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Roles of long chain fatty acids and carnitine in mitochondrial membrane permeability transition

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Abstract

Palmitoyl-CoA (Pal-CoA) lowered the respiratory control ratio (RCR), and induced mitochondrial membrane permeability transition (MPT) and cytochrome c (Cyt. c) release from isolated rat liver mitochondria. L-Carnitine suppressed the Pal-CoA-induced dysfunction, MPT, and Cyt. c release of isolated mitochondria. This suppression was inhibited by cephaloridine, an inhibitor of carnitine uptake into mitochondria. Cyclosporin A (CsA), an inhibitor of MPT, and BSA also suppressed the Pal-CoA-induced MPT. In the presence of inorganic phosphate (P_i), Ca^{2+}-induced MPT was suppressed by BSA, L-carnitine, and chlorpromazine, an inhibitor of phospholipase A_2. In the presence of a low concentration of Ca^{2+}, 3,3′,5-triiodothyronine, long chain fatty acids, salicylic acid, and diclofenac induced MPT by a mechanism that was suppressed by BSA, L-carnitine, or chlorpromazine. During the incubation of mitochondria on ice, their respiratory competence decreased; L-carnitine and BSA also prevented this decrease. Mitochondrial depolarization in pheochromocytoma PC12 cells was induced by either serum deprivation or arachidonic acid by a mechanism that was suppressed by acetyl-L-carnitine. These results indicate that some MPTs may be regulated by fatty acid metabolism and that the Pal-CoA-induced MPT plays an important role in the induction of apoptosis.

Keywords: Apoptosis; Carnitine; Cyclosporin A; Cytochrome *c*; Membrane permeability transition; Mitochondria; Phospholipase A_2

1. Introduction

Biochemical studies indicate a decline of electron transport and some bioenergetic activities of mitochondria during aging and ischemia–reperfusion [1–3]. The decrease in membrane potential, respiratory control, and cardiolipin levels of mitochondria is prevented by supplementation of the diet with acetyl-L-carnitine [1,4]. Administration of acetyl-L-carnitine to aged rats restores the decreased functions and cardiolipin levels of mitochondria to those of young rats [4]. These results indicate that accumulation and/or abnormal metabolism of fatty acids may induce the age-associated dysfunction of mitochondria. L-Carnitine facilitates mitochondrial transport and β-oxidation of LCFAs through formation of their acyl-esters [5].

Depolarization of the mitochondrial inner membrane coupled with MPT has been shown to release Cyt. c from mitochondria and induce apoptosis in various types of cells [2]. CsA [6] and L-carnitine [7,8] suppress MPT. L-Carnitine also suppresses Fas-induced apoptosis and the production of ceramide in cells [9]. Acetyl-L-carnitine inhibits apoptosis of teratocarcinoma cells induced by serum deprivation [10]. Thus, it is possible that the inhibitory effect of L-carnitine on cellular apoptosis may be due to inhibition of mitochondrial MPT [11,12]. PLA_2 in mitochondrial membranes is activated by MPT induced by Ca^{2+} plus t-butylhydroperoxide, whereas MPT can be inhibited by PLA_2 inhibitors [13]. Furthermore, in the presence of Ca^{2+}, large amplitude swelling of mitochondria induced by P_i and T_3 releases free

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Abbreviations: CsA, cyclosporin A; Cyt. c, cytochrome *c*; FBS, fetal bovine serum; Pal-CoA, palmitoyl-CoA; P_i, inorganic phosphate; LCFA, long chain fatty acid; MPT, membrane permeability transition; PLA_2, phospholipase A_2; RCR, respiratory control ratio; and T_3, 3,3′,5-triiodothyronine.

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fatty acids [14]. These results suggest that L-carnitine, fatty acids, and PLA_2 play important roles in the induction of mitochondrial MPT, which releases Cyt. c and triggers apoptosis.

Thus, we studied the effects of Pal-CoA, fatty acids, L-carnitine, BSA, and chlorpromazine on mitochondrial functions and MPT induced by various reagents. We also studied the effect of acetyl-L-carnitine on mitochondrial membrane depolarization and apoptosis of PC12 cells induced by serum deprivation and arachidonic acid.

2. Materials and methods

2.1. Chemicals

Atractyloside, L-carnitine, acetyl salicylate, chlorpromazine, CsA, diclofenac, Cyt. c, T_3, Pal-CoA, palmitic acid, cephaloridine, bongkrekic acid, and trifluoperazine were obtained from the Sigma Chemical Co. Anti-Cyt. c antibody was obtained from PharMingen. 3,3′-Dipropyl-2,2′-thiodicarbocyanine iodide [diS-C_3-(5)], a cyanine dye, was obtained from the Kanko-Shikiso Research Institute. MitoSensor (ApoAlert™) was purchased from Clontech.

2.2. Isolation and in vitro *aging of mitochondria*

Mitochondria were isolated from Wistar rat liver by the method of Hogeboom [15] using sucrose density gradient centrifugation as described in a previous paper [16]. Isolated mitochondria (0.5 mg protein/mL) were subjected to *in vitro* aging at 4°C for 24 hr in 0.2 M sucrose containing 5 mM Tris–HCl buffer (pH 7.4), 3 mM $MgCl_2$, and 10 mM KCl.

2.3. Assay for oxidative phosphorylation, swelling, and membrane potential of mitochondria

Mitochondria (0.5 mg protein/mL) were incubated in 0.2 M sucrose containing 5 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, and 10 mM KCl at 25°C. Oxygen uptake was assayed by a polarographic method [17]. Mitochondrial swelling was measured in 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl at 25° at a protein concentration of 0.1 mg/mL. The change in absorbancy was recorded at 540 nm by a spectrophotometer (Shimadzu UV-3000) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer [16]. The membrane potential was measured by using 0.15 μg/mL of diS-C_3-(5) in the same medium containing mitochondria (0.1 mg protein/mL); fluorescence intensity was recorded at 670 nm (excitation at 622 nm) in a fluorescence spectrophotometer (Hitachi 650–10LC) [18].

