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Regulation of transmembrane ion transport by reaction products of phospholipase A_2. II. Effects of arachidonic acid and other fatty acids on mitochondrial Ca^{2+} transport

Ingo Rustenbeck and Sigurd Lenzen

Institute of Pharmacology and Toxicology, University of Gottingen, Gottingen (F.R.G.)

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The effects of arachidonic acid and other fatty acids on mitochondrial Ca^{2+} transport were studied. *Cis*-unsaturated fatty acids generally strongly inhibited mitochondrial Ca^{2+} uptake, induced a net Ca^{2+} efflux, and thereby increased the extramitochondrial Ca^{2+} concentration, whereas *trans*-unsaturated fatty acids were ineffective. Saturated fatty acids exhibited slight activity at chain lengths from C_{16} to C_{14} only. The structure–activity relationship and the inability of some of the effective fatty acids such as palmitoleic and myristoleic acid to be metabolized to eicosanoids suggest that Ca^{2+} release was induced by the fatty acids themselves and resulted from changes in the mitochondrial membrane bilayer structure. There was a correlation between Ca^{2+}-releasing potency and reduction of mitochondrial membrane potential, which is the main driving force for mitochondrial Ca^{2+} uptake. There were, however, considerable differences compared with the effects of lysophospholipids on the membrane potential. The mechanism of action of fatty acids may be that of a fluidizing effect on the hydrophobic core of the membrane, thereby modulating the activity of integral membrane proteins of the respiratory chain.

Introduction

A variety of recent reports on the relationship between cell stimulation and phospholipid metabolism have described the production of lysophospholipids, indicating activation of phospholipase A_2 (PLA_2) in different tissues [1–4]. These and other observations have led to the hypothesis that a PLA_2-mediated signalling pathway might exist [5], in analogy to the phospholipase-C-mediated signalling pathway which generates the intracellular second messengers inositol 1,4,5-trisphosphate [6] and diacylglycerol [7].

PLA_2 removes the fatty acid from the C_2-atom of the phospholipid glycerol backbone [8]. Fatty acids esterified in this position are mainly unsaturated. The other reaction product – the lysophospholipid – is determined by the specificity of the intracellular PLA_2 for the head-group of the parent phospholipid. As a single type of phospholipid consists of several molecular species with different fatty acids [9,10], the action of the PLA_2 will probably generate more than one type of fatty acid.

The interest in fatty acid liberation by PLA_2 has focused mainly on arachidonic acid [11,12] due to its further metabolism in the cyclooxygenase and lipoxygenase pathways. Many biological effects which can occur after activation of PLA_2 are usually not ascribed to the fatty acids themselves but to the generation of eicosanoid metabolites. Earlier reports describing effects of unsaturated fatty acids on intracellular Ca^{2+} mobilization [13] have been criticized from this point of view [14] or have been interpreted as not representing biologically relevant phenomena [15].

It is unsettled at present whether fatty acids, generated by the action of PLA_2, can influence intracellular Ca^{2+} homeostasis without requiring further metabolism. Previously we have demonstrated that the release of intracellularly stored Ca^{2+} induced by arachidonic acid acting on permeabilized cells results primarily from its action on the mitochondria [16]. Ca^{2+} release from endoplasmic reticulum also contributed somewhat to the increase in the free Ca^{2+} concentration but far less

Abbreviations: PLA_2, phospholipase A_2 (EC 3.1.1.4); TPP^+, tetraphenylphosphonium; PLC, phospholipase C; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

Correspondence: S. Lenzen, Institut für Pharmakologie und Toxikologie, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, F.R.G.

than mitochondria, and it did not further increase the free Ca^{2+} concentration when co-incubated with mitochondria [16]

In the present study the effects of saturated, *cis*- and *trans*-unsaturated fatty acids on mitochondrial Ca^{2+} transport were investigated to elucidate their mechanism of action and evaluate a possible second messenger role of these substances The effects of lysophospholipids are described in a preceding paper [17]

Materials and Methods

Chemicals Arachidonic acid and all other fatty acids were obtained from Sigma $^{45}CaCl_2$ was from Amersham International All other chemicals of analytical grade were from Sigma (St Louis MO), Boehringer (Mannheim, F R G), Serva (Heidelberg, F R G), or Merck (Darmstadt, F R G)

Preparation of mitochondrial fractions Liver was obtained from Wistar rats homogenized in homogenization medium (210 mM mannitol/70 mM sucrose/20 mM Hepes, adjusted to pH 7 0 with KOH) and maintained on ice [18,19] The sediment obtained after centrifugation for 15 min at 660 × *g* was discarded The mitochondrial pellet was obtained by centrifugation of the supernatant for 15 min at 4000 × *g* at 4 °C [18,19] After resuspension and recentrifugation the pellet was resuspended in test medium (125 mM KCl/2 mM KH_2PO_4/5 mM succinate/0 3 mM $MgATP^{2-}$/25 mM Hepes adjusted to pH 7 0 with KOH) [19–21] Protein was determined according to McKnight [22] The protein content of 1 µl of the resuspended mitochondrial fractions was 27 ± 1 µg ($N = 23$)

