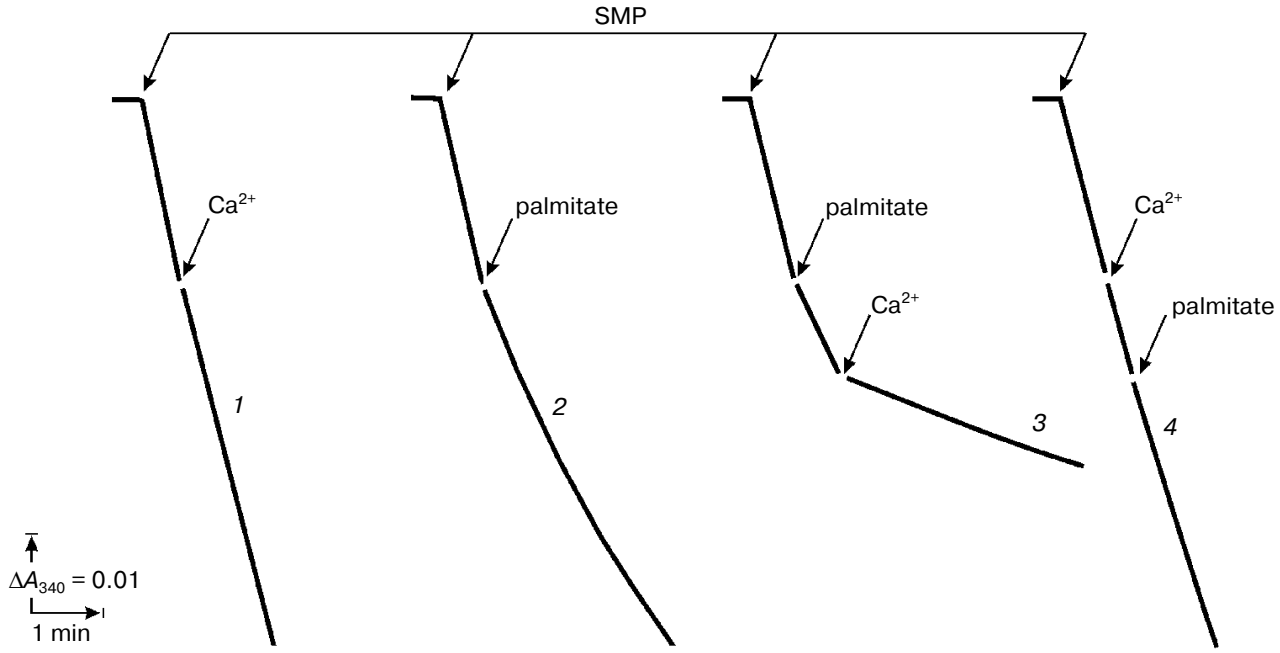

Fig. 3. Inhibitory effect of Ca^{2+} and added palmitate on NADH oxidase (10 μg of SMP protein per ml, pH 8.9, 30°C, 10 mM $CaCl_2$, and 50 μM palmitate).

could be proposed that time-dependent Ca^{2+}-promoted inhibitory effect of fatty acids is due to stabilization of the D-form, thus preventing its turnover-dependent activation. The de-activation process is extremely temperature dependent (the activation energy barrier was estimated as 230 and 260 kJ/mol for brain and heart enzyme, respectively [14, 33]). If the enzyme de-activation is responsible for the time-dependent inhibition, strong temperature

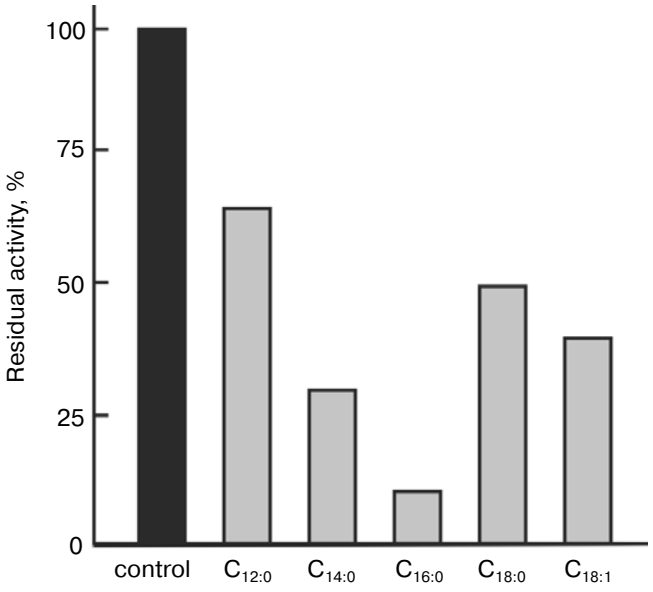

Fig. 4. Specificity of Ca^{2+}-dependent inhibition of NADH oxidase by free fatty acids (at 50 μM concentration). Experimental conditions were as in Fig. 3, curve *3*.

