Journal of Bioenergetics and Biomembranes, Vol. 33, No. 4, 2001

Palmitic and Stearic Acids Bind Ca^{2+} with High Affinity and Form Nonspecific Channels in Black-Lipid Membranes. Possible Relation to Ca^{2+}-Activated Mitochondrial Pores[1]

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Received December 28, 2000; revised May 1, 2001

A mitochondrial hydrophobic component that forms Ca^{2+}-induced nonspecific ion channels in black-lipid membranes (Mironova *et al.*, 1997) has been purified and its nature elucidated. It consists of long-chain saturated fatty acids—mainly palmitic and stearic. These fatty acids, similar to the mitochondrial hydrophobic component, bind Ca^{2+} with high affinity in comparison with unsaturated fatty acids, saturated fatty acids with shorter aliphatic chains, phospholipids, and other lipids. Ca^{2+}-binding is inhibited by Mg^{2+} but not by K^+. For palmitic acid, the K_d for Ca^{2+} was 5 μM at pH 8.5 and 15 μM at pH 7.5, with the B_{max} of 0.48 $\pm$ 0.08 mmol/g. This corresponds to one Ca^{2+} ion for eight palmitic acid molecules. The data of IR spectroscopy confirm that Ca^{2+} does not form ionic bonds with palmitic and stearic acids under hydrophobic conditions. It has been found that in the presence of Ca^{2+}, palmitic and stearic acids, but not unsaturated FFA induce a nonspecific permeability in black-lipid membranes. Addition of Ca^{2+} in order to induce the permeability transition, increases the extractable amount of palmitic and stearic acids, the effect being prevented by a phospholipase A_2 inhibitor. The possible involvement of palmitic and stearic acids in the mitochondrial nonspecific permeability is discussed.

KEY WORDS: Palmitic acid; calcium binding; mitochondria; CsA-insensitive pore; ion channel; black-lipid membrane; phospholipase A_2.

INTRODUCTION

The calcium ion is a regulator of many processes in the cell. As a rule, this property of Ca^{2+} is connected with its ability to bind to different proteins. Earlier, we reported that the inner mitochondrial membrane contained a hydrophobic component with low molecular weight, probably of lipid nature, which bound Ca^{2+} and formed ion channels in black-lipid membranes (BLM) (Mironova *et al.*, 1997). These channels were nonspecific, but Ca^{2+} was necessary for their formation. The Ca^{2+}-binding channel-forming component (CaBC) was purified and characterized (Gateau-Roesch *et al.*, 2000). At pH 8.5, it exhibited high-affinity Ca^{2+}-binding sites with lower affinity at pH 7.2. One calcium ion bound maximally about 8 units of CaBC. Incubation of mitochondria with $CaCl_2$ prior to extraction of CaBC increased the

[1] Key to abbreviations: ANT, adenine nucleotide translocator, BLM, black-lipid membranes; CaBC, Ca^{2+}-binding channel-forming component; FFA, free fatty acids; CsA, cyclosporin A; GC, gas chromatography; MPT, mitochondrial permeability transition; PLA_2, phospholipase A_2; PVDF, polyvinylidene difluoride; TPP^+, tetraphenylphosphonium; BHT, butyl hydroxytoluene.

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Ca^{2+}-binding capacity of the partially purified component and probably its amount in mitochondria. Cyclosporin A (CsA) decreased Ca^{2+}-binding capacity of the partially purified CaBC to that obtained from control mitochondria (Gateau-Roesch *et al*., 2000).

In this paper, we report that the purified CaBC is composed mainly of palmitic and stearic acids, which have the highest calcium affinity of the mitochondrial lipids studied. The affinity of long-chain saturated free fatty acids (FFA) was significantly higher than that of unsaturated FFA and other lipids. Addition of palmitic and stearic acids to total beef brain or mitochondrial lipids used for BLM formation, increased ion permeability of BLM in the presence of Ca^{2+}. Incubation of mitochondria with a high concentration of Ca^{2+} increased both the Ca^{2+}-binding capacity of the FFA fraction and the amount of palmitic and stearic acids in mitochondria, while a phospholipase A_2 (PLA_2) inhibitor prevented these effects. The involvement of nonesterified long-chain saturated FFA in mitochondrial functions is discussed. This is of physiological interest, since FFA content is increased during ischemia-reperfusion (Johnston and Lewandowski, 1991) and palmitic acid has recently been shown to promote apoptosis and necrosis by liberating some factors from mitochondria (Kong and Rabkin, 2000).

MATERIALS AND METHODS

Rat liver mitochondria were isolated as previously described (Gateau-Roesch *et al*., 2000) and purified on a discontinuous sucrose gradient (Gasnier *et al*., 1989). The contact site-enriched fractions were prepared from purified mitochondria using the swelling procedure (Ardail *et al*., 1990). The inner membrane fraction was layered on a discontinuous sucrose gradient (39 ml) varying from 59 to 36.5% (w/v). The fractions (1 ml) were collected and characterized by marker enzyme activities. Cytochrome *c* oxidase (EC 1.9.3.1) was used as an inner membrane marker and monoamine oxidase (EC 1.4.3.4) as a marker for the outer membrane (Wharton and Tzagoloff, 1967; Caman *et al*., 1965).

CaBC was extracted from mitoplasts or purified fractions of mitochondrial membranes with ethanol and then according to the Floch procedure (Folch *et al*., 1957) and purified on an aminopropyl-bonded silica column (Pietsch and Lorenz, 1993). CaBC was eluted with diethylether/acetic acid (98:2, v/v) as a part of the FFA fraction. The Ca^{2+}-binding capacity of CaBC and various lipids was measured with $^{45}Ca^{2+}$ (Amersham Radiochemical Co., U.K.) using the protocol previously described (Gateau-Roesch *et al*., 2000).

Dissociation constants (K_d) and the maximal number of binding sites (B_{max}) were calculated using a computer-assisted nonlinear regression analysis (Motulsky, 1995).

For infrared (IR) spectroscopy, freshly distilled organic solvents and dehydrated $CaCl_2$ were used. The IR spectra were obtained using the FTIR spectrophotometer Nicolet Magna OR-750. Solid-state samples were cast from chloroform solution on the surface of crystals that were resistant to solvents and IR transparent. Potassium bromide crystals were used for registration of the palmitic acid spectrum. KRS-5 plates were used for sodium palmitate (Zeiss, Jena). Spectra of the Ca^{2+} complexes were obtained using the following mixture: One part of 1.2 M $CaCl_2$ in methanol and six parts of 1.2 M palmitic acid in chloroform. This mixture was applied to the surface of a potassium bromide crystal, dried, dissolved in chloroform, and redried to remove traces of methanol. The last procedure was repeated twice.