Measurement of free Ca^{2+} concentration Ca^{2+} uptake and efflux by isolated mitochondria were measured in test medium at 25 °C with a newly designed Ca^{2+}-ion-sensitive minielectrode and microincubation chamber (40 µl volume) as described recently in detail [18] Addition of fatty acids to the test medium did not affect electrode recordings in control experiments

Measurement of $^{45}Ca^{2+}$ fluxes $^{45}Ca^{2+}$ uptake by isolated mitochondria incubated for 30 s in 20 µl labelled test medium at 25 °C was measured at a free Ca^{2+} concentration of 10 µM as described [23] The free Ca^{2+} concentration in the medium was adjusted with the Ca^{2+} electrode Separation of mitochondria from incubation medium for measurement of $^{45}Ca^{2+}$ uptake by liquid scintillation counting was performed with a newly designed microfiltration device [23]

Measurement of mitochondrial Ca^{2+} content Mitochondrial Ca^{2+} content was measured by atomic absorption spectroscopy with a Massmann cuvette from Beckman Instruments [18]

Measurement of mitochondrial membrane potential Membrane potential of isolated mitochondria was determined from the distribution of the lipophilic cation, TPP^+ (Aldrich) between the incubation medium and the mitochondrial matrix [21] Measurement of the TPP^+ concentration in the incubation medium was performed in a microincubation chamber [18] using a TPP^+-ion-sensitive membrane [24] mounted on the tip of the exchangeable membrane support inset of the minielectrode which has also been used for free Ca^{2+} ion measurements [18] and calculated as recently described [19]

Results

Effects of arachidonic acid on mitochondrial Ca^{2+} transport

Isolated liver mitochondria (9 6 ± 1 6 nmol/mg protein Ca^{2+} content) incubated in a test medium of an

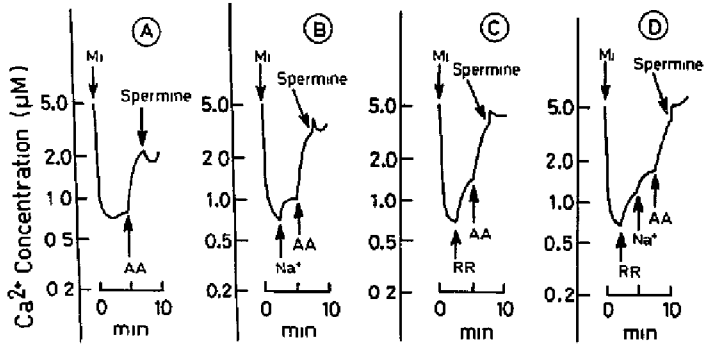

Fig 1 Effect of arachidonic acid (AA) (50 µM) on the regulation of the free Ca^{2+} concentration by rat liver mitochondria and its modulation by Ruthenium red (RR) (250 nM), Na^+ (5 mM), and spermine (400 µM) Mitochondria (suspended in 1–2 µl test medium) were added to the test medium with an initial Ca^{2+} concentration of 5 µM in the microincubation chamber at min 0 AA RR, Na^+, spermine or test medium only (control) were added after 2 5, 5, 7 5 and 10 min, respectively The curves represent typical recordings which were repeated five times

ionic composition simulating the composition of the cytosol [19–21] are able to maintain a steady-state free Ca^{2+} concentration well below 1 μM (Fig 1A) Addition of arachidonic acid (50 μM) to the incubation medium increased the free Ca^{2+} concentration to values above 1 μM Thereafter, spermine (400 μM), an activator of the mitochondrial Ca^{2+} uniporter [21], had virtually lost its ability to decrease the free Ca^{2+} concentration through stimulation of mitochondrial Ca^{2+} uptake

Addition of Na^+ (5 mM), an activator of mitochondrial Ca^{2+} efflux via Na^+–Ca^{2+} exchange [25] (Fig 1B), as well as Ruthenium red (250 nM), an inhibitor of the mitochondrial Ca^{2+} uniporter [26], to the incubation medium induced the typical Ca^{2+} efflux from liver mitochondria (Fig 1C) The effect of Ruthenium red (250 nM) on Ca^{2+} efflux was enhanced by Na^+ (5 mM) (Fig. 1D) Addition of arachidonic acid (50 μM) to the incubation medium further increased the free Ca^{2+} concentration in all three situations (Fig. 1B–D)