dependence of the process would be expected. When the rates of NADH oxidase inhibition by 50 μM palmitate initiated by 1 mM Ca^{2+} were measured at 25 and 37°C, only two-fold acceleration of the apparent first-order rate constant was observed. To follow the rate of Ca^{2+}-induced inhibition, the experiments were carried out at pH 8.7, i.e. under the conditions where the time dependence could be measured. We conclude that no de-activation of complex I proceeds during Ca^{2+}-promoted fatty acid-induced inhibition.

Although the data described above clearly demonstrate a synergism in the inhibitory effects of Ca^{2+} and free fatty acids, they are hard to quantitate because of their prominent time dependence. To avoid this difficulty the inhibitory effect of Ca^{2+} was titrated under the conditions where SMP were preincubated with palmitate for 30 min (the time presumably needed for equilibration), and the residual NADH oxidase activities in the presence of increasing Ca^{2+} concentration were measured. Under those conditions apparent affinity for Ca^{2+} was dramatically increased, and half-maximal inhibition was seen at 0.3 mM Ca^{2+}. Further increase of preincubation time (with fatty acid) did not affect the titration curve (Fig. 5a). The titration NADH oxidase activity by added palmitate in the presence of high (10 mM) Ca^{2+} showed half-maximal inhibition at 5 μM (0.5 μmol/mg protein) (Fig. 5b).

As noted in the introduction, divalent cations and alkaline pH strongly retard the turnover-dependent D-to-A-form transition of complex I [17]. On the other hand, palmitate was also shown to inhibit the turnover-dependent activation of de-activated complex I [30]. If these findings are to be placed side by side with the data described in this study, an obvious hypothesis arises: the