The ion-transporting properties of FA were measured using the BLM technique (Mueller *et al*., 1962). A Teflon cell, with a circular hole (0.975×10^{-2} cm^2) in the plastic sheet, separated the two chambers. The cell contained 4 ml of 20mM Tris–HCl buffer, pH 8.5, in the *cis* and *trans* compartments. For formation of BLM, 1.2 mg of total brain lipids, 100 μg of cardiolipin, and 6 μg of palmitic or stearic acids were mixed, dried, and dissolved in 60 μl of *n*-decane. Conductance was estimated by the voltage clamp method. Current was measured with an operational amplifier MAX406 connected to an IBM-compatible computer.

The FFA content in mitochondria and the Ca^{2+}-binding capacity of CaBC were assayed by suspending mitochondria in a medium containing 150 mM sucrose, 50 mM KCl, 10 mM Tris, and 3 mM KH_2PO_4, pH 7.4. $CaCl_2$ and aristolochic acid, an inhibitor of PLA_2, were added as indicated below. The mixture was incubated at 28°C for 10 min and diluted by the addition of four volumes of the incubation medium without KH_2PO_4, and centrifuged for 15 min at 5000 × g. CaBC was isolated as described above.

The mitochondrial membrane potential ($\Delta\Psi$) was measured with 1 μM TPP^+ using a TPP^+-sensitive electrode (Kamo *et al*., 1979).

For gas chromatographic (6c) analysis of FFA, lipid fractions were methylated with one volume of 5% sulfuric acid/methanol (v/v) at 100°C for 3 h. The reaction was stopped by the addition of three volumes of 5% K_2CO_3 (v/v). Methyl esters were extracted with four volumes of isooctane and then evaporated to dryness under nitrogen. Then they were dissolved in isooctane and injected into a SP 2380 capillary column (0.32 mm × 30 m, Supelco). Analysis was performed with a Perkin Elmer Autosystem

gas chromatograph equipped with a flame ionization detector coupled with a PE Nelson 1020 integrator. The initial oven temperature (50°C) was programmed from 50 to 150°C at 32°C/min, held for 1 min at 150°C, and then raised from 150 to 215°C at 2°C/min. The injection and detection temperatures were fixed at 220 and 250°C, respectively. The pressure of carrier gas was 12 psi. For identification of FFA, known standards were used. To correct for procedural losses between samples, peak areas were normalized to the area of the methylheptadecanoate internal standard peak. The injection of each sample was repeated at least three times and each peak area was measured and expressed as micrograms of FFA.

All chemicals were from Sigma. Aminopropyl-bonded silica columns were from Waters Associates, silica gel plates from Merck, and polyvinylidene difluoride (PVDF) Immobilon-P from Millipore.

RESULTS

The Nature of the Mitochondrial Ca^{2+}-Binding Component

Using [^{1}H] NMR and IR spectroscopy, it was demonstrated that CaBC contains aliphatic chains with carbonyl groups (Gateau-Roesch *et al*., 2000). Gas chromatograms of the purified CaBC (Fig. 1A) show two peaks corresponding to palmitic acid (C16:0) and stearic acid (C18:0). Moreover, this purified component was not stained with cupric acetate, which stains almost all lipids, including unsaturated FFA, but does not stain saturated ones. However, CaBC was stained with bromocresol green, a reagent for acids. These data indicate that mitochondrial CaBC is composed of long-chain saturated FFA.

GC analysis of FFA isolated from the whole mitochondria (Fig. 1B) shows that palmitic and stearic acids are the main FFA in mitochondria. In thin-layer chromatography saturated FFA and CaBC, visualized by bromocresol green, were colocalized (data not shown).

We compared the technique of FFA extraction with ethanol (Mironova *et al*., 1997; Gateau-Roesch *et al*., 2000) with some classical protocols that make use of other organic solvents (Folch *et al*., 1957; Bligh and Dyer, 1959; Bowman and Beroza, 1966) and did not find any significant differences in the amounts of FFA extracted from mitochondria.

Ca^{2+}-Binding Properties of Different Lipids

The data of Table I indicate that saturated FFA have higher Ca^{2+}-binding capacity than unsaturated ones, phospholipids, and lysophospholipids. The Ca^{2+} affinity of FFA varies with the length of the aliphatic chain, with palmitic, stearic, and eicosanoic acids exhibiting the highest Ca^{2+}-binding capacity.

The finding that saturated FFA have the highest Ca^{2+} affinity is confirmed by the results on Ca^{2+}-binding properties of different lipid classes from mitochondria. As shown in Table II, the FFA-containing fraction had the highest Ca^{2+} binding.

Ca^{2+}-Binding Properties of Palmitic and Stearic Acids

Since palmitic and stearic acids were identified as the substances responsible for the Ca^{2+} binding to CaBC (Mironova *et al*., 1997; Gateau-Roesch *et al*., 2000), their Ca^{2+}-binding properties were studied in more detail. The binding of Ca^{2+} to CaBC was shown to be clearly pH dependent (Gateau-Roesch *et al*., 2000). In the present work, we analyzed the pH dependence for the Ca^{2+}-binding properties of palmitic and stearic acids. The maximal binding of Ca^{2+} to palmitic acid was observed at alkaline pH (Fig. 2), while almost no Ca^{2+} binding is found at pH 6.5. The same was found for stearic acid (data not shown). These data are analogous to those obtained for CaBC (Gateau-Roesch *et al*., 2000).

Figure 3 shows data used for the estimation of K_d and B_{max} for the complex of Ca^{2+} with palmitic acid at pH 8.5 and 7.5. The calculated K_d values are 5 μM at pH 8.5 and 15 μM at pH 7.5, with B_{max} being 0.48 $\pm$ 0.08 mmol Ca^{2+}/g of palmitic acid at both pH's.

Additional information on the nature of Ca^{2+}–palmitic acid complex was obtained by comparing its IR spectra with those of free palmitic acid and sodium palmitate (Fig. 4). The spectrum of palmitic acid is a typical spectrum of a FFA dimer. For acid monomers, there is a characteristic band at 1760 cm^{-1} in polar solvents ascribed to the stretching vibration of the carbonyl group, while for dimers of FFA with even number of carbon atoms, the band of carbonyl involved in intermolecular hydrogen bonding is at 1701-1698 cm^{-1} (Nakanishi, 1962). In the sodium palmitate spectrum, the band of carbonyl group is shifted from 1705 to 1558 cm^{-1}, which is typical for carboxyl ions of FFA salts. The fact that, under anhydrous conditions, such a spectral shift is not observed in the presence of Ca^{2+}, indicates that Ca^{2+} does not form ionic bonds with palmitic acid under hydrophobic conditions. At the same time, the appearance of a more narrow and intensive band at 3403 cm^{-1} testifies to the formation of a complex of palmitic acid with Ca^{2+}, this occurring in such a way that hydrogen bonds of the OH-group becomes more