The ability of spermine (400 μM) to decrease the free Ca^{2+} concentration through stimulation of mitochondrial Ca^{2+} uptake was lacking after previous addition of Na^+ (5 mM) plus arachidonic acid (50 μM) (Fig. 1B) as well as after previous addition of Ruthenium red (250 nM) plus arachidonic acid (50 μM) without (Fig 1C) or with (Fig 1D) concomitant presence of Na^+ (5 mM), but a further increase in the free Ca^{2+} concentration was attenuated

Effects of arachidonic acid and other fatty acids on mitochondrial Ca^{2+} uptake and Ca^{2+} efflux

The rates of Ca^{2+} uptake by liver mitochondria which increased in dependence on the Ca^{2+} concentration were decreased by arachidonic acid (100 μM) (Fig. 2). Initial free Ca^{2+} concentrations in the incubation medium which was supplemented with 5 mM succinate as a mitochondrial substrate were adjusted to 0 5, 1 0, 2 5, 5 0, 7 5, or 10 μM Ca^{2+} Ca^{2+} uptake by the mitochondria was initiated by injection of mitochondria into the microincubation chamber (Fig 2) The degree of inhibition of mitochondrial Ca^{2+} uptake by arachidonic acid was not constant, but increased with higher Ca^{2+} loading of the mitochondria

Irrespective of the initial free Ca^{2+} concentration mitochondria rapidly decreased the free Ca^{2+} concentration in the incubation medium in control experiments, achieving a steady state in the range well below 0 5 μM, which was reached after 15–20 s, even in the presence of the highest initial free Ca^{2+} concentration of 10 μM (Fig. 3) Arachidonic acid affected the kinetics of Ca^{2+} uptake and Ca^{2+} efflux by isolated rat liver mitochondria in a concentration-dependent manner Up to concentrations of 10 μM, arachidonic acid reduced only marginally the amount of Ca^{2+} taken up by mitochondria during a 30 s incubation period At higher concentrations of arachidonic acid (25, 50 or 100 μM),

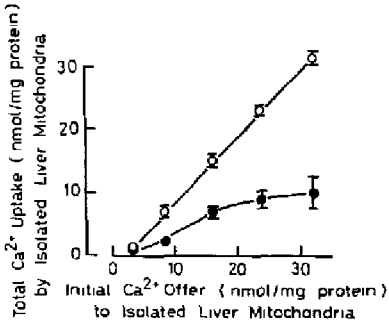

Fig 2 Effects of a single concentration (100 μM) of arachidonic acid (AA) on Ca^{2+} uptake by isolated rat liver mitochondria at different initial Ca^{2+} concentrations The test medium in the microincubation chamber contained spermine (100 μM) and AA (●——●) in a concentration of 100 μM Before the beginning of the experiment the different Ca^{2+} concentrations in the microincubation chamber were adjusted (0 5 1 2 5 5 7 5 or 10 μM) These initial Ca^{2+} concentrations under control conditions are presented as initial Ca^{2+} offered to the mitochondria and expressed as nmol Ca^{2+} per mg protein (2 4 4 7 11 8 23 5 35 3 or 47 0 nmol Ca^{2+} per mg protein) At time zero the experiment was started by addition of the mitochondria (suspended in 0 5 μl test medium) The minimal Ca^{2+} concentration achieved after addition of the mitochondria during a 30 s incubation period was registered The difference between this value and the initial Ca^{2+} concentration at the beginning of the experiment was calculated and Ca^{2+} uptake was expressed in nmol Ca^{2+} per mg protein The amount of Ca^{2+} taken up by mitochondria in control experiments (○——○) at each initial Ca^{2+} concentration was compared with the amount of Ca^{2+} taken up by mitochondria under the influence of AA (●——●) The values represent means ± S E from four experiments AA significantly ($P < 0.01$) (analysis of variance) reduced mitochondrial Ca^{2+} uptake

the ability of the mitochondria to take up Ca^{2+} gradually deteriorated After uptake of a reduced amount of Ca^{2+} with a nadir after 5–15 s, mitochondria released Ca^{2+}, so that the initial Ca^{2+} concentration of 10 μM was achieved again within 30 s of incubation with 100 μM arachidonic acid (Fig 3)

Rates of mitochondrial Ca^{2+} uptake (Fig. 4A) and Ca^{2+} efflux (Fig 4B) were measured in dependence on the concentration of arachidonic acid and a variety of other fatty acids according to the protocol described in Fig. 3 for arachidonic acid

In Fig 4A, the amount of Ca^{2+} maximally taken up by the isolated liver mitochondria within a 30 s incubation period under the influence of increasing fatty acid concentrations is expressed as a percentage of the Ca^{2+} uptake value obtained in the absence of any fatty acid All fatty acids, with the exception of palmitic acid (16 0), showed a significant concentration-dependent decrease of mitochondrial Ca^{2+} uptake

