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Inhibitory effect of Mg^{2+} on the protonophoric activity of palmitic acid

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Abstract

To discriminate whether fatty acids are uncouplers that cause acceleration of State-4 respiration, associated with a decrease in the protonmotive force, or decouplers that increase respiration without associated decrease in the protonmotive force, we examined the effect of palmitate on functions of rat-liver mitochondria under various conditions. We found that palmitate itself increases State-4 respiration, releases oligomycin-inhibited State-3 respiration, inhibits ATP synthesis and ATP $\rightleftharpoons$ P_i exchange reaction, and increases H^+ permeability in mitochondrial and model bilayer phospholipid membranes. Thus, palmitate is a classical uncoupler of oxidative phosphorylation. However, these effects were inhibited by Mg^{2+}, due to rapid formation of a stable complex between palmitate and Mg^{2+}.

Keywords: Palmitic acid; Protonophore; Mitochondrial membrane; Phospholipid bilayer; Oxidative phosphorylation; Magnesium ion

1. Introduction

Uncouplers of oxidative phosphorylation inhibit ATP synthesis in mitochondria without direct interaction with the respiratory chain or ATP synthase. They stimulate State-4 respiration, release oligomycin-inhibited State-3 respiration, inhibit ATP $\rightleftharpoons$ P_i exchange reaction, and activate ATPase [1,2]. These effects of uncouplers are always accompanied by dissipation of $\Delta\mu_{H^+}$, and they are interpreted to be due to an increase in the H^+-permeability of the inner mitochondrial membrane by their protonophoric action. However, recently, inhalation anesthetics such as chloroform [3] and halothane [3], the tertiary amine local anesthetic bupivacaine [4,5], and long-chain fatty acids [6,7] were reported to induce uncoupling-related reactions without dissipation of $\Delta\mu_{H^+}$. Uncoupling without associated dissipation of $\Delta\mu_{H^+}$ is referred to as decoupling [7,8], and is postulated to be due to the formation of an intramembrane H^+ pathway [8] or slipping of the primary pump [9] in energy-transducing membranes, and the compounds that exhibit decoupling are referred to as decouplers. However, the decoupling effect of fatty acids such as palmitic acid and oleic acid has been disputed, and they were proposed to be uncouplers [10–13].

Rottenberg and Hashimoto [7] reported that these fatty acids released State-4 respiration in mitochondria without remarkable decrease in the $\Delta\Psi$. Others had reported that fatty acids induce uncoupling in association with a normal decrease in the $\Delta\Psi$ [10]. Recently, Luvisetto et al. [14] reported that the discrepancy resides in the difference in composition of the incubation medium: in the medium of Rottenberg and Hashimoto [7], valinomycin but not K^+ was present. In Luvisetto's report, although the possible involvement of molecular slip in the action of palmitate was discussed, essentially, they concluded that palmitate is a classical uncoupler, increasing membrane conductance to H^+ and decreasing $\Delta\Psi$, like protonophoric uncouplers [14].

It is of importance to know whether these compounds really cause decoupling, or act as decouplers under specified conditions, or they are normal uncouplers. First, we studied the effect of palmitate on oxidative phosphorylation under various conditions. We found that palmitic acid acts as a protonophoric uncoupler of oxidative phosphorylation in rat-liver mitochondria, but its effectiveness is

Abbreviations: $\Delta\mu_{H^+}$, electrochemical proton gradient; $\Delta\Psi$, transmembrane electrical potential; BLM, bilayer lipid membrane; TPP^+, tetraphenylphosphonium ion; Hepes, *N*-(2-hydroxyethyl)- piperazine-*N'*-2-ethanesulfonic acid; LCFAs, long chain fatty acids.

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affected by Mg^{2+} in the incubation medium because it forms a tight complex with palmitic acid.

2. Materials and methods

Palmitic acid, valinomycin, antimycin A and SF 6847 were purchased from Nacalai Tesque (Kyoto), Sigma Chemical Co. (St Louis) and Wako Pure Chemical Industry Co. (Osaka), respectively. Palmitic acid was dissolved into analytical grade of ethanol to make a final concentration of 25 mM, and this solution was used as a stock solution. Other reagents were of the highest grade commercially available.