2.4. Assay of mitochondrial membrane potential in PC12 cells

Pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Life Technologies Inc.) on Type I collagen-coated dishes. These cells were grown in a humidified incubator at 37° under 5% CO_2/95% air and used in their logarithmic phase. PC12 cells were incubated in an 8-well culture slide (0.8×10^5 cells/well) for the analysis of mitochondrial membrane potential. The cells were preincubated with 1 mM L-carnitine for 3 days. After incubation in serum-deprived culture medium for 6 hr, cells were washed with serum-free medium, and stained with MitoSensor (ApoAlert™) for 20 min at 37°. Stained cells were observed by fluorescence microscopy.

2.5. Western blot analysis of Cyt. c

Mitochondria (0.1 mg protein/mL) were incubated in 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl at 25° for 10 min, and centrifuged at 7000 g for 10 min at 4°. The supernatant was added to ½ vol. of SDS–PAGE sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue] and boiled at 100° for 5 min. Then the samples (20 μL) were subjected to SDS–PAGE. After transfer of the proteins on the gel to an Immobilon filter (Millipore Co.), the filter was incubated with primary antibody (1:1000 dilution) and then with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution), and analyzed by using an ECL plus kit (Amersham Co.) [19]. Protein concentrations were determined by Bio-Rad Protein Assay reagent (Bio-Rad) using BSA as a standard.

3. Results

3.1. Effect of Pal-CoA on the phosphorylation of mitochondria

Figure 1A shows the effect of Pal-CoA and L-carnitine on mitochondrial respiration. Pal-CoA strongly inhibited the succinate-dependent phosphorylating respiration (state 3 respiration) in a concentration-dependent manner but slightly increased the state 4 respiration. Half-maximum inhibition occurred at a Pal-CoA concentration of 2 μM. The inhibitory effect of Pal-CoA was suppressed by L-carnitine in a concentration-dependent manner.

3.2. Effects of Pal-CoA and L-carnitine on mitochondrial MPT

In agreement with its inhibition of mitochondrial oxidative phosphorylation, Pal-CoA induced MPT in a concentration-dependent manner (Fig. 2) [20–22]. Pal-CoA also induced the swelling and depolarization of the mitochondrial inner membrane (Fig. 2, A and B). The concentration of Pal-CoA required for the induction of half-maximum swelling was about 30 nmol/mg protein.

The Pal-CoA-induced swelling and depolarization of mi-

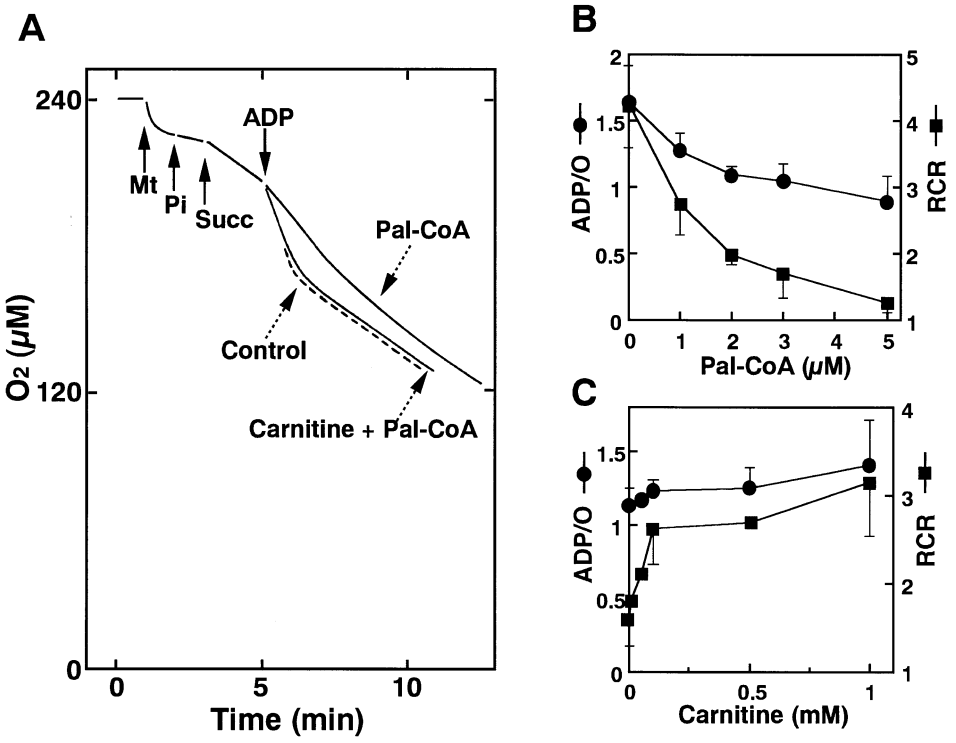

Fig. 1. Inhibitory effect of Pal-CoA on mitochondrial oxidative phosphorylation and its prevention by L-carnitine. Mitochondria (0.5 mg protein/mL) were suspended in 0.2 M sucrose containing 10 mM KCl, 3 mM $MgCl_2$, and 5 mM Tris–HCl buffer (pH 7.4) at 25°. Pal-CoA (3 μM) and L-carnitine (1 mM) were added to the medium before the addition of mitochondria. Reagents used were 2 mM P_i, 5 mM succinate, and 150 μM ADP. (A) Mitochondrial respiration measured polarographically. The dotted line shows control respiration without Pal-CoA and L-carnitine. (B) Concentration-dependent effects of Pal-CoA. (C) Concentration-dependent effects of L-carnitine in the presence of 3 μM Pal-CoA. Data are expressed as the means ± SD from five separate experiments.