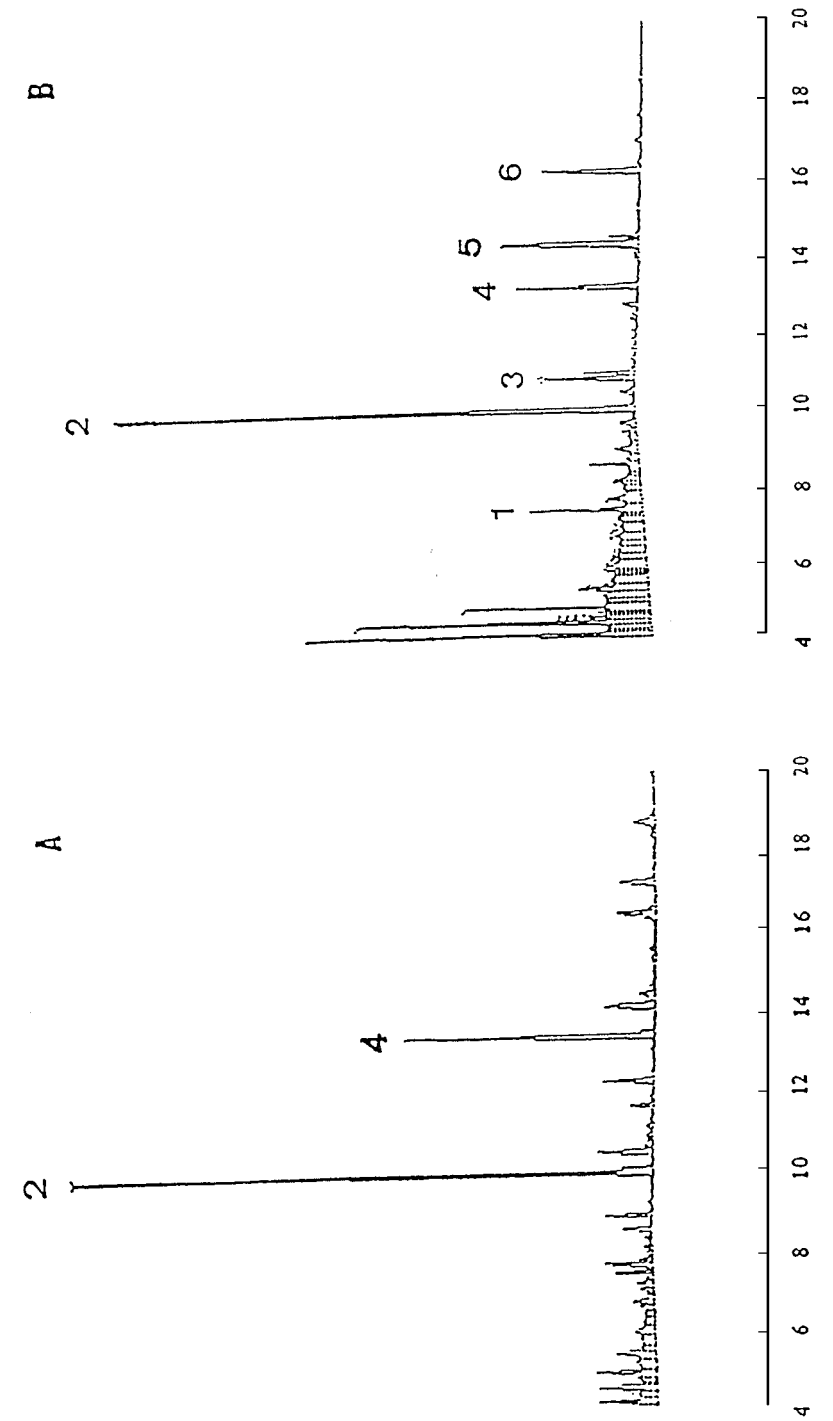

Fig. 1. Gas-liquid chromatogram of CaBC. (A) Pure CaBC was analyzed by GC (as described in Materials and Methods). (B) The ethanol extract from 20 mg of mitochondria (6 mg of protein/ml) was fractionated on a NH_2–silicic acid column (see Materials and Methods), and the resulting FFA fraction methylated and analyzed by GC. The FFA chain length and the quantity of unsaturated bonds are: **1**, C14:0; **2**, C16:0; **3**, C16:1; **4**, C18:0; **5**, C18:1; **6**, C18:2.

Table I. Binding of Ca^{2+} to Various Lipids[a]

Lipid	Relative Ca^{2+} binding
Lauric acid (12:0)	0.50 ± 0.03
Myristic acid (14:0)	7.30 ± 0.25
Palmitic acid (16:0)	83.00 ± 0.72
Stearic acid (18:0)	100.00
Eicosanoic acid (20:0)	73.00 ± 2.5
Docosanoic acid (22:0)	44.00 ± 1.2
Lignoceric acid (24:0)	15.00 ± 0.35
Palmitoleic acid (16:1)	1.90 ± 0.08
Oleic acid (18:1)	5.70 ± 0.12
Linoleic acid (18:2)	0.65 ± 0.04
Linoleinic acid (18:3)	0.87 ± 0.05
Arachidonic acid (20:4)	1.10 ± 0.05
1-Palmitoyl-lysophosphatidylcholine	0.43 ± 0.02
1-Stearoyl-lysophosphatidylcholine	0.47 ± 0.02
1-Lauroyl-lysophosphatidylcholine	0.43 ± 0.01
Lysophosphatidylserine	0.54 ± 0.03
1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine	0.40 ± 0.2
1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine	0.22 ± 0.01
1-Palmitoyl-*sn*-glycero-1-3-phosphatidylethanolamine	0.76 ± 0.03
Palmitoyl-CoA	0.43 ± 0.02
Cardiolipin	0.60 ± 0.03
L-α-phosphatidic acid	19.50 ± 0.8
Cholesterol	0.33 ± 0.01
Cerebrosides	0.20 ± 0.01
Sphingomyelin	0.30 ± 0.01

[a] Five microliters of 2 mM solution of each lipid were applied on a PVDF membrane and binding of Ca^{2+} to the sample was estimated at pH 8.5 in the presence of 5 μM^{45} $CaCl_2$ (see Materials and Methods). Ca^{2+} binding was measured in counts per minute. Stearic acid was taken as a reference to calculate the relative Ca^{2+} binding. Data are expressed in percentage and as the means ± SD of four experiments.

regular than in the dimer of palmitic acid (Pimental and McClellan, 1960). The same characteristics were obtained for stearic acid (data not shown). However, it should be noted that in a water solution at pH 6.8, one Ca^{2+} ion forms a salt with two molecules of palmitic acid (Waters *et al.*, 1984).