Mitochondria were isolated from the liver of adult male Wistar rats, and the amount of mitochondrial protein was determined by the biuret method with bovine serum albumin as a standard.

The respiration of mitochondria (1.0 mg protein/ml) was monitored polarographically with a Clark-type oxygen electrode (Yellow Spring, YSI 5331) at 25°C with succinate (final concentration of 10 mM) plus rotenone (0.6 μg/mg protein) in a total volume of 2.5 ml. We used three types of incubation medium: medium A consisted of 200 mM sucrose, 1 mM EDTA-2Na, 2 mM $MgCl_2$, 10 mM sodium phosphate buffer, pH 7.4, and medium B consisted of 200 mM sucrose, 50 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 5 mM Hepes buffer (pH 7.4), 5 mM sodium phosphate buffer (pH 7.4) and 0.1 μM valinomycin according to the medium used by Rottenberg and Hashimoto [7]. Medium C had the same composition as medium A but the $MgCl_2$ concentration was 5 mM.

The $\Delta\Psi$ was estimated from the distribution of the membrane- permeable ion TPP^+ in mitochondria [15]. The free concentration of TPP^+ was measured with a TPP^+ electrode, prepared according to the method of Satake et al. [16], concomitantly with O_2 uptake, or with a use of [^{3}H]TPP^+ as a tracer. In latter case, the mitochondria were precipitated by centrifugation at 13500 rpm for 1 min and then solubilized with 0.5 ml of 2% SDS. The amount of [^{3}H]TPP^+ accumulated in the matrix space of mitochondria was determined in an Aloka liquid scintillation counter, LSC-700. In both cases, the initial concentration of TPP^+ in the incubation medium was 0.8 μM. TPP^+ at this concentration showed only a slight effect on the action of palmitic acid as reported by Schönfeld [17]. The values of $\Delta\Psi$ were corrected for the adsorption of TPP^+ as reported previously [18]. The volume of mitochondrial matrix space used for the calculations of $\Delta\Psi$ was 0.82 μl/mg protein which was measured from the distribution of [^{3}H]water and [^{14}C]sucrose [19].

The proton conductivity induced by palmitate in the presence of valinomycin in nonrespiring mitochondria was determined from the decrease in optical absorbance associated with swelling of mitochondria [4]. Mitochondria (1.0 mg protein/ml) were suspended in a medium consisting of 145 mM potassium acetate, and 5 mM Tris-HCl buffer (pH 7.4) in the presence of rotenone (0.6 μg/mg protein) in a total volume of 2.5 ml, and valinomycin (0.12 μg/mg protein) and palmitate (80 nmols/mg protein) were added at the desired time. The optical absorbance at 520 nm was monitored in a Shimadzu dual-wavelength spectrophotometer, model UV-3000.

A BLM consisting of soybean phosphatidylcholine and cholesterol in a molar ratio of 1:1 was formed by applying a solution in n-decane to a hole (about 1.0 mm diameter) in the wall of a Teflon test-tube, as described previously [4]. The medium consisted of 150 mM KCl and 100 mM Tris-HCl buffer (pH 7.4). Palmitate was added on one side of the membrane, and the membrane conductance was monitored by application of an electrical potential of 60 mV.

Complex formation between palmitate and Mg^{2+} was determined by monitoring optical absorbance at 520 nm in a Shimadzu dual-wavelength spectrophotometer, model UV-3000.

3. Results

3.1. Incubation medium dependent action of palmitate

When palmitic acid (final concentration 80 nmols/mg protein) was added to State-4 mitochondria, suspended in the commonly used incubation medium A with succinate (plus rotenone) as a respiratory substrate, the respiration increased accompanied by a decrease in $\Delta\Psi$ as determined from the concentration change of the permeant ion TPP^+ in the medium [15,16] (see Fig. 1, trace A). It also released oligomycin-inhibited State-3 respiration (data not shown). However, with mitochondria suspended in medium B, essentially the same medium used by Rottenberg and Hashimoto [7] containing valinomycin but not K^+, the release of respiration and decrease in $\Delta\Psi$ were smaller, as shown in trace B, consistent with the observation of Rottenberg and Hashimoto [7] and of Luvisetto et al. [14].