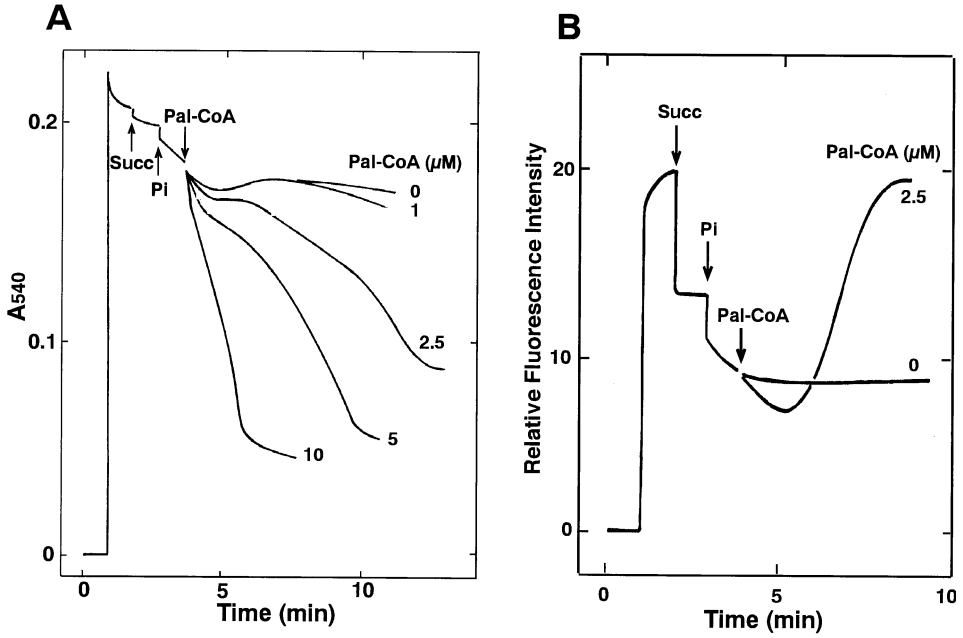

Fig. 2. Induction of MPT by Pal-CoA. Mitochondria (0.1 mg protein/mL) were incubated in 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl at 25°. (A) Mitochondrial swelling was monitored by absorption at 540 nm. (B) Membrane potential was monitored by measuring the fluorescence of diS-C_3-(5) (0.15 μg/mL). The reagents used were 5 mM succinate, 2 mM P_i, and various concentrations of Pal-CoA. Similar results were obtained in three separate experiments.

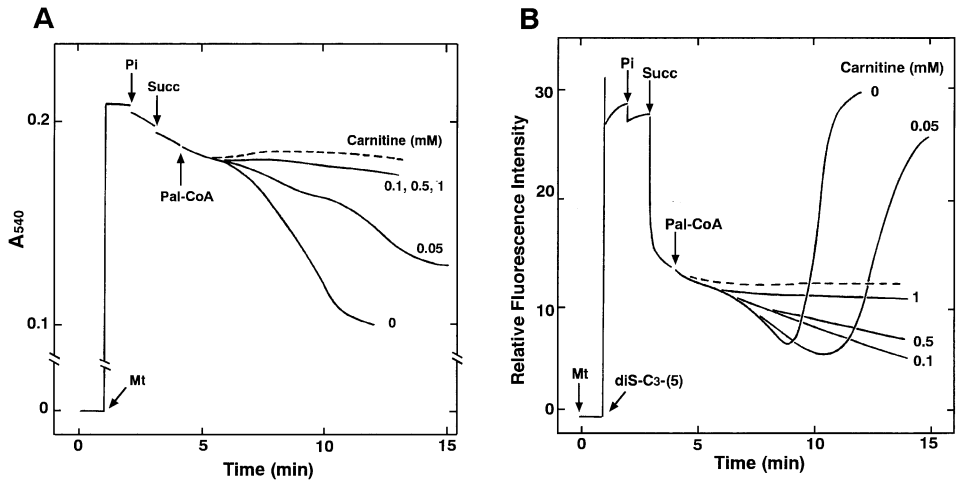

Fig. 3. Suppression of Pal-CoA-induced MPT by L-carnitine. Experimental conditions were as described in the legend of Fig. 2. L-Carnitine (50 μM to 1 mM) was added to the medium before the mitochondria were added. Dotted lines show the change in the absence of Pal-CoA (3 μM). Panels A and B show the swelling and membrane potential changes, respectively. Similar results were obtained in three separate experiments.

tochondria were suppressed by L-carnitine in a concentration-dependent manner (Fig. 3). The concentration of L-carnitine required for 50% suppression of the MPT was 50 μM. LCFAs also induced mitochondrial swelling and depolarization by an L-carnitine-inhibitable mechanism (data not shown) [23].