Influence of Different Cations on the Binding of Ca^{2+} to Palmitic Acid

Figure 5 shows that 0.1–1 mM $MgCl_2$ inhibits binding of Ca^{2+} to palmitic acid. Similar results were obtained for stearic acid. Since this concentration of free Mg^{2+} is in the physiological range for mitochondria, the inhibition may play a physiological role. The K^+ ions at 50–500 mM do not influence binding of Ca^{2+} to palmitic or stearic acids.

Table II. Binding of Ca^{2+} to Fractions from the Aminopropyl-Bonded Silica Column[a]

Fraction	Relative Ca^{2+}-binding
1 Neutral lipids–cholesterol	5.0 ± 0.08
2 Free fatty acids	100.0
3 Phosphatidylcholine	1.5 ± 0.05
4 Phosphatidylethanolamine	0.0
5 Phosphatidylserine	15.0 ± 0.09
6 Phosphatidylinositol	5.0 ± 0.06

[a] The ethanol extract from 20 mg of mitochondria was fractionated on an aminopropyl-bonded silica column (see Materials and Methods) to separate different lipid classes. Each fraction was dried and dissolved in chloroform/methanol (2:1, v/v). An aliquot was then applied to a PVDF membrane and incubated with 5 μM $^{45}CaCl_2$ at pH 8.5 and Ca^{2+} binding measured in counts per minute. The FFA fraction was taken as a reference to calculate the relative Ca^{2+} binding. Data are expressed in percentage and as the means ± SD of four experiments.

Localization of the Ca^{2+}-Binding Component in Mitochondria

Mitoplasts were subjected to a hypotonic treatment and two fractions were obtained after centrifugation in a sucrose gradient. One fraction, IM_1, was enriched in monoamine oxidase (81.5% of the total activity), indicating it to be a contact site-enriched fraction (Table III). Moreover, this fraction contained a high level of cholesterol, which is preferentially localized in the contact sites (Levrat and Louisot, 1992). The second fraction, IM_2, was enriched in cytochrome *c* oxidase (78% of the total activity) and, thus, was an inner membrane-enriched fraction. Measurement of Ca^{2+} binding to the CaBC-containing fractions extracted from IM_1 and IM_2 indicated that the IM_1 had a much higher Ca^{2+}-binding capacity than the IM_2 (Table III). Thus, it may be concluded that CaBC is mainly located in the contact sites.

Influence of Palmitic and Stearic Acids on BLM Permeability

The addition of 0.5% (w/w) palmitic or stearic acid to the total brain lipids/cardiolipin mixture used for formation of BLM increases ion permeability of the membrane in the presence of $CaCl_2$ (Table IV). One can see that in the absence of Ca^{2+}, modification of the membrane by these FFA does not change permeability, even in the presence of 100 mM KCl. However, the addition of 1 mM Ca^{2+} leads to a 30-fold increase in membrane conductance. The subsequent addition of 100 mM KCl caused a further conductance increase. Figure 6 shows that addition

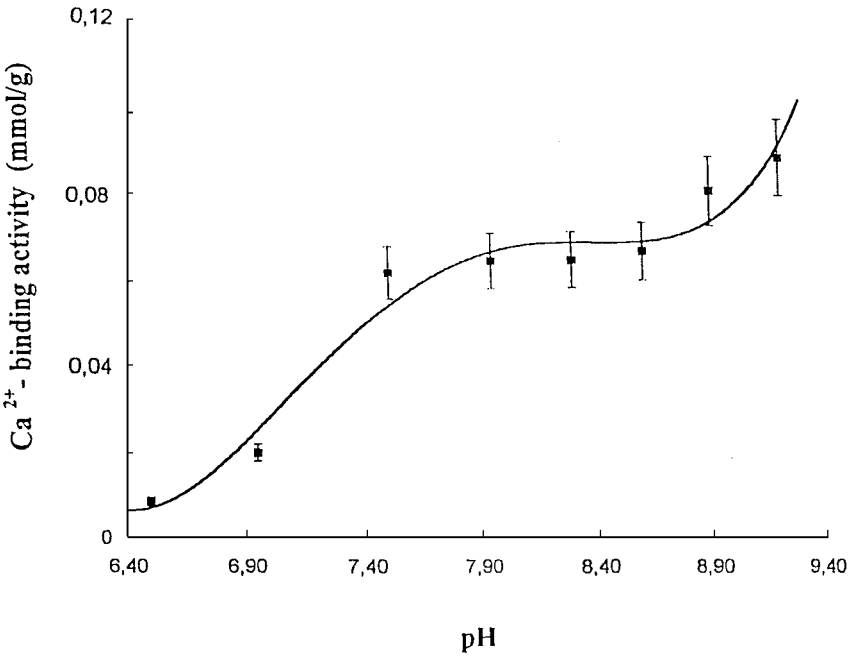

Fig. 2. pH dependence of Ca^{2+} binding to palmitic acid. Palmitic acid (5 μl of 2 mM) were applied on a PVDF membrane and Ca^{2+} binding of the sample measured in the presence of 3 μM^{45} $CaCl_2$ (see Materials and Methods). Data are from six experiments.

of 0.5 mM Ca^{2+} induced channel activity in the palmitic acid-modified membrane. In the presence of a threefold Ca^{2+} gradient, the membrane potential was only 3 mV. This indicates the channels to be nonspecific. Analogous experiments with modification of BLM by 0.5–2% (w/w) unsaturated palmitoleic or oleic acids, which bind Ca^{2+} with low affinity, showed no changes in membrane permeability in the presence of Ca^{2+} and K^+ (Table IV).

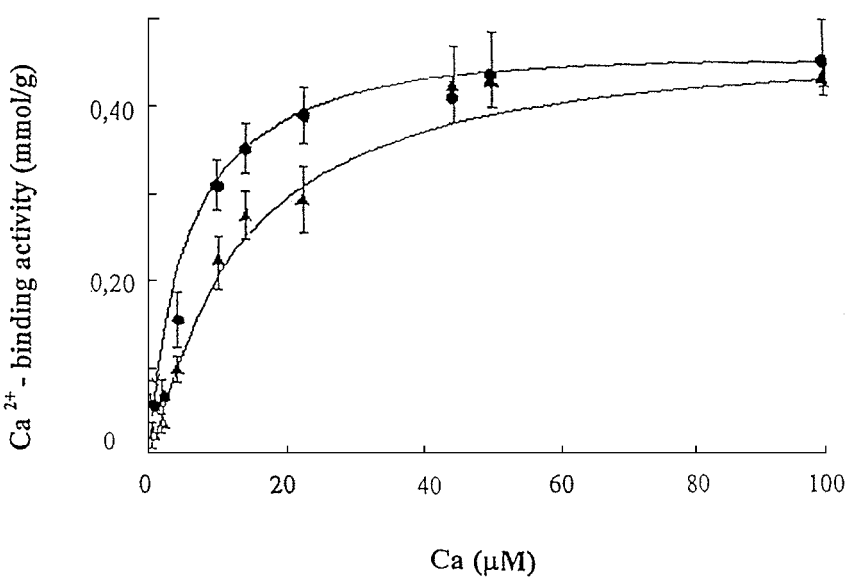

Fig. 3. Binding of Ca^{2+} to palmitic acid. Measurements were performed at pH 7.5 (triangles) and pH 8.5 (circles) and at different concentrations of Ca^{2+}. Experimental details are as in Fig. 2. Data from four experiments.