The dependence of the respiratory rate (J_0) and $\Delta\Psi$ of mitochondria suspended in both media on the concentration of palmitate is summarized in Fig. 2. In medium A, J_0 increased with increasing concentration of palmitate, and attained the maximal level (about 6-fold that in State-4) at about 80 μM (Fig. 2A). Because the further addition of typical protonophoric uncoupler SF 6847 to the mitochondria treated with this concentration of palmitate was ineffective in inducing the complete accerelation of mitochondrial respiration (data not shown), palmitate is suggested to have a slight inhibitory effect on the respiratory chain. The increase in J_0 with palmitate concentration was associated with decrease in $\Delta\Psi$, and at maximal J_0, $\Delta\Psi$ was decreased from -185 mV to -140 mV (Fig. 2A). This decrease was also less than that induced by SF 6847 at full uncoupling (-100 mV). These results indicate that

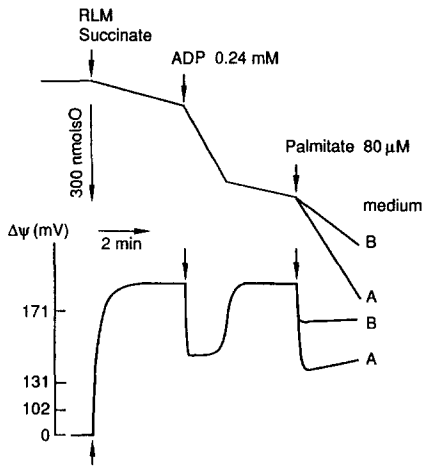

Fig. 1. Effect of palmitate on State-4 mitochondria suspended in medium A or B. Changes in the respiration (upper traces) and $\Delta\Psi$ (lower traces) were measured at 25°C in medium A and B. Rat-liver mitochondria (RLM, at 1.0 mg/ml) were suspended in the incubation medium in a total volume of 2.5 ml, and respiration was initiated by 10 mM succinate plus rotenone (0.6 μg/mg protein). First 0.24 mM ADP was added and then palmitate at a final concentration of 80 μM. The value of $\Delta\Psi$ was determined concomitantly with Jo from the change in the TPP^+ concentration in the incubation medium, which contained initially 0.8 μM TPP^+, using a TPP^+ electrode.

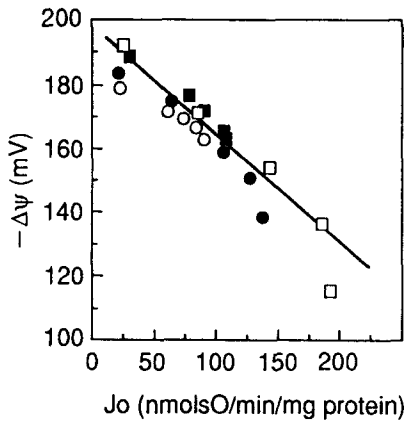

Fig. 3. Linear relationship between $\Delta\Psi$ and J_0 induced by palmitate and SF 6847. Effect of various concentrations of palmitate on the rate of respiration (J_0) and $\Delta\Psi$ of rat-liver mitochondria suspended in medium A (closed circles), B (open circles) or C (closed squares) were determined, and these values were plotted. The effect of SF 6847 was studied under similar conditions. As its effects were independent of the incubation medium, all the values for SF 6847 are represented by open squares. Experimental conditions were as for Fig. 1.

palmitic acid acts as a classical uncoupler in medium A, although its effectiveness is less than that of the typical protonophoric uncouplers, such as SF 6847.