3.3. Effect of cephaloridine on Pal-CoA-induced MPT

To gain further insight into the inhibitory mechanism of L-carnitine, the effect of cephaloridine, an inhibitor of the mitochondrial carnitine transporter, was examined [24]. The suppression of Pal-CoA-induced mitochondrial swelling by

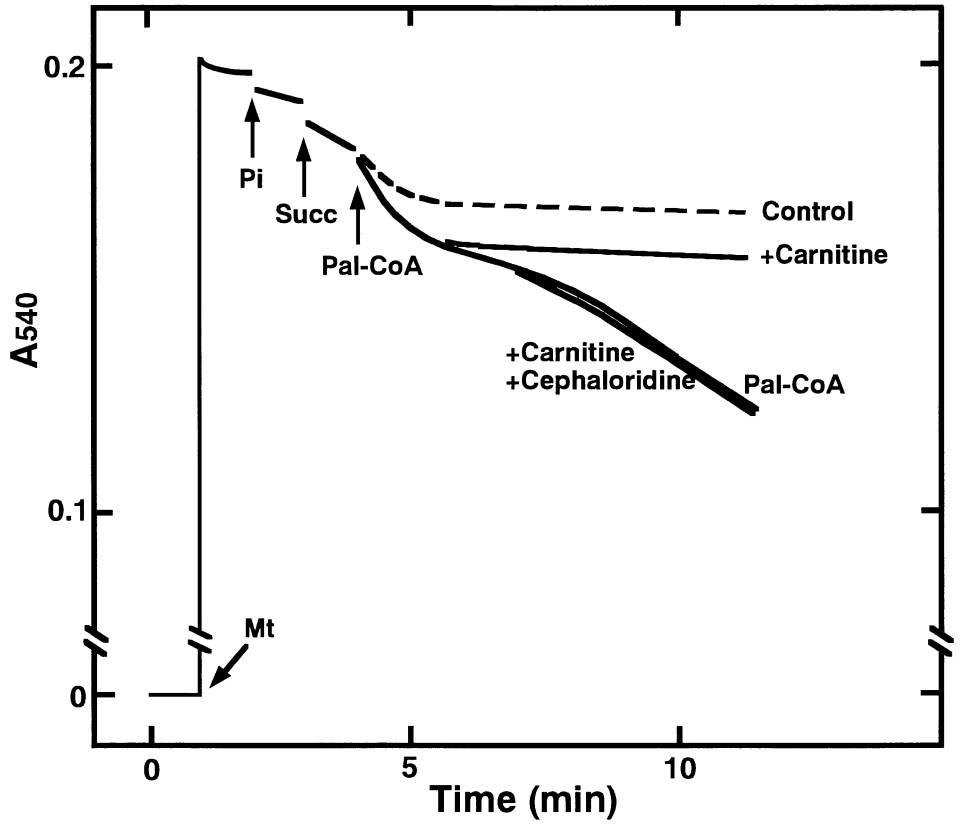

Fig. 4. Effect of cephaloridine and L-carnitine on Pal-CoA-induced swelling of mitochondria. Experimental conditions were as described in the legend of Fig. 2. L-Carnitine (0.1 mM) and cephaloridine (2.5 mM) were added to the medium before the addition of mitochondria. The concentration of Pal-CoA was 5 μM. Similar results were obtained in three separate experiments.

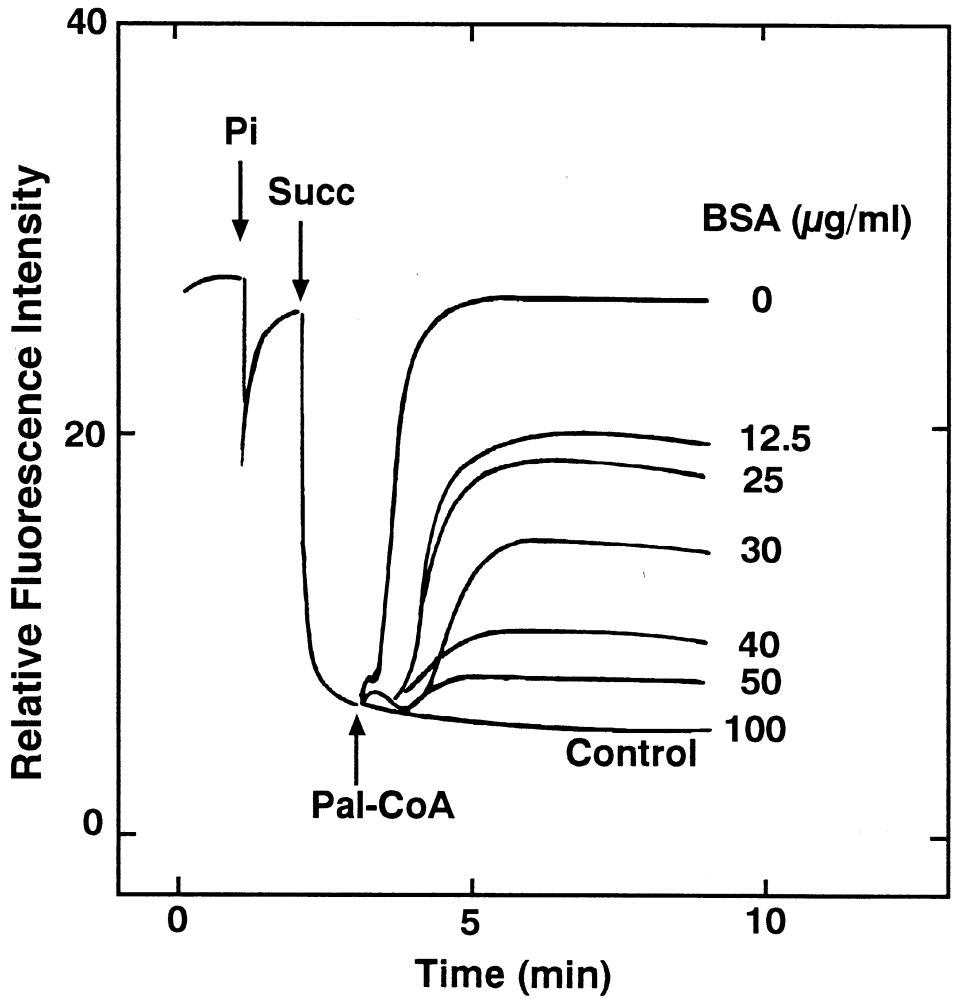

Fig. 5. Effect of BSA on Pal-CoA-induced depolarization of mitochondria. Experimental conditions were as described in the legend of Fig. 2. The concentrations of Pal-CoA and BSA were 5 μM and 12.5 to 100 μg/mL, respectively. Similar results were obtained in three separate experiments.