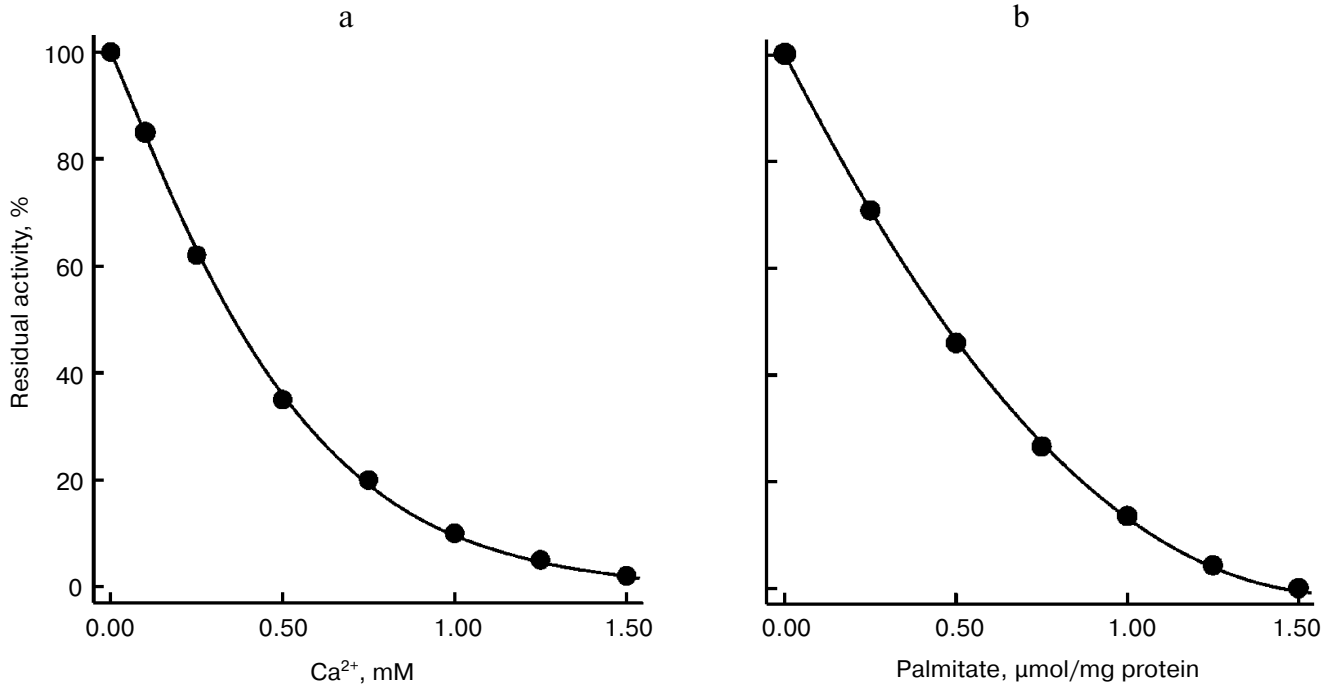

Fig. 5. "Equilibrium" titration of Ca^{2+}/fatty acid-induced inhibition of NADH oxidase: a) SMP were preincubated for 30 min (pH 8.7, 22°C) in the presence of palmitate (1 μmol per mg protein) and the residual activity was measured in the presence of Ca^{2+}; b) SMP were preincubated for 30 min (pH 8.7, 22°C) in the presence of palmitate, and the residual activity was measured in the presence of 1.5 mM $CaCl_2$.

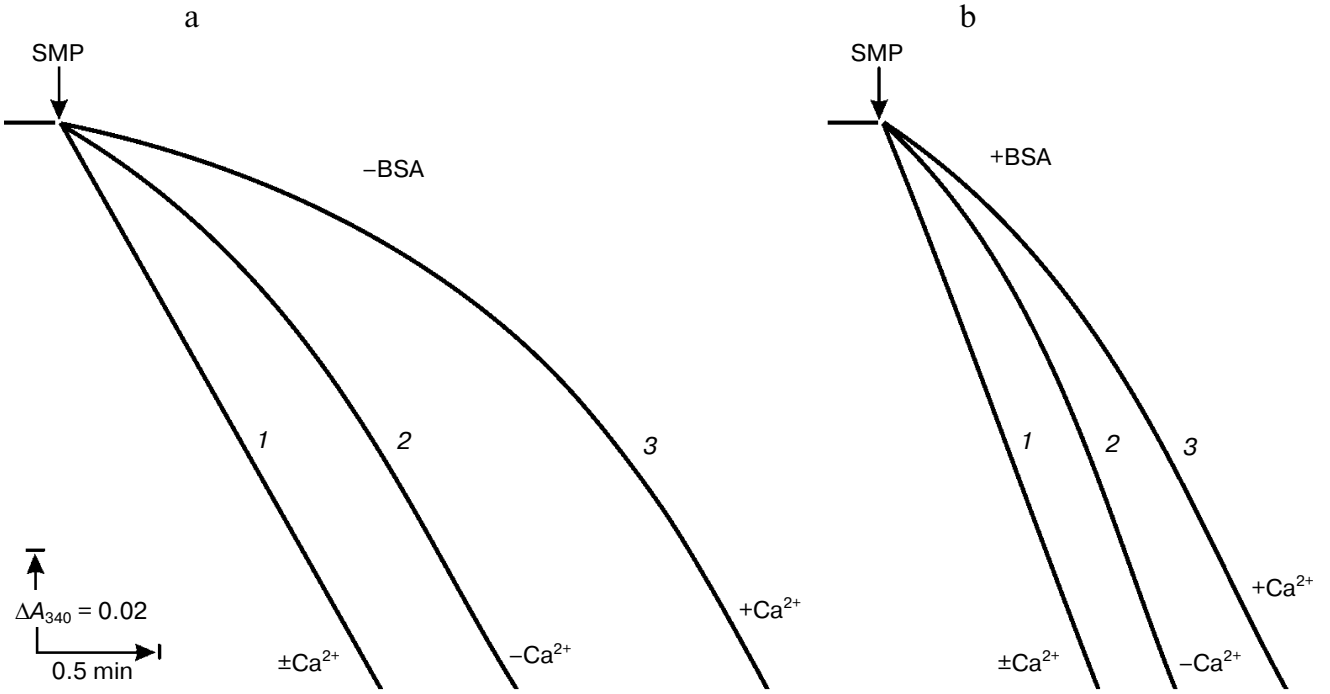

Fig. 6. Effect of albumin on activation of thermally de-activated NADH oxidase. SMP (4 mg/ml) were de-activated by preincubation for 15 min at 37°C. NADH oxidation at 30°C was initiated by the addition of SMP (20 μg/ml). Assays in the absence (a) or presence (b) of BSA (2 mg/ml), respectively. Curves: *1*) active SMP (no preincubation at 37°C); *2*) de-activated SMP; *3*) as in (*2*), 0.5 mM $CaCl_2$ was added to the assay mixture.

inhibitory effects of divalent cations and alkaline pH on D-to-A-form transition of complex I are due to the presence of endogenous free fatty acids in the membranes of SMP. We checked this hypothesis in the experiments shown in Fig. 6. Complex I in SMP was de-activated by preincubation at 37°C, and the time course of its activity was followed in the presence of BSA added to deplete endogenous fatty acids. As expected, BSA abolished the inhibitory effect of Ca^{2+} on activation of complex I.