In contrast, in medium B, the increase in J_0 with increasing palmitate concentration was smaller than that in medium A. This increase in J_0 was also associated with a decrease in $\Delta\Psi$, though the changes were smaller than those observed in medium A (Fig. 2B). These results are essentially in accordance with the results of Luvisetto et al. [14]. It is worthwhile to note that the change in J_0 was dependent on the decrease in $\Delta\Psi$ in the same manner in both incubation media (Fig. 3). Palmitate activated ATPase and inhibited the ATP $\rightleftharpoons$ P_i exchange reaction, like SF 6847. However, these activities were always greater in medium A than in medium B (data not shown). These results indicate again that palmitate itself is an uncoupler of oxidative phosphorylation.

The major difference between medium A and B is the absence and presence of valinomycin and NaCl as pointed out by Luvisetto et al. [14]. They reported that the valinomycin is essential for the constancy of $\Delta\Psi$ upon addition of palmitate. Although we also examined this 'protective effect' of valinomycin on the decrease of $\Delta\Psi$ induced by the addition of palmitate, its effect was not significant, as shown in Table 1. Thus, the smaller J_0 increase and $\Delta\Psi$ decrease by palmitate in medium B was not ascribed to valinomycin. We also examined the effect of the concentration of NaCl in the incubation medium and concluded that it was also not major effector for the action of palmitic acid (data not shown).

3.2. Effect of Mg^{2+} on the effect of palmitate

Besides valinomycin and NaCl, the concentration of Mg^{2+} is different in medium A and B. Thus, we next examined the effect of Mg^{2+} on the effect of palmitate.

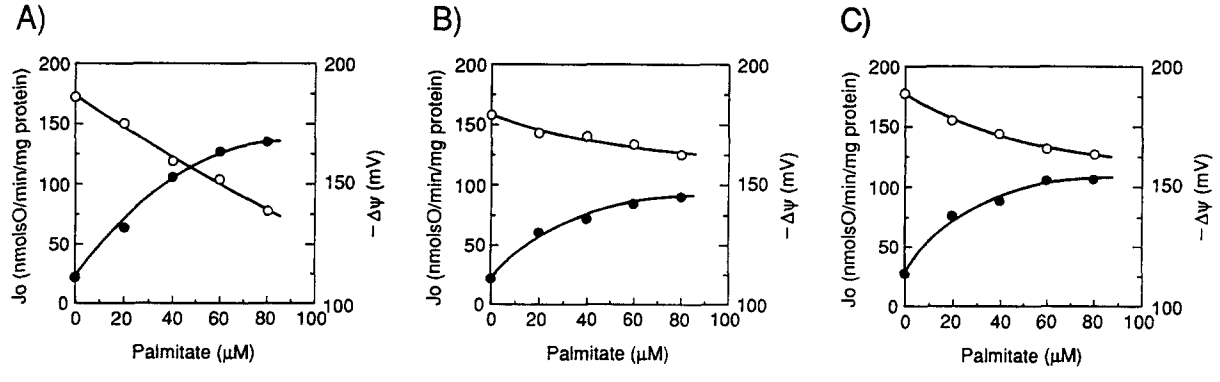

Fig. 2. Effect of various concentration of palmitate on J_0 and $\Delta\Psi$ of mitochondria suspended in medium A, B or C. A, B and C represent the results obtained in medium A, B and C, respectively. Experimental conditions were as for Fig. 1.

Table 1
Effect of valinomycin and palmitate on the magnitude of $\Delta\Psi$ in state-4 mitochondria [a]

Palmitate (μM)	$\Delta\Psi$ (mV)	
	−valinomycin	+valinomycin
0	−182	−186
40	−158	−160
80	−135	−141

[a] To State-4 mitochondria (1.0 mg protein/ml) suspended in medium A, either in the absence or presence of valinomycin (0.12 μM) with succinate as a respiratory substrate in the presence of rotenone (0.6 μg/ml) in a total volume of 1.0 ml, palmitate was added. The value of $\Delta\Psi$ was determined from the TPP^+ concentration in the incubation medium with the use of [^{3}H]TPP^+ as a tracer at 25° C. The initial TPP^+ concentration in the incubation medium was 0.8 μM.