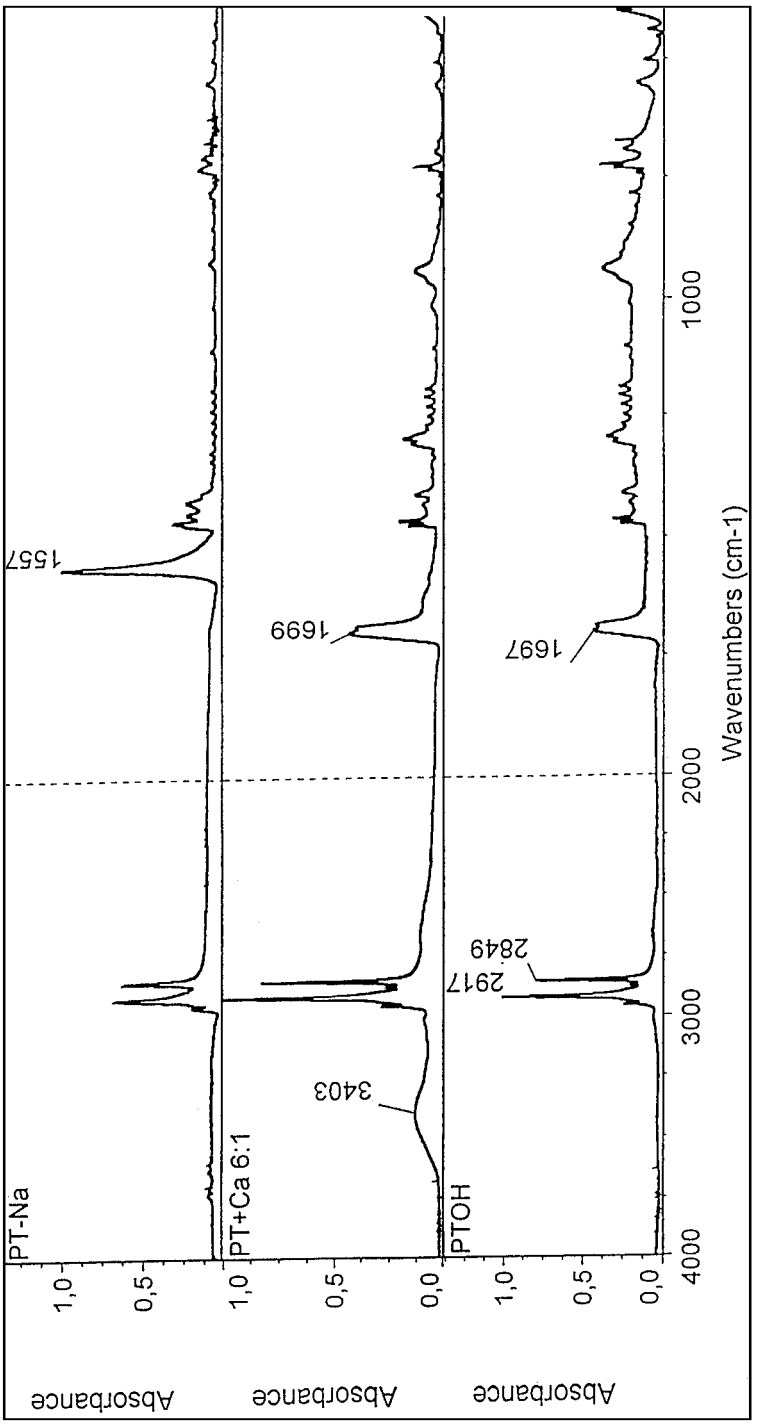

Fig. 4. IR spectra of free palmitic acid and its complex with Ca^{2+} and sodium palmitate. The solid-state samples were cast from organic solvents on IR transparent plates. (A) palmitic acid; (B) palmitic acid in the presence of Ca^{2+}; (C) sodium palmitate. For experimental details, see Materials and Methods.