The extent of inhibition of Ca^{2+} uptake expressed as a percentage by these and several other fatty acids is given in Table I When the extent of inhibition by the

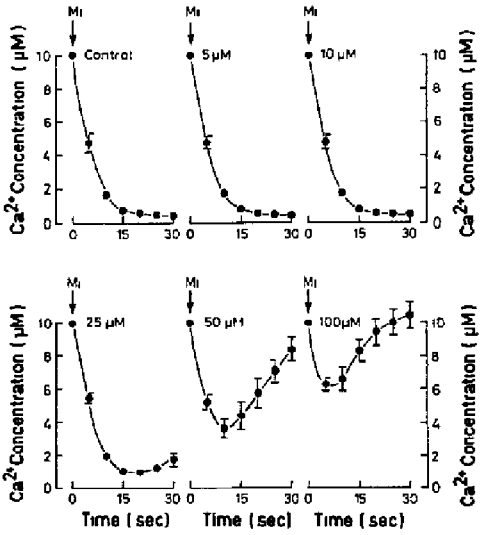

Fig. 3 Concentration-dependent effects of arachidonic acid (AA) on the kinetics of Ca^{2+} uptake and Ca^{2+} efflux by isolated rat liver mitochondria The test medium in the microincubation chamber contained spermine (100 μM) and increasing AA concentrations (5, 10, 25, 50 or 100 μM) The initial Ca^{2+} concentration in the incubation medium was adjusted to 10 μM at the beginning of the experiment At time zero the experiments were initiated through addition of mitochondria (suspended in 0.5 μl test medium) to the test medium in the microincubation chamber The values represent means±S E from 4–9 experiments

fatty acid allowed the calculation of a half-maximally effective concentration, this figure is also given in Table I From the group of saturated fatty acids, only lauric acid (12 0) (Fig 4) and myristic acid (14 0) slightly reduced the rate of mitochondrial Ca^{2+} uptake (Table I) However, even at a 100 μM concentration, the inhibition of mitochondrial Ca^{2+} uptake did not reach 50% Thus, maximally effective concentrations of these two saturated fatty acids were far above 100 μM (Table I) Saturated fatty acids with a shorter chain length – caproic acid (6 0), caprylic acid (8 0), capric acid (10 0) – and with a longer chain length – palmitic acid (16 0), stearic acid (18 0), and arachidic acid (20 0) – were completely ineffective as inhibitors of mitochondrial Ca^{2+} uptake (Table I)

Cis-unsaturated fatty acids, monounsaturated as well as polyunsaturated, were more effective than saturated fatty acids (Table I), while all *trans*-unsaturated fatty acids tested ((elaidic acid (18 $1(n-9)$), linolelaidic acid (18 $2(n-9,12)$), *trans*-vaccenic acid (18 $1(n-11)$) were completely ineffective The *cis*-monounsaturated fatty acids with a shorter chain length, myristoleic acid (14 $1(n-9)$) and palmitoleic acid (16 $1(n-9)$) (Fig 4), were more effective inhibitors of mitochondrial Ca^{2+} uptake, with half-maximal effective concentrations around 70 μM (Table I), than *cis*-monounsaturated fatty acids with a longer chain length oleic acid (18 $1(n-9)$) (Fig 4), petroselinic acid (18 $1(n-6)$), and *cis*-vaccenic acid (18 $1(n-11)$) with half-maximal effective concentrations above 100 μM (Table I) The *cis*-monounsaturated fatty acids with an even longer chain length, eicosenoic acid (20 $1(n-11)$) and erucic acid (22 $1(n-13)$) were completely ineffective (Table I)

The *cis*-polyunsaturated fatty acids showed a differential effectivity, depending on the relation between the chain length and the number and position of the double bonds in the chain (Table I) An increase in the inhibition of mitochondrial Ca^{2+} uptake by linoleic acid (18 $2(n-9,12)$) was achieved when a third double bond was introduced in a position proximal to the two others, as shown by the higher degree of mitochondrial Ca^{2+} uptake inhibition and the lower half-maximal inhibitory concentration of γ-linolenic acid (18 $3(n-6,9,12)$) (Fig. 1) but not when the third double bond was introduced in a position distal to the two others, as in the case of linolenic acid (18 $3(n-9,12,15)$) (Fig 1) (Table I) An increase in the chain length as in the case of eicosatrienoic acid (20 $3(n-11,14,17)$) and homo-γ-linolenic acid (20 $3(n-8,11,14)$) (Table I) attenuated the efficiency However, the introduction of an additional double bond in a position proximal to the three other ones as in the case of arachidonic acid (20 $4(n-5,8,11,14)$) (Fig 1) as shown by the higher degree of mitochondrial Ca^{2+} uptake inhibition and the lower half-maximal inhibitory concentration increased the efficiency (Table I) Introduction of further additional double bonds in a position distal to the other ones, as in the case of eicosapentaenoic acid (20 $5(n-5,8,11,14,17)$) and docosahexaenoic acid (22 $6(n-4,7,10,13,16,19)$) was accompanied by decreased effectivity (Table I)