For this we used another medium, C, which had the same composition as medium A but a higher $MgCl_2$ concentration, of 5 mM. As shown in Fig. 2C, the effectiveness of palmitate in increasing State-4 respiration with succinate as substrate and decreasing $\Delta\Psi$ was smaller in medium C than in medium A. Inhibition of ATP synthesis and ATP $\rightleftharpoons P_i$ exchange reaction by palmitate was also dependent on the Mg^{2+} concentration (data not shown). In contrast, Mg^{2+} had no effect on the uncoupling induced by SF 6847 (data not shown). Thus, Mg^{2+} was concluded to be responsible for suppression of uncoupling action of palmitate. It is noteworthy that the change in J_0 with $\Delta\Psi$ induced by palmitate in medium C, and that by SF 6847 in both medium A and C were linear, and followed the same relation shown in Fig. 3.

3.3. Effect of Mg^{2+} on the palmitate-induced H^+ transport

The protonophoric action of compounds can be examined by swelling of mitochondria associated with efflux of H^+ mediated by the protonophoric compound in exchange with influx of K^+, mediated by valinomycin in potassium acetate [4]. We examined the effect of palmitate on swelling of non-energized mitochondria by monitoring the change in absorbance at 520 nm in the presence of various amounts of Mg^{2+}. As shown in Fig. 4, palmitate at 80 μM after addition of valinomycin (left traces) caused a significant decrease in the absorbance associated with swelling of mitochondria in the absence of Mg^{2+}. However, the extent of absorbance change became smaller with increasing in Mg^{2+} concentration. Addition of palmitate and valinomycin in a different order caused similar results (right traces). In contrast, swelling of mitochondria induced by SF 6847 was essentially independent of the Mg^{2+} concentration (data not shown).

Next, we examined the effect of palmitate on the electrical conductance of BLM consisting of phosphatidylcholine and cholesterol in a molar ratio of 1:1. Palmitate (10 μM) caused an increase in the membrane conductivity

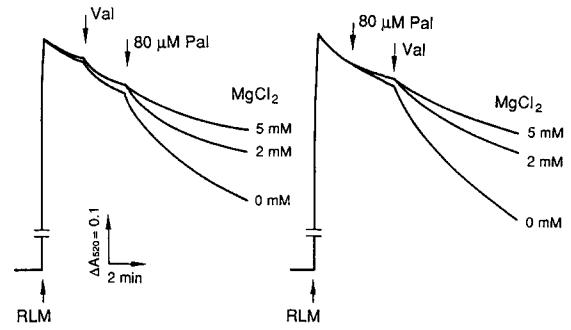

Fig. 4. Effect of Mg^{2+} on the passive swelling of mitochondria induced by valinomycin and palmitate. Rat-liver mitochondria (RLM, at 1.0 mg/ml) were suspended in a medium consisting of 145 mM potassium acetate and 5 mM Tris-HCl buffer (pH 7.4), in the presence of rotenone (0.6 μg/mg protein) in a total volume of 2.5 ml in the presence of various concentrations of $MgCl_2$. Then 0.12 μM valinomycin and 80 μM palmitate were added in this order (traces on the left) or in the reverse order (traces on the right). Swelling was monitored as a decrease in absorbance at 520 nm.

to a level similar to that induced by SF 6847, the permeability being more than 15-fold that in the absence of palmitate (Fig. 5, columns 1, 2 and 6). Further addition of 5 mM Mg^{2+} decreased the conductance to the level about 1.8-fold that without palmitate (column 3). Mg^{2+} alone did not increase the electrical conductivity at all (column 4), and the subsequent addition of palmitate increased it slightly (column 5) to a level similar to that induced by palmitate and subsequent addition of Mg^{2+} (column 3). These results again indicate the protonophoric action of palmitate, the effectiveness being influenced by Mg^{2+}. It was shown that Mg^{2+} itself does not modify the membrane structure.