L-carnitine was abolished in the presence of cephaloridine (Fig. 4). This indicates that the inhibition by L-carnitine is mediated through a mechanism involving the inner mitochondrial membrane.

3.4. Effect of BSA on Pal-CoA-induced MPT

Since plasma albumin strongly binds hydrophobic anions, such as fatty acids and their metabolic derivatives, Pal-CoA-induced MPT might be suppressed by BSA. To test this possibility, the effect of BSA on Pal-CoA-induced mitochondrial depolarization was investigated. BSA inhibited the depolarization of mitochondria in a concentration-dependent manner (Fig. 5). BSA also inhibited the swelling of mitochondria (data not shown).

3.5. Effects of various inhibitors of MPT on Pal-CoA-induced mitochondrial swelling

Since CsA is a specific inhibitor of MPT like bongkrekic acid, the effect of CsA on Pal-CoA-induced mitochondrial swelling was examined. The Pal-CoA-induced swelling and depolarization of mitochondria were suppressed by CsA, BSA, and L-carnitine (Fig. 6). However, CsA-insensitive swelling was induced by Pal-CoA at concentrations higher than 20 μM, as was the case of arachidonic acid (data not shown) [25].

3.6. Inhibition of Pal-CoA-induced Cyt. c *release*

It has been reported that Cyt. c is released from mitochondria during MPT by a CsA-inhibitable mechanism [26]. Hence, we tested the effects of L-carnitine, BSA, and CsA on the Pal-CoA-induced release of Cyt. c. All three agents suppressed the release of Cyt. c (Fig. 7). The amount of Cyt. c released from mitochondria increased following swelling, while, concomitantly, intramitochondrial content diminished. As a consequence, succinate oxidation gradually decreased during the time after swelling.

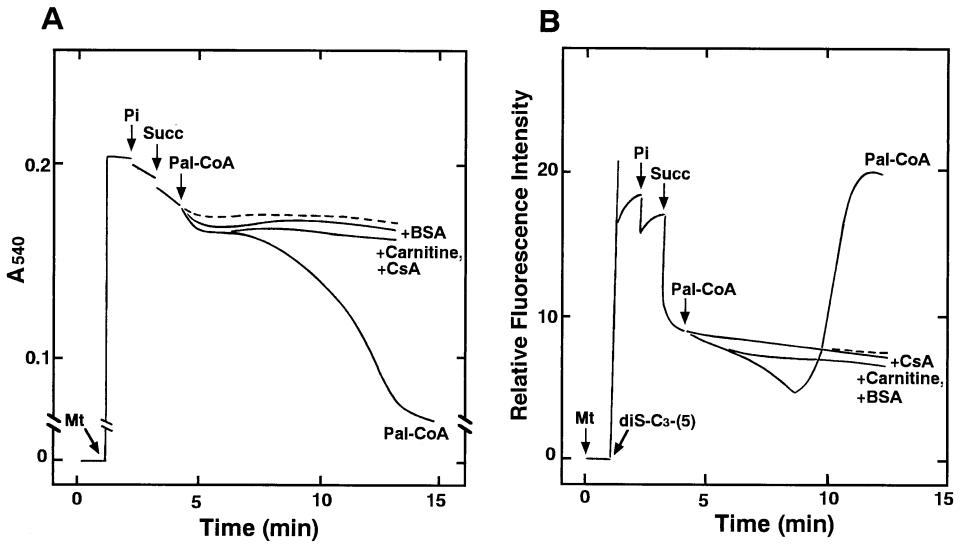

Fig. 6. Effects of various reagents on Pal-CoA-induced MPT. Experimental conditions were as described in the legend of Fig. 2. Panels A and B show mitochondrial swelling and membrane potential change, respectively. Swelling and membrane potential changes were induced by 3 μM Pal-CoA in the presence or absence of 1 mM L-carnitine, 0.1 mg protein/mL of BSA, and 1 μM CsA. Dotted lines show traces in the absence of Pal-CoA. Similar results were obtained in three separate experiments.

3.7. Effects of CsA, BSA, and L-carnitine on MPT induced by various reagents

In the presence of P_i, Ca^{2+} induces swelling and depolarization of mitochondria by a CsA-inhibitable mechanism [27]. BSA, L-carnitine, and chlorpromazine also suppressed the Ca^{2+}-induced swelling (Fig. 8). The concentration of chlorpromazine used was similar to that required for the inhibition of mitochondrial PLA_2.

Because L-carnitine inhibited Pal-CoA-induced MPT, its effect on MPT induced by other reagents was also investigated. In the presence of 2 μM Ca^{2+}, palmitic acid [7], T_3 [28], acetyl salicylate [29], and diclofenac [30] also induced swelling and depolarization of mitochondria by a mechanism that was suppressed by L-carnitine, BSA, chlorpromazine, and CsA (data not shown).