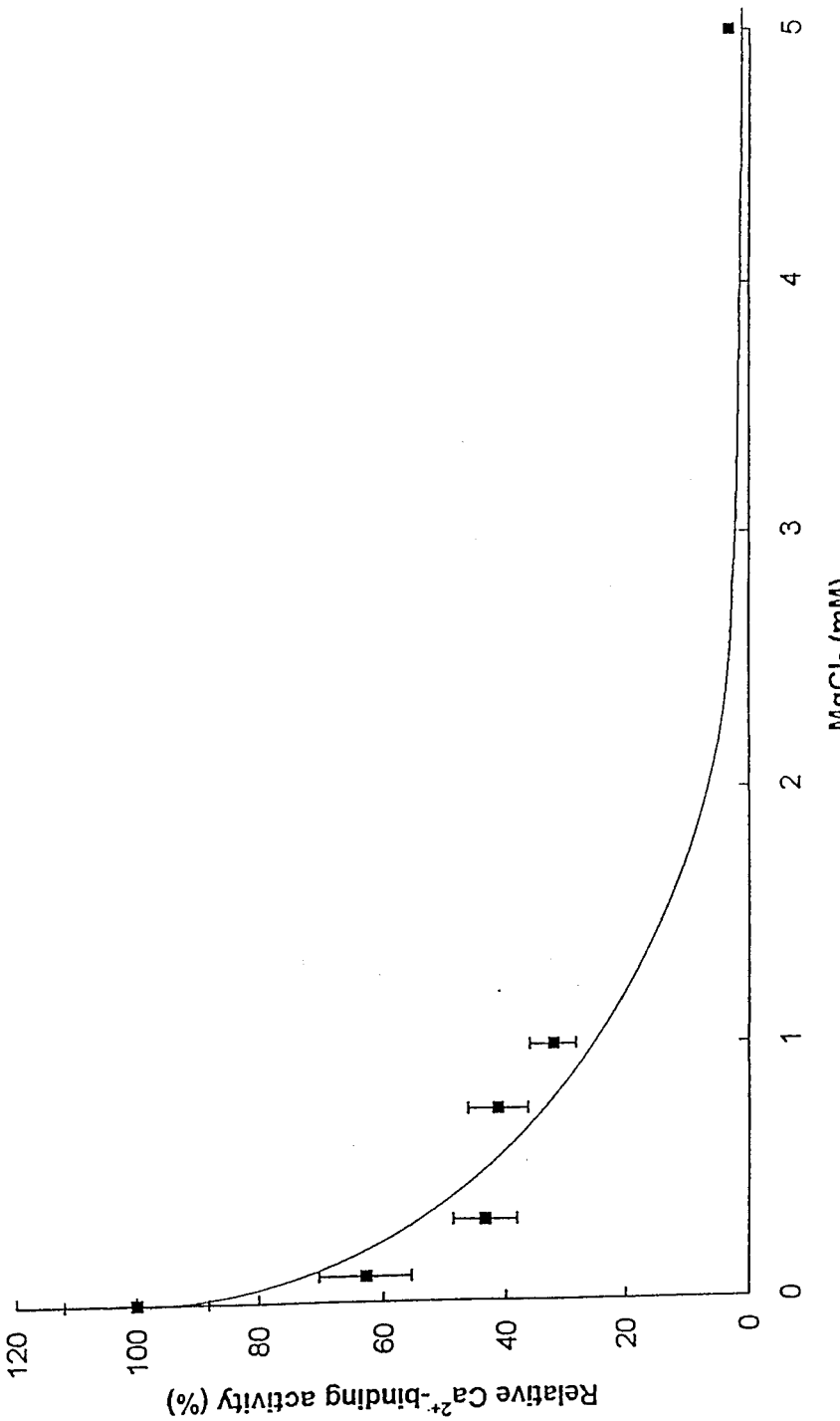

Fig. 5. Influence of Mg^{2+} on the binding of Ca^{2+} to palmitic and stearic acid. Ca^{2+}-binding was measured in the presence of different concentrations of $MgCl_2$. The data are from three experiments. Experimental details are as in Fig. 2.

Table III. Localization of Hydrophobic Ca^{2+}-Binding Component in Mitochondria[a]

	Contact site-enriched fraction	Inner membrane-enriched fraction
Relative monoamine oxidase activity (%)	81.5	18.5
Relative cytochrome *c* oxidase activity (%)	21.6	78.4
Cholesterol (μg/mg of protein)	6.54	2.85
Relative Ca^{2+} binding (%)	86	14

[a] Preparation of the contact site-enriched fraction (IM1) and the inner membrane-enriched fraction (IM_2), assay of the activity of marker enzymes, and Ca^{2+}-binding were performed as described in Materials and Methods.

When mitochondrial lipids, instead of total brain lipids, were used for BLM formation, channels could form with a lower content of palmitic or stearic acid in the membrane and at a lower concentration of Ca^{2+} in the medium (Table IV).

Influence of Ca^{2+} and a PLA_2 Inhibitor on the Content of Saturated Fatty Acids in Rat Liver Mitochondria

The additon of Ca^{2+} to mitochondria is known to activate PLA_2 resulting in FFA release (Pfeiffer *et al*., 1979). In our experiments, preincubation of mitochondria with 83 nmol $CaCl_2$/mg of mitochondrial protein in presence of nonorganic phosphate, which from our and literature data would lead to the opening of MPT (Pfeiffer *et al*., 1979; Broekemeier and Pfeiffer, 1995), increased the Ca^{2+}-binding capacity of the partially purified hydrophobic fraction extracted from mitochondria with ethanol (Fig. 7a and b). In the presence of aristolochic acid, an inhibitor of PLA_2 (Pastorino *et al*., 1996), the Ca^{2+}-binding capacity (Fig. 7c) approached control levels (Fig. 7a). The content of palmitic and stearic acids in the mitochondrial membranes in these conditions is correlated with the Ca^{2+}-binding capacity the mitochondrial fractions (Table V).

DISCUSSION

The main finding in this study is that the mitochondrial hydrophobic component, which has high affinity to Ca^{2+} and in the presence of Ca^{2+} forms nonspecific channels in BLM (Mironova *et al*., 1997; Gateau-Roesch *et al*., 2000), is composed of long-chain saturated FFA, mainly palmitic and stearic acids. The ability of these FFA to form ion channels in the presence of Ca^{2+} is confirmed experimentally. Another important finding is that among mitochondrial lipids, palmitic and stearic acids have the highest Ca^{2+} affinity. Of special physiological interest are the growth of affinity over the pH range 7.0–7.5 and a

Table IV. Influence of Free Fatty Acids on BLM Conductance[a]

	BLM conductance (10^{-9} S/cm^2)			
	Palmitic acid			
Additions	Total brain lipids	Mitochondrial lipids	Stearic acid total brain lipids	Palmitoleic acid total brain lipids
None	1 ÷ 1.5	4.08 ÷ 6.1	1.02 ÷ 2.04	1 ÷ 1.6
0.1 mM $CaCl_2$	1 ÷ 1.5	10.2 ÷ 14.3	1 ÷ 1.5	—
0.5 mM $CaCl_2$	10 ÷ 50	40.8 ÷ 48.9	28.5 ÷ 53.1	—
1 mM $CaCl_2$	30 ÷ 150	581 ÷ 618	53.14 ÷ 114.6	1.1 ÷ 3.04
0.1 mM $CaCl_2$ +30 mM KCl	10 ÷ 12	51 ÷ 81	12 ÷ 15	—
1 mM $CaCl_2$ +30 mM KCl	—	15600 ÷ 30000	122.14 ÷ 341.06	1.05 ÷ 1.9
1 mM $CaCl_2$ +100 mM KCl	300 ÷ 500	40700 ÷ 40900	—	—
100 mM KCl	1 ÷ 1.5	9 ÷ 6.5	1.02 ÷ 2.04	—

[a] The experimental cell contained 20 mM Tris–HCl buffer, pH 8.5. BLM was formed from the mixture of 1.2 mg of total brain lipids, 100 μg of cardiolipin, and 6 μg of palmitic or stearic acid dissolved in 60 μl of *n*-decane, or from the mixture of 1.2 mg of mitochondrial lipids and 3.9 μg of palmitic acid dissolved in 60 μl of *n*-decane (see Materials and Methods).