In Fig. 4B, the amount of Ca^{2+} released again during the 30 s incubation period by the isolated liver mitochondria under the influence of increasing fatty acid concentrations is expressed as a percentage of the maximal amount of Ca^{2+} taken up during the first 10–20 s of the incubation period

All fatty acids with a half-maximal inhibitory concentration for mitochondrial Ca^{2+} uptake below 100 μM, i e, all fatty acids which reduced mitochondrial Ca^{2+} uptake by more than 50% at a 100 μM concentration (Table I), also induced a potent release of Ca^{2+} from mitochondria, so that the initial Ca^{2+} concentrations of 10 μM were regained within less than 30 s, as shown for several of these fatty acids in Fig 4B In addition, all fatty acids which reduced mitochondrial Ca^{2+} uptake by more than 10% at a 100 μM concentration (Table I) also induced a potent release of Ca^{2+} from mitochondria, which in each case accounted for

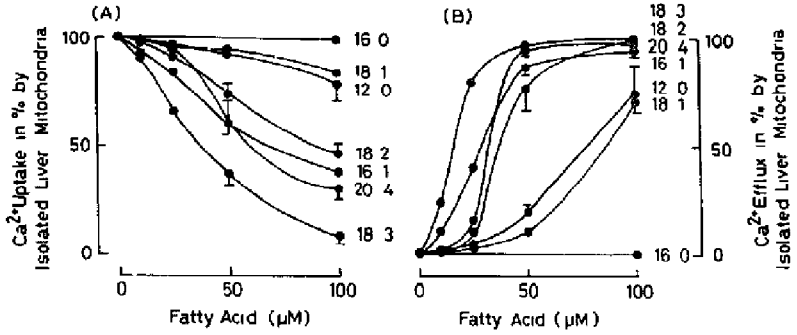

Fig 4 Comparison of the concentration-dependent effects of arachidonic acid (AA) and some other fatty acids on Ca^{2+} uptake (A) and Ca^{2+} efflux (B) by isolated rat liver mitochondria The fatty acids shown in this figure are lauric (12 0) palmitic (16 0) oleic (18 1) palmitoleic (16 1) linoleic (18 2) γ-linolenic (18 3) and arachidonic (20 4) acids In the (A) mitochondrial Ca^{2+} uptake is presented in dependence on the fatty acid concentration (10, 25, 50 or 100 μM) and expressed as percent of control The test medium in the microincubation chamber was supplemented with spermine (100 μM) and the initial Ca^{2+} concentration was adjusted to 10 μM at the beginning of the experiment In control experiments (see Fig. 3) mitochondria in the incubation chamber decreased the Ca^{2+} concentration within 30 s below 0 5 μM The amount of Ca^{2+} taken up in these control experiments was 21 9±0 9 nmol Ca^{2+} per mg protein Thus amount was set 100% and compared with the amount of Ca^{2+} taken up by the mitochondria at increasing fatty acid concentrations In (B) mitochondrial Ca^{2+} efflux is presented in dependence on the fatty acid concentration (10, 25, 50 or 100 μM) and expressed as percent of control In control experiments (Fig. 3) mitochondria in the incubation chamber did not release Ca^{2+} during a 30 s incubation period Therefore Ca^{2+} release of these mitochondria was set at 0% and compared with the amount of Ca^{2+} released by the mitochondria at the increasing fatty acid concentrations The values represent means±S E. from four experiments All fatty acids, with the exception of palmitic acid (16 0) significantly decreased mitochondrial Ca^{2+} uptake ($P < 0.01$) and significantly increased Ca^{2+} efflux ($P < 0.01$) (analysis of variance)

more than 50% of the Ca^{2+} originally taken up by these mitochondria, as shown for two of these fatty acids in Fig 4B Fatty acids which did not significantly inhibit Ca^{2+} uptake (not more than 1%) (Table I) also failed to induce release of Ca^{2+} from mitochondria, as shown for palmitic acid (16·0) in Fig 4B