3.4. Interaction of palmitate with Mg^{2+}

It could be that the difference in uncoupling activity of palmitate arose by complex formation between palmitate and Mg^{2+}, and that this complex is ineffective in inducing proton permeability. Formation of such a complex was

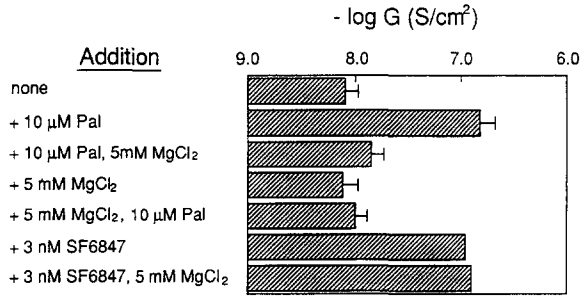

Fig. 5. Effect of Mg^{2+} on the electrical conductance of BLM under various conditions. Membrane conductance (G) of a BLM composed of egg-yolk phosphatidylcholine and cholesterol in a molar ratio of 1:1 after the addition of various reagents was monitored by application of an electrical potential of 60 mV. The incubation medium consisted of 150 mM KCl and 100 mM Tris-HCl buffer (pH 7.4).

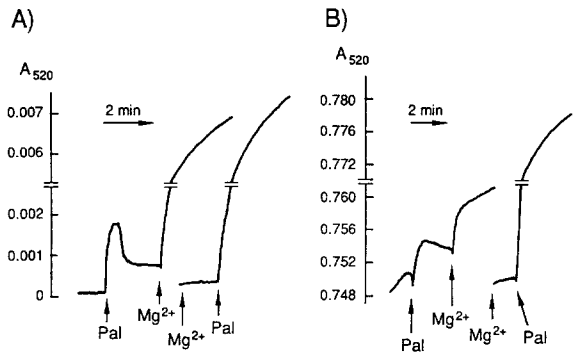

Fig. 6. Complex formation of palmitate and Mg^{2+}. Palmitate (Pal, 80 μM) and $MgCl_2$ (Mg^{2+}, 2 mM) were added to Mg^{2+}-free medium A in the absence (traces in A) or presence (traces in B) of rat-liver mitochondria at 1.0 mg/ml in a total volume of 2.5 ml, and the absorbance change at 520 nm was monitored. The effect of the order of addition of palmitate and $MgCl_2$ was examined in both cases.

determined by monitoring the turbidity change in a solution containing palmitate and Mg^{2+}. The absorbance changes at 520 nm associated with complex formation between palmitate and Mg^{2+} examined in the absence and presence of mitochondria are shown in Fig. 6. In the presence of Mg^{2+} in the incubation medium, addition of palmitate caused a rapid and remarkable increase of absorbance both in the absence and presence of mitochondria (right traces in Fig. 6A and 6B, respectively). In the absence of Mg^{2+}, little absorbance change was observed in both cases. However, there was a marked difference on

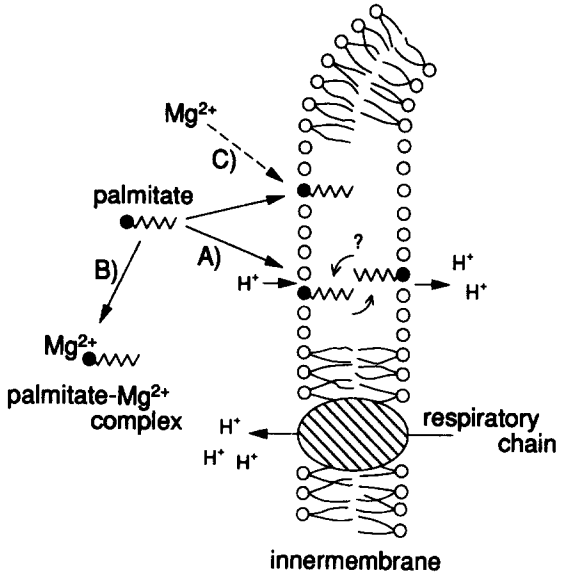

Fig. 7. Schematic representation of the protonophoric action of palmitate on the mitochondrial membrane in the presence of Mg^{2+}. The palmitate molecules in principle act as protonophore in the membrane, as observed in rat-liver mitochondria and in BLM (case A). In the presence of a high concentration of Mg^{2+} in the incubation medium, palmitate forms a metal soap, and thus, it can not act as protonophore (case B). Once the palmitate molecule has been transferred into the membrane (in the presence of a low concentration of Mg^{2+}), further added Mg^{2+} is not so effective in trapping the palmitate molecule (case C).