DISCUSSION

Numerous studies on effects of free fatty acids on mitochondria have been published. The vast majority of these studies are focused on the uncoupling, or uncoupling-related phenomena (see ref. [34-37] for reviews) as they are seen in isolated intact mitochondria oxidizing succinate in the presence of rotenone. The succinate oxidase activity of isolated mitochondria in the presence of externally added succinate hardly imitates any conceivable physiological situation: succinate is formed and utilized mostly within the mitochondrial matrix, and complex II (succinate:ubiquinone oxidoreductase) provides only one-fifth of the total reducing equivalents entering the respiratory chain during complete oxidation of pyruvate in the Krebs cycle. We [30] and others [21-29] have shown that free fatty acids are potent inhibitors of complex I, the major contributor of energy production in mitochondria. Two effects of palmitate and other fatty acids were observed: it inhibits the NADH oxidase activity of heart SMP, and (with higher affinity) it prevents the turnover-dependent activation of the de-activated complex I [30]. Here we show that Ca^{2+} drastically potentiates the inhibitory effects of fatty acids. Two aspects of those phenomena merit brief discussion: as they concern possible physiological relevance of fatty acid-induced inhibitory effects, and the mechanism of Ca^{2+}–fatty acid synergism.

The normal mitochondrially located intermediates of fatty acid metabolism are their carnitine esters (the inner membrane transportable species) and acyl-CoA esters (the substrate of β-oxidation). The steady-state free fatty acid content in the mitochondrial membranes, which are assumed to increase under a pathological condition termed metabolic syndrome (imbalance between nutrient uptake and utilization), is not well known and may be variable in different tissues. The other sources of free fatty acids are phospholipids hydrolyzed by phospholipases, whose activities are stimulated by divalent cations [38]. The potentiating effect of Ca^{2+} on the inhibitory effect of fatty acids as reported here was particularly evident at alkaline non-physiological pH. This does not however eliminate its possible physiological significance. The synergetic inhibitory effect is time-dependent and is expected to proceed at lower pH, although on a longer time scale.

All the data reported here were obtained using brain SMP. In light of great similarity (in fact identity) between catalytic properties of heart and brain complexes I [14], it is safe to conclude that the Ca^{2+}-potentiating effect would be seen in other mammalian tissues. We do not derogate the significance of uncoupling proteins (UCP) or/and adenine nucleotide translocator-mediated effects of free fatty acids [35]. However, their complex I-directed inhibitory effects should not be ignored.

What is the mechanism of Ca^{2+}-promoted (accelerated) fatty acid-induced inhibition? The most plausible explanation seems to lie in ionic pair (fatty $acid_2$–Ca^{2+}) formation that affects complex equilibrium in the system composed of phospholipid membrane, membrane bound fatty acid (hydrophobic tail immersed into lipid bilayer), and hydrophilic Ca^{2+}. The original Bjerrum's ion pair formation concept has been greatly developed in modern organic chemistry [39], and the "ion-pair absorption hypothesis" has been the rationale for longstanding attempts to improve penetration of biological membranes by cationic drugs (reviewed in [40, 41]). Recently, concerted action of penetrating cations and fatty acids was found to result in conversion of pH gradient to electrical potential across planar bilayer phospholipid membrane (presumably via ion-pair formation mechanism) [42]. Mironova et al. have identified palmitate as a "transporter" of Ca^{2+} across artificial phospholipid membrane [43]. IR spectroscopy data in their studies suggest that no Ca^{2+}–carboxylate group complex formation takes place during fatty acid-promoted Ca^{2+} transport [43]. In the framework of the classical Bjerrum's model, the ion pair of monovalent ions is considered as formed when the distance between them is 3.6 Å in water and 120 Å (!) in benzene [39]. Needless to say, because of extreme complexity of the system it is hard if not impossible to give a simple explanation for the phenomenon described here. Ion-pair formation that would increase intramembrane fatty acid concentration thus increasing accessibility of the inhibitor-sensitive site of complex I seems to be just a first approximation level hypothesis. A possibility that Ca^{2+} directly binds to the quinone reactive site, thus increasing its affinity to inhibitory fatty acid, cannot be excluded. The question of what part of the inhibitory fatty acid, its carboxylic group or hydrophobic tail binds to the enzyme quinone-binding site remains open.

Whatever the mechanistic explanation of Ca^{2+}-dependent inhibitory effect of free fatty acids, the data presented in Fig. 6 elucidate a previously described inhibitory effect of Ca^{2+} (and other divalent cations) on activation of complex I [17]. We believe now that this phenomenon is due to an increased potency of the endogenous inhibitory fatty acid by Ca^{2+}.

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