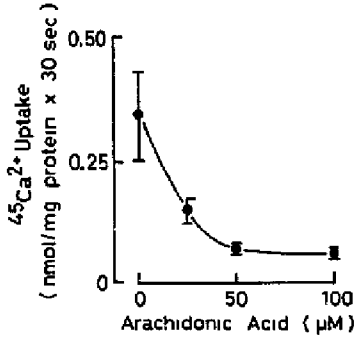

Fig 5 Concentration-dependent effects of arachidonic acid (AA) on $^{45}Ca^{2+}$ accumulation by isolated rat liver mitochondria The test medium contained spermine (100 μM) and increasing AA concentrations (25, 50 or 100 μM) The initial Ca^{2+} concentration was adjusted to 10 μM After a 30 s incubation period mitochondria were separated from incubation medium by microfiltration and $^{45}Ca^{2+}$ content was determined by scintillation counting. The points in the curve represent rates of $^{45}Ca^{2+}$ accumulation by the mitochondria and are means± S E of 20 experiments AA significantly decreased the $^{45}Ca^{2+}$ accumulation in dependence on the concentration ($P < 0.01$, Anova)

Effects of arachidonic acid on mitochondrial $^{45}Ca^{2+}$ uptake

Arachidonic acid reduced the rate of $^{45}Ca^{2+}$ accumulation by isolated liver mitochondria in a concentration-dependent manner (Fig 5), indicating that arachidonic acid inhibited Ca^{2+} uptake rather than inducing Ca^{2+} efflux by unspecific leakage from mitochondria

Effects of arachidonic acid and other fatty acids on mitochondrial membrane potential

Arachidonic acid reduced the membrane potential of isolated liver mitochondria in a concentration-dependent manner (Fig. 6) The reduction of the mitochondrial membrane potential by arachidonic acid was accentuated by increasing the initial free Ca^{2+} concentration in the incubation medium, leading to a higher Ca^{2+} load of the mitochondria (Fig 6)

A variety of other fatty acids were also tested for their ability to affect the mitochondrial membrane potential (Table I) In fact, a correlation between the ability of all tested fatty acids at a 100 μM concentration to reduce the mitochondrial uptake of TPP^+ as a measure of the ability to reduce mitochondrial membrane potential and mitochondrial Ca^{2+} uptake was observed (Table I) Thus, all fatty acids which did not inhibit mitochondrial Ca^{2+} uptake also failed to inhibit mitochondrial TPP^+ uptake (Table I) And all fatty acids which reduced mitochondrial Ca^{2+} uptake also

TABLE I

Inhibition of rat liver mitochondrial Ca^{2+} uptake and of mitochondrial TPP^+ uptake as a measure of the mitochondrial membrane potential

All fatty acids were tested at a concentration of 100 μM for their ability to inhibit mitochondrial Ca^{2+} uptake according to the protocol described in legends to Figs 2 and 3 and for their ability to inhibit mitochondrial TPP^+ uptake as a measure of the mitochondrial membrane potential according to the protocol described in legend to Fig 6 The degree of inhibition of uptake was expressed as percentage reduction of the control rate of uptake Mitochondria not exposed to a fatty acid se ved as controls The mitochondria decreased the free Ca^{2+} concentration in the incubation medium from 10 μM to 0 36±0 02 μM This decrease of the free Ca^{2+} concentration in the incubation medium of 9 64±0 02 μM corresponded to a mitochondrial Ca^{2+} uptake of 24 1±1 6 nmol of Ca^{2+} per mg protein ($n = 43$) The mitochondria decreased the TPP^+ concentration in the incubation medium from 8 μM to 2 47±0 06 μM This decrease of the TPP^+ concentration in the incubation medium of 5 53±0 06 μM corresponded to a mitochondrial membrane potential of 201 5 mV in controls ($n = 43$) Half-maximal inhibitory concentrations of the various fatty acids expressed in μM for mitochondrial Ca^{2+} uptake and mitochondrial TPP^+ uptake as a measure of the mitochondrial membrane potential were calculate r for all fatty acids which reduced uptake by more than 50% at a 100 μM concentration These values were obtained in experiments where the concentration-dependent effects of the fatty acids were determined on Ca^{2+} uptake according to the protocol described in legends to Figs 2 and 3 and on TPP^+ uptake according to the protocol described in legend to Fig 6 All values represent means±S E. of four experiments All concentration dependent inhibitory effects of fatty acids on mitochondrial Ca^{2+} uptake and membrane potential greater than 1% were significant ($P < 0.01$) (analysis of variance)