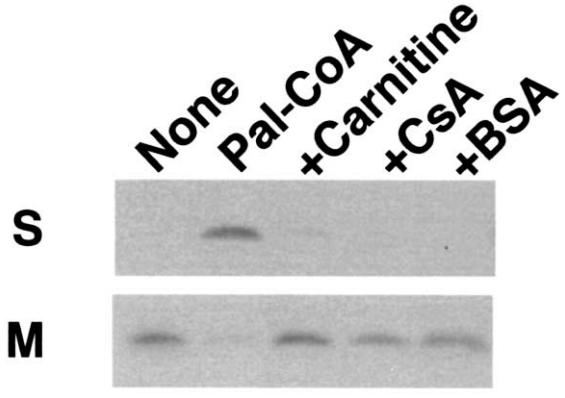

Fig. 7. Effects of various reagents on Pal-CoA-induced Cyt. c release. Experimental conditions were as described in the legend of Fig. 2. Western blot analysis of Cyt. c release during Pal-CoA-induced MPT of mitochondria is shown. Mitochondria were treated with 5 μM Pal-CoA for 12 min in the presence or absence of various reagents. Mitochondrial (M) and supernatant (S) fractions were assayed for the presence of Cyt. c following treatment with 1 mM L-carnitine, 0.1 mg/mL of BSA, or 1 μM CsA. Similar results were obtained in three separate experiments.

3.8. Effects of L-carnitine, BSA, and CsA on mitochondrial aging

It is well known that mitochondrial functions decrease during storage even at a low temperature. To test the possible involvement of free fatty acids released from membranes in the mechanism of *in vitro* aging of mitochondria, we studied the effects of L-carnitine, CsA, and BSA. L-Carnitine and BSA but not CsA suppressed the decrease in the RCR in mitochondria stored at 4° (Fig. 9). The protective effects were seen only when L-carnitine and BSA were present during storage.

3.9. Effect of L-carnitine on the depolarization of PC12 cells by serum deprivation

Serum deprivation activates PLA_2, thereby enhancing the release of free fatty acids in various cells including PC12 [31,32]. Thus, free fatty acids might underlie the mechanism of apoptosis induced by serum deprivation. To test this hypothesis, the effect of serum deprivation on mitochondrial membrane depolarization in PC12 cells was investigated in the presence or absence of L-carnitine. The presence of L-carnitine inhibited depolarization of PC12 cells induced by serum deprivation (Fig. 10). L-Carnitine also inhibited the apoptosis of PC12 cells induced by serum deprivation (data not shown). LCFAs also induced apoptosis of PC12 cells. In this case, mitochondrial membrane

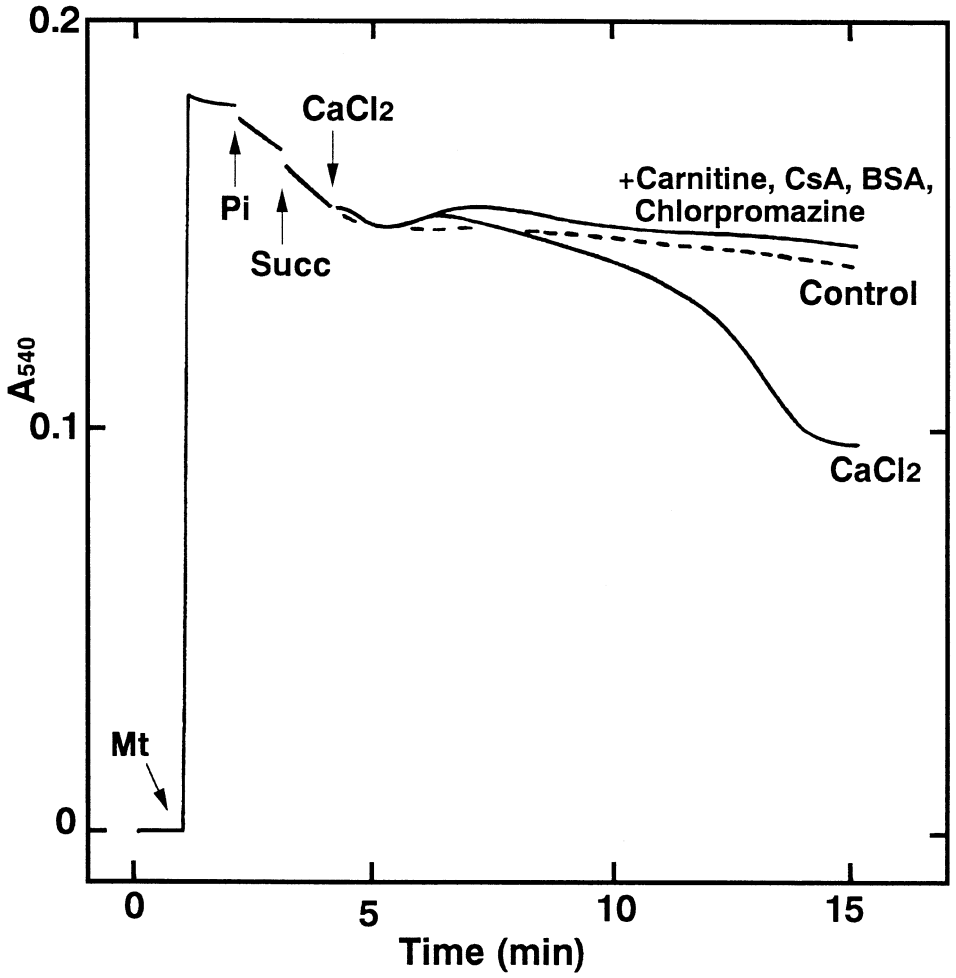

Fig. 8. Calcium-induced MPT and its inhibition by various reagents. Experimental conditions were as described in the legend of Fig. 2. The concentrations of $CaCl_2$, BSA, L-carnitine, chlorpromazine, and CsA were 5 μM, 0.1 mg/mL, 1 mM, 10 μM, and 1 μM, respectively. The dotted line shows the result in the absence of $CaCl_2$. Similar results were obtained in three separate experiments.

depolarization in PC12 cells was induced by LCFAs by an L-carnitine-inhibitable mechanism (data not shown).