further addition of Mg^{2+}; absorbance increased remarkably in the absence of mitochondria but not in the presence of mitochondria. The smaller extent of absorbance change on addition of Mg^{2+} in the presence of mitochondria was attributed to the fact that palmitate had been incorporated in the membrane and can hardly be trapped by Mg^{2+} in the incubation medium. This result accords well with a fact that the uncoupling effect of palmitate observed in the presence of low concentration of Mg^{2+} is not so much affected by the further addition of Mg^{2+} (data not shown).

4. Discussion

Uncoupling of oxidative phosphorylation by LCFAs has been well studied [6,7,10–14,20–34]. However, their mechanism of action has not been well defined, due to the composite effects in their interaction with energy-transducing membranes. Rottenberg and Hashimoto [7] found that LCFAs increased State-4 respiration of mitochondria, but did not decrease $\Delta\mu_{H^+}$. They concluded that LCFAs belong to a class of uncouplers which stimulate electron transport in the respiratory chain without decreasing $\Delta\mu_{H^+}$, i.e. decouplers [8]. However, LCFAs were reported by others to be protonophoric uncouplers by stimulating State-4 respiration with a concomitant decrease in $\Delta\mu_{H^+}$ [10,13] and by increasing H^+-conductivity in artificial phospholipid membranes [27]. Furthermore, an involvement of the adenine nucleotide translocator in the action of LCFAs has been suggested [28–30]. It would be of importance to understand why the action of LCFAs differs between laboratories.

In this study we show that the LCFA palmitate itself is a protonophore with a slight inhibitory activity on the respiratory chain. It stimulates electron transfer in the respiratory chain with a concomitant decrease in $\Delta\Psi$, activates ATPase and inhibits ATP $\rightleftharpoons$ P_i exchange. However, these effects are significantly dependent on the composition of the incubation medium. We found that this can be ascribed to the fact that palmitate easily forms a complex with Mg^{2+} in the incubation medium, and this complex formation causes a decrease in the free palmitate concentration, thus leading to an apparent reduced activity of palmitate. It has long been known that LCFAs form complexes with metals such as Ca^{2+}. The metal soap thus formed is hardly soluble in water. A similar complex formation between palmitate and Mg^{2+} takes place in the usual incubation medium as shown in this study and in a previous study [34]. In view of the findings that the protonophoric action of palmitate differs in incubation media containing 2 mM (medium A) or 5 mM $MgCl_2$ (medium B), and the ability of Mg^{2+} to trap palmitate after it has been transferred into the membrane is small (data not shown), the formation constant of the palmitate-Mg^{2+} is expected to be not great. The proposed action of palmitate in the incubation medium containing Mg^{2+} is

schematically depicted in Fig. 7. If the palmitate is added in a medium which contains a low concentration of Mg^{2+}, it would transfer into membrane and act as a protonophoric uncoupler (case A). Indeed, although the medium A contains 2 mM $MgCl_2$, the free concentration of Mg^{2+} can be estimated as low as 0.24 mM from the stability constants of metal complexes [35]. Thus, the effect of palmitate observed in medium A would correspond to this case. In the presence of a high concentration of Mg^{2+}, the added palmitate would form a water-insoluble metal complex, and hence, it could not act as a protonophore any longer (case B). However, if the palmitate already has been transferred into the membrane, further addition of Mg^{2+} is ineffective to trap the palmitate molecule present in the membrane (case C). The different behavior of palmitate in case A and B is concluded to be a main reason for the different observations in different laboratories. In addition to the protonophoric activity, a possible induction of molecular slip of the respiratory chain [14] or permeability transition of the mitochondrial inner membrane by LCFAs [36] were suggested. In this study, we did not examine such possibilities. However, to understand the physiological role of LCFAs in vivo, not only the presence or absence of these effects of LCFAs but also the dependence of such effects on the experimental conditions must be examined carefully.

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