	Half-maximal inhibitory concentration of test agent (A) and % uptake inhibition at 100 μM of test agent (B) for			
	mitochondrial Ca^{2+} uptake		mitochondrial TPP^+ uptake	
	A (μM)	B (%)	A (μM)	B (%)
I Saturated fatty acids				
1 Caproic (6 0)		0± 0		1±0
2 Caprylic (8 0)		1± 0		1±0
3 Capric (10 0)		7± 2		11±1
4 Lauric (12 0)	>100	22± 8	>100	25±1
5 Myristic (14 0)	>100	16± 5	>100	18±4
6 Palmitic (16 0)		1± 0		0±0
7 Stearic (18 0)		0± 0		1±1
8 Arachidic (20 0)		0± 0		0±0
II Unsaturated fatty acids				
(a) *trans*-unsaturated fatty acids				
1 Elaidic (18 1($n-9$))		1± 1		1±0
2 Linolelaidic (18 2($n-9,12$))		1± 0		1±0
3 *trans*-Vaccenic (18 1($n-7$))		1± 1		1±0
(b) *cis*-unsaturated fatty acids				
(ba) Monounsaturated fatty acids				
1 Myristoleic (14 1($n-9$))	76± 5	71± 3	85± 3	63±5
2 Palmitoleic (16 1($n-9$))	67± 4	70± 6	56± 7	77±5
3 Oleic (18 1($n-9$))	>100	16± 1	130±11	36±3
4 Petroselinic (18 1($n-6$))	>100	21± 4	121± 9	40±4
5 *cis*-Vaccenic (18 1($n-11$))	>100	11± 1	>100	24±3
6 Eicosenoic (20 1($n-11$))		1± 1		1±0
7 Erucic (22 1($n-13$))		1± 1		1±0
(bb) Polyunsaturated fatty acids				
8 Linoleic (18 2($n-9,12$))	95± 7	54± 5	81± 5	62±3
9 Linolenic (18 3($n-9$ 12 15))	93± 8	58± 8	77± 9	71±6
10 γ-Linolenic (18 3($n-6$ 9 12))	43± 7	91± 4	49± 0	74±1
11 Eicosatrienoic (20 3($n-11,14$ 17))	>100	38± 7	98± 6	52±3
12 Homo-γ-linolenic (20 3($n-8$ 11 14))	99±11	50± 9	100± 7	49±6
13 Arachidonic (20 4($n-5,8,11$ 14))	75± 4	66± 5	46± 3	77±3
14 Eicosapentaenoic (20 5($n-5,8,11,14,17$))	95± 9	54± 7	100± 9	51±5
15 Docosahexaenoic (22 6($n-4$ 7 10 13 16,19))	97±15	57±11	112±18	49±8

reduced mitochondrial TPP^+ uptake to a similar extent (Table I)

However, a discrepancy between influence on membrane potential and Ca^{2+} uptake becomes apparent, comparing chemically closely related fatty acids such as linolenic acid and γ-linolenic acid Both are comparably effective in decreasing the membrane potential, but the former has a half-maximally effective concentration of 93 μM for inhibition of Ca^{2+} uptake, while the latter has one of 43 μM This indicates that the correlation is not linear for each substance

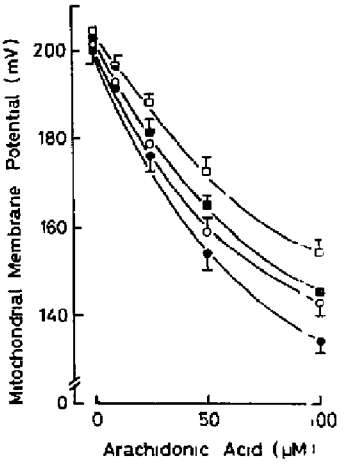

Fig. 6 Concentration-dependent effect of arachidonic acid (AA) on the mitochondrial membrane potential of isolated rat liver mitochondria at different initial Ca^{2+} concentrations (□ 0.01 ■ 1 ○ 5 ● 10 μM) The test medium in the microincubation chamber was supplemented with 100 μM spermine, 8 μM TPP^+ and AA in increasing concentrations (5, 25, 50 or 100 μM) The experiment was started by addition of the mitochondria (suspended in 1–2 μl test medium) to the test medium in the microincubation chamber Shown are the mitochondrial membrane potential values which were measured during a 1 min incubation period The points represent means ± S E of four experiments AA significantly decreased the mitochondrial membrane potential at all initial Ca^{2+} concentrations ($P < 0.01$) (analysis of variance)

Discussion

Unesterified fatty acids have profound effects on mitochondrial Ca^{2+} handling They inhibit Ca^{2+} uptake and lower mitochondrial membrane potential and at higher concentrations lead to a net Ca^{2+} efflux from mitochondria While *cis*-unsaturated fatty acids were mostly effective, *trans*-unsaturated and saturated fatty acids were much less effective or even ineffective As shown by Karnovsky et al. [27] *cis*-unsaturated fatty acids have a fluidizing effect [28] on cellular membranes. A slight activity of saturated fatty acids with chain lengths between C_{10}–C_{14} in the present investigation indicates that saturated fatty acids of these chain lengths may also have some fluidizing effects in membranes The general conclusion is in accordance with the observation of Takenaka et al [29] that these saturated fatty acids – apart from *cis*-unsaturated fatty acids – inhibit transmembrane K^+-conductance This structure–activity relationship is at variance from recently published data describing the effects of fatty acids on the anion transport protein of erythrocyte membranes [30], indicating that a generalized model for the actions of fatty acids cannot be set up at present