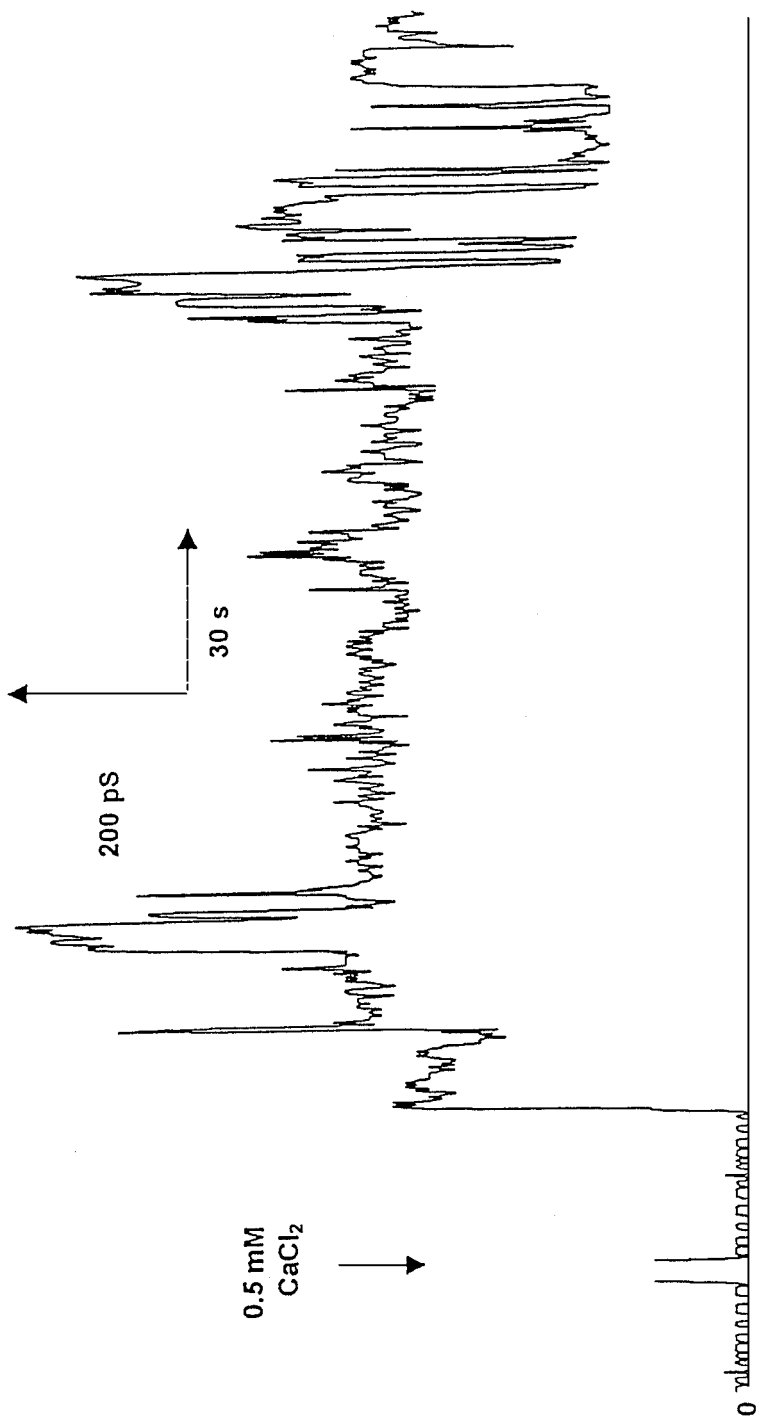

Fig. 6. Influence of Ca^{2+} on the conductance of BLM modified by palmitic acid. The chamber solution contained 20 mM Tris–HCl buffer, pH 8.5, and 0.5 mM Ca^{2+}. The applied voltage was 100 mV. For BLM formation and reconstitution, see Materials and Methods.

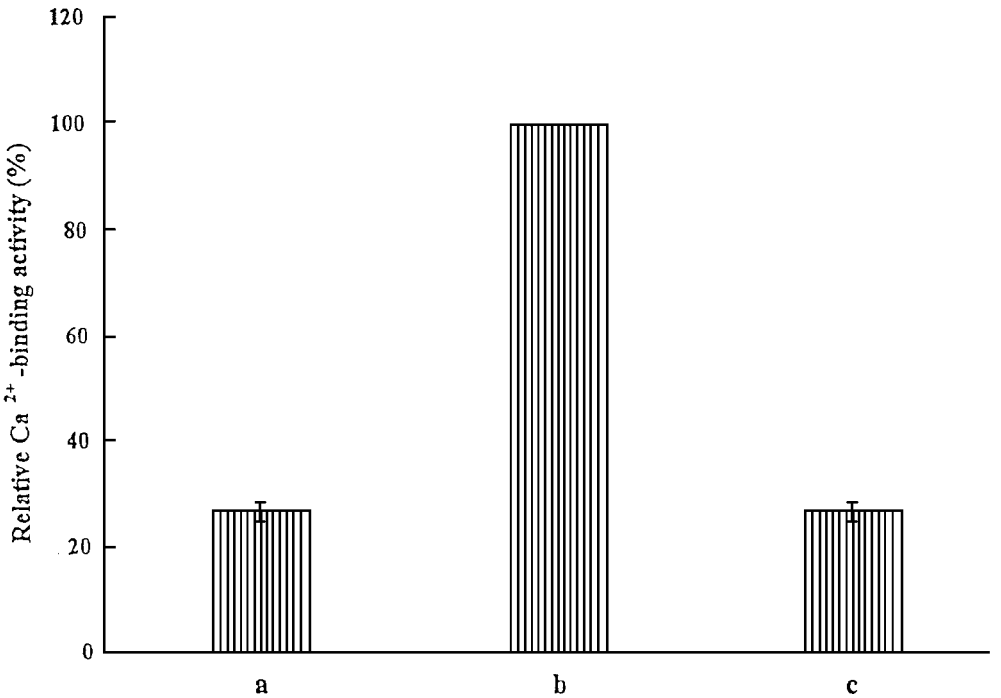

Fig. 7. Influence of a PLA_2 inhibitor on Ca^{2+} binding to the partially purified mitochondrial channel-forming component. Twenty mg of mitochondria (6 mg of protein/ml) were incubated at 28°C for 10 min in a medium containing 150 mM sucrose, 50 mM KCl, and 3 mM KH_2PO_4 in 10 mM Tris–HCl buffer, pH 7.4. (a) Control without any additions; (b) 83 nmol of Ca^{2+}/mg of protein added; (c) 82 nmol of Ca^{2+}/mg of protein and 10 μM aristolochic acid added. Ca^{2+} binding capacity was measured in counts per minute. Results obtained from (b) were taken as a reference to calculate the relative Ca^{2+} binding. Data are expressed in percentage and as the means ± SD of five experiments.

high-affinity value at pH 8.5. Indeed, the uptake of Ca^{2+} by mitochondria is accompanied by the matrix alkalization (Saris, 1963), which may promote the MPT pore opening (Bernardi, 1999; Haworth and Hunter, 1980). It is also worth mentioning that palmitic and stearic acids have an optimal chain length for Ca^{2+} binding. Table I shows that palmitic, stearic and eicosanoic acids have the highest affinity to Ca^{2+}. In mitochondria, however, the content of palmitic acid is two to three times higher than that of stearic acid, while eicosanoic acid is present only in trace amounts.