4. Discussion

The present work showed that both L-carnitine and CsA effectively suppressed mitochondrial MPT, comparable to the action of bongkrekic acid [22], and that L-carnitine suppressed the mitochondrial dysfunction induced by Pal-CoA. L-Carnitine also suppressed the mitochondrial swelling and depolarization induced by LCFAs, Pal-CoA, Ca^{2+} plus P_i, T_3, acetyl salicylate, and diclofenac, as well as the dysfunction induced by means of cold storage. L-Carnitine facilitates the transport of LCFAs into the mitochondrial matrix, thereby enhancing β-oxidation. Because cephaloridine, a potent inhibitor of the carnitine transporter, abolished the inhibitory effect of L-carnitine, the protective effect of L-carnitine may occur in and around the mitochondrial membranes.

The transport of acyl-L-carnitine is mediated by carnitine palmitoyltransferase (CPT) I and II in the mitochondrial inner membrane [33]. Because the free form of LCFAs inhibits the activity of CPT I [34] and Pal-CoA inhibits the ADP/ATP carrier [21,22], LCFAs as well as Pal-CoA may modulate mitochondrial functions particularly when the concentration of L-carnitine in mitochondria is low. Hence, the inhibitory effects of L-carnitine on the occurrence of MPT, Cyt. c release, and apoptosis of PC12 cells may relate to the removal of LCFAs and Pal-CoA and the enhancement of β-oxidation that facilitates ATP formation. Consistent with this hypothesis is the finding that both defatted BSA and chlorpromazine also inhibited mitochondrial MPT.

The inhibitory effect of defatted BSA against Pal-CoA-induced swelling and depolarization occurred in a stoichiometric manner; 1 mol of BSA effectively inhibited the toxic effect of 6.6 mol of Pal-CoA. This ratio of BSA to Pal-CoA is in good agreement with the binding capacity of albumin for fatty acids [35]. Thus, fatty acids might be generated in and around mitochondrial membranes prior to the occurrence of MPT. In fact, polyunsaturated fatty acids were found to accumulate in mitochondria concomitant with

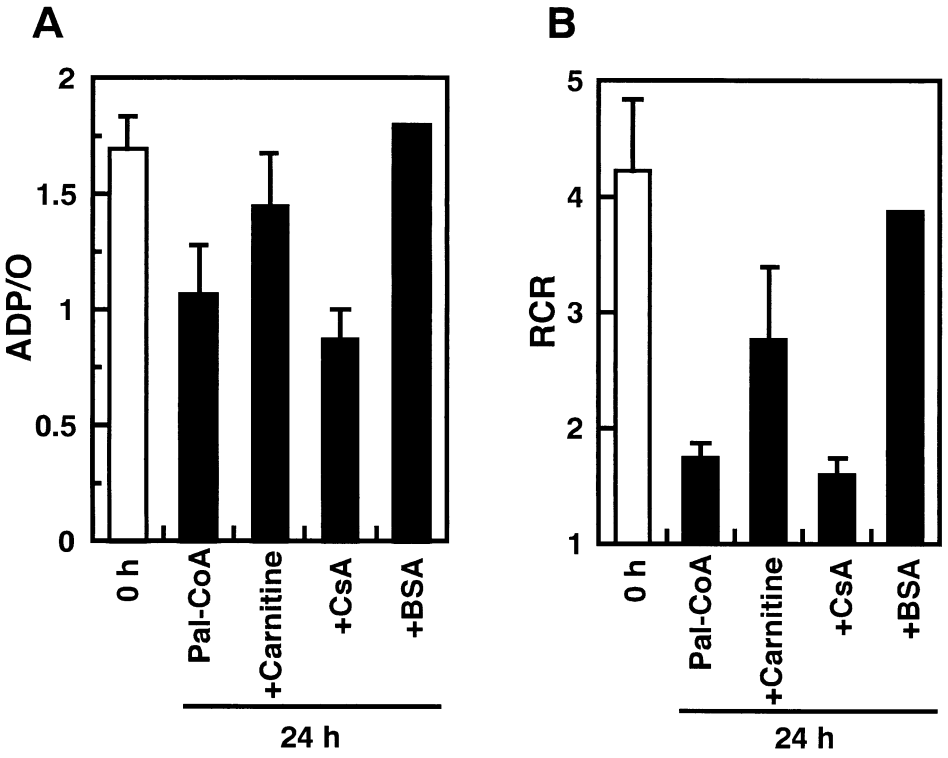

Fig. 9. Effects of L-carnitine, BSA, and CsA on mitochondrial functions after cold storage. Experimental conditions were as given in the legend of Fig. 1. Mitochondria were stored in 0.2 M sucrose containing 3 mM $MgCl_2$, 10 mM KCl, and 5 mM Tris–HCl buffer (pH 7.4) at 4° for 24 hr in the absence or presence of 1 mM L-carnitine, 0.5 mg/mL of BSA, or 1 μM CsA. Panels A and B show the ADP/O ratio and the respiratory control ratio, respectively. Values are means ± SD, N = 3.