Table V. Content of Palmitic and Stearic Acids in Mitochondria Incubated under Various Conditions[a]

Incubation conditions	μgC16:0/mg protein	μ g C18:0/mg protein
Control	1.10 ± 0.04	0.30 ± 0.01
+82 nmol $CaCl_2$/mg protein	2.20 ± 0.07	0.46 ± 0.02
+82 nmol $CaCl_2$/mg protein + 10 μM aristolochic acid	1.22 ± 0.04	0.30 ± 0.01

[a] Mitochondria (6 mg of protein/ml) were incubated at 28°C for 10 min in a medium containing 150 mM sucrose, 50 mM KCl, 3 mM KH_2PO_4, and 10 mM Tris, pH 7.4. Ethanol extracts of mitochondrial samples were fractionated on an aminopropyl-bonded silica columns and the methylated FFA fraction analyzed by gas chromatography (see Materials and Methods). Data are expressed as the means ± SD of four experiments.

Like CaBC, pure palmitic and stearic acids form nonselective channels in BLM-made total brain extract only in the presence of Ca^{2+} (Fig. 6). In contrast, unsaturated FFA, such as palmitoleic and oleic acids, which have a low affinity to Ca^{2+}, are unable to form ion channels in BLM. When mitochondrial lipids, instead of those from total brain extract, are used for BLM formation, the same level of membrane permeability is achieved at a smaller content of FFA in the lipid mixture and at a lower concentration of Ca^{2+} in the medium (Table IV). Apparently, the composition of mitochondrial lipids is more favorable for the formation of complexes of Ca^{2+} with plamitic or stearic acid, which are responsible for the increase in membrane permeability.

The B_{max} value for binding of Ca^{2+} to palmitic acid and its molecular weight (256 Da) gives a palmitic acid/Ca^{2+} ratio of 8. It should be noted that in coordination complexes, Ca^{2+} usually has six to eight bonds (Williams, 1976). Thus, maximally, eight molecules of palmitic acid

can be bound to one calcium ion. Analogous results were obtained for stearic acid. The IR spectra of dry films of palmitic acid, sodium palmitate, and Ca^{2+}–palmitic acid complexes cast from hydrophobic solvents (Fig. 4) support the conclusion that the bonds between Ca^{2+} and fatty acid molecules are coordination ones. The formation of Ca^{2+}–palmitic acid complexes could well cause perturbations in the membrane, resulting in induction of ion channels or leaks. The BLM data (Fig. 6, Table IV) indicate that complexes of Ca^{2+} with plamitic acid may be directly involved in the increase in nonspecific membrane permeability. For BLM and mitochondrial experiments, the amount of calcium for pore opening needs significant more than for Ca^{2+}-binding in mitochondria, for example. Ions of Ca need not only for binding of palmitic acids but for PLA_2 activation.

The rise of nonspecific permeability of the inner mitochondrial membrane in the presence of Ca^{2+} could be ascribed to FFA released by the Ca^{2+}-activated PLA_2 (Pfeiffer *et al*., 1979). This enzyme has been shown to induce an increase in membrane permeability in liposomes (Eriksson and Saris, 1989). In our experiments, the Ca^{2+}-induced rise in the content of palmitic and stearic acids in mitochondria (Table V), as well as an increase in Ca^{2+}-binding capacity of the partially purified ethanol fraction (Fig. 7), were prevented by inhibition of PLA_2 activation. Moreover, earlier we found that CsA at a concentration that inhibits MPT decreased the Ca^{2+}-binding capacity of the mitochondrial FFA-contained fraction (Gateau-Roesch *et al*., 2000). It seems likely that this effect is due to a modification of the state of phospholipids in the presence of CsA (Thuren *et al*., 1984), which may, in turn, modulate the PLA_2 activity.

It has been suggested that activation of PLA_2 and, thereby, production and accumulation of free fatty acids, might also promote opening of the CsA-insensitive pore (Gudz *et al*., 1997). This may also be responsible for the CsA-insensitive increase in permeability induced by the signal peptide (Sokolove and Kinnally, 1996).

Recently it was found that palmitic acid opens a CsA-insensitive pore in the inner mitochondrial membrane (Sutlan and Sokolove, 2001a) and, in the presence of modest Ca^{2+} concentrations (75 mmol/mg protein), medium-chain FFA (C12:0-C18:0) were more effective in eliciting CsA-insensitive mitochondrial swelling than were shorter or longer saturated and unsaturated FFA (Sutlan and Sokolove, 2001b). This is in good agreement with the data presented in Table I and show that Ca^{2+}-binding properties are important for the formation of the CsA-insensitive pore in the inner mitochondrial membrane.

Schönfeld and Bohnensack (1997) found palmitic acid to compete with [^{3}H] atractyloside for binding to the ANT, whereas lauric acid was less effective. It was suggested that FFA could promote MPT pore opening by stabilizing the cytosolic conformation of ANT—one strong candidate for a CsA-sensitive pore component (Halestrap *et al*., 1998). A study of lipid–protein interaction in ANT has shown stearic acid to be bound to ANT with a higher specificity than phospholipids (Horvath *et al*., 1990). The relatively high Ca^{2+} affinity of palmitic and stearic acids and their ability to bind to ANT, may provide a mechanism for making the conformation of ANT sensitive to changes in the Ca^{2+} concentration. These FFA may thus be potential Ca^{2+} sensors for MPT. In this context it is of some interest that Mg^{2+} inhibits both the Ca^{2+} binding to palmitic acid (Fig. 5) and the MPT pore opening (Zoratti and Szabo, 1995). Cardiolipin also is bound to ANT and was proposed to be a potential Ca^{2+} sensor for MPT (Brustovetsky and Klingenberg, 1996). However, the higher Ca^{2+} affinity of palmitic and stearic acid in comparison with cardiolipin (Table 1), makes them better potential candidates for such a function. As long as the composition of the pore is not unanimously known (Bernardi, 1999), it is too early to make conclusions on which Ca^{2+}-binding sites actually act as sensors.

FFA have a number of well-described biological properties, some of which are directly related to their effects on mitochondria. It has recently been reported that palmitic acid induces mitochondria to release proapoptotic factors (cytochrome *c* and the other apoptois-inducing factors) which causes apoptosis or necrosis and cell death (Kong and Rabkin, 2000). The data presented in this paper give more information on the mechanism of mitochondrial pore stimulation by palmitic acid and, thereby, of apoptosis and necrosis.

ACKNOWLEDGMENTS

We are grateful to Prof. Yu A. Lazarev for fruitful discussions. We are grateful to Mrs. Margarita Nesterova and Mrs. Galina Partala for expert technical assistance. This work was supported by Institut National de la Santé et de la Recherche Médicale INSERM 189, France (to P.L.), by a grant from the Russian government RFBR N 01-04-48551 (to G.D.M.), and by the Academy of Finland, the Finnish Society of Sciences and Letters (to N.-E.L.S.).

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