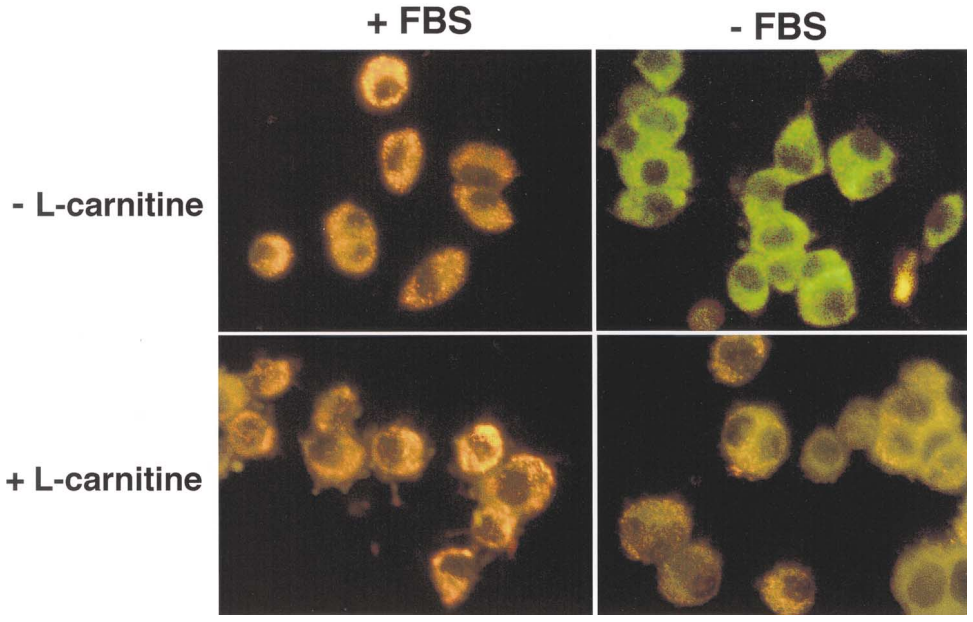

Fig. 10. Effect of L-carnitine and serum deprivation on mitochondrial depolarization in PC12 cells. Cells were preincubated with or without 1 mM L-carnitine for 3 days, and then the culture medium was deprived of serum (FBS). After 6 hr, the cells were stained with MitoSensor and observed under a fluorescence microscope. + L-Carnitine or –L-carnitine: cells preincubated with or without 1 mM L-carnitine, respectively. + FBS or –FBS: cells with or without serum, respectively.

their swelling [14]. Preliminary experiments in this laboratory also showed the accumulation of free fatty acids in mitochondria during MPT. Isolated mitochondria undergo aging and lose their functions during storage even at low temperature. Mitochondrial dysfunction occurring during cold storage has been shown to be caused by an increase in LCFAs [14,36]. Thus, the protective effect of BSA and L-carnitine could be explained by their ability to remove toxic LCFAs through the formation of either dissociable complexes or their esters, respectively. In contrast, CsA failed to suppress mitochondrial dysfunction during cold storage. This may be due to the inability of CsA to suppress the accumulation of LCFAs. Consistent with this hypothesis is the finding that PLA_2 inhibitors, such as chlorpromazine and mepacrine, also suppressed mitochondrial MPT. In this context, MPTs induced by either T_3, acetyl salicylate, diclofenac, LCFAs, or Pal-CoA required Ca^{2+}. These results indicate that Ca^{2+}-dependent activation of PLA_2 in mitochondria is essential for the induction of certain MPTs. Mitochondrial PLA_2 is localized predominantly in the inner membranes, and its activity at or near the contact sites of the outer and inner membranes is about two times higher than that at other sites of the inner membrane [37]. A wide variety of toxic compounds release mitochondrial Ca^{2+} by increasing the permeability of the inner membrane [38]. It has been reported that anoxia impairs mitochondrial functions by depleting ATP and releasing Ca^{2+} and free fatty acids through activation of PLA_2 [39]. These findings suggested that free fatty acids and lysophospholipids may accumulate in mitochondria through activation of Ca^{2+}-dependent PLA_2 prior to the induction of certain MPTs [40].

It has been reported that the membranes of cells undergoing apoptosis are highly susceptible to the action of secretory PLA_2 ($sPLA_2$). For example, the accumulation of arachidonic acid followed by apoptosis occurred when PC12 cells were deprived of nerve growth factor and serum, when mast cells were deprived of hematopoietic cytokines, and when Fas-antigen treated monocytic U937 cells were stimulated via Fas antigen [30]. These facts indicate that the cell membranes of apoptotic cells are the potential targets for extracellular type II $sPLA_2$ and that LCFAs and carnitine play critical roles in certain types of apoptotic cell death. Consistent with this hypothesis is the finding that L-carnitine delayed the depolarization of mitochondrial membranes in PC12 cells induced to undergo apoptosis by serum deprivation and arachidonic acid. Similar suppression of apoptotic cell death was observed with acetyl-L-carnitine [41,42].

At present, the molecular mechanism by which Pal-CoA and LCFAs enhance mitochondrial MPT is not clear. The effects of Pal-CoA and LCFAs are similar to that of carboxyatractyloside, a specific inhibitor of an ADP/ATP carrier [23]. In this case, LCFAs induce MPT not only by their protonophilic action (mediated by mitochondrial anion carriers) but also by interacting with the proteins of the MPT pore [43]. Pal-CoA and carboxyatractyloside bind to ADP/ATP carrier proteins with high affinity, thereby inhibiting anion-conducting channels [20–23,44].

Broekemeier and Pfeiffer [45] reported that a more negative membrane surface potential favors pore opening, whereas a more positive potential favors a closed pore. Furthermore, Starkov *et al*. [7] suggested that L-carnitine might have a sealing effect against MPT pore opening. Quite recently, it was also reported that L-carnitine inhibits Fas ligand-induced apoptosis of Jurkat cells through the inhibition of caspase-3, -7, and -8 activities [46]. These observations suggest that MPT and apoptosis of certain cells are regulated, in part, by a dynamic balance between LCFAs and L-carnitine in and around mitochondrial membranes. The molecular mechanism of LCFA-induced MPT should be studied further.

Acknowledgments

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