The capability of *cis*-unsaturated fatty acids to interfere with mitochondrial Ca^{2+} uptake increases with the number of double bonds and decreases with chain length γ-Linolenic acid has apparently an optimal configuration, while longer chains, even when containing six double bonds (e g , docosahexaenoic acid), proved to be less active This structure–activity relationship supports the assumption that fatty acids act by perturbance of the hydrophobic core of the phospholipid bilayer [31] Double bonds in acyl chains induce disorder in neighbouring acyl chains due to their bulkiness, as has been detected with ESR studies [32] Compared with saturated or *trans*-unsaturated fatty acids, the shape of the acyl chain is changed to a shorter and more lateral expanding form The strongest shortening occurs with 18 3 chains [33], which conforms with the measured interference with the mitochondrial Ca^{2+} uptake Again, with monounsaturated fatty acids, reduction of acyl chain length without changing the position of the double bond results in a higher activity Palmitoleic acid is thus much more active than oleic acid, close to arachidonic acid As the overall molecular shape will be different from that of polyunsaturated fatty acids, the common critical property might as well be an acyl chain end with high motional freedom at a certain distance from the carboxyl group Nevertheless since palmitoleic acid was found to be a potent fusogen of vesicles as were polyunsaturated fatty acids [34], the lipid bilayer is probably the primary target of action, rather than a direct interaction with a membrane protein

Furthermore, as palmitoleic acid and myristoleic acids are not substrates for cyclo- or lipoxygenases [35], their activity supports the view that it is a property of a given fatty acid as such and not the metabolism to eicosanoids that determines the inhibitory effect on mitochondrial Ca^{2+} uptake The recently described Ca^{2+}-releasing effect of HPETEs and HETEs on mitochondria [14] may be a consequence of the same membrane perturbance as exerted by unsaturated fatty acids, because they have about the same molar potency Prostaglandins did not show any effect in our system (unpublished results) However variations in the degree of fatty acid incorporation into phospholipids during deacylation and reacylation cycles may also be responsible for some of the differences in potency

As in the case of lysophospholipids [17] the ability of fatty acids to inhibit mitochondrial Ca^{2+} uptake was accompanied by its ability to lower the mitochondrial membrane potential, which constitutes the main driving force for the mitochondrial Ca^{2+} uniporter and thus for Ca^{2+} uptake [36] It has been argued by Rottenberg and Hashimoto [37] that fatty acids do not act as protonophores as do the classical 'uncouplers', but rather interfere with the function of an integral membrane protein of the respiratory chain, probably subunits of the F_0-ATPase which act as proton capacitors In contrast to

lysophospholipids [17], there are discrepancies between the ability to lower the membrane potential and to inhibit Ca^{2+} uptake, as has been mentioned for the comparison of linolenic acid and γ-linolenic acid Furthermore, arachidonic acid and lysophosphatidylcholine [17] have nearly identical half-maximal concentrations for inhibition of Ca^{2+} release, but those for decrease of membrane potential are rather divergent. While interference with the membrane potential seems to be a prerequisite for effects on mitochondrial Ca^{2+} transport, additional mechanisms may be involved Another difference between lysophospholipids and fatty acids is the time-course of action Whereas the action of lysophosphatidylcholine vanishes within a few minutes [17] the effect of arachidonic acid is longer lasting (Fig 1A) The prolonged presence of arachidonic acid in the mitochondrial membrane is a likely explanation for the reduced ability of spermine to activate Ca^{2+} uptake (Fig 1A)

In contrast to lysophospholipids, unesterified fatty acids can be generated by mechanisms other than the action of PLA_2, e g , a sequential action of a PLC and a diacylglycerolipase [11] As the absolute amount of unsaturated fatty acids, particularly of arachidonic acid, esterified to PE and PC is far greater than the one esterified to PI [38], and PE and PC are more likely to be metabolized by PLA_2 than by PLC, the potential of PLA_2 as a liberator of unsaturated fatty acids is probably higher than that of PLC PLA_2 of liver mitochondria has a preference for PE over PC which, however, can express considerable activity towards PC in dependence on the composition of the phospholipid membrane [39,40] Thus, an activation of mitochondrial PLA_2 will produce a mixture of active and inactive fatty acids (Table I) as well as active and inactive lysophospholipids [17] with effects on Ca^{2+} transport depending on the composition of the mixture Recent measurements of arachidonic acid generation during cell stimulation gave values of 50 to 100 μM intracellular concentration [41] Even if some accumulation of fatty acids from the incubation medium into the mitochondrial membranes may occur, which will be limited during short-term incubation (Fig 3), a physiological role in mitochondrial Ca^{2+} transport regulation may be considered in view of these results [41]

Whether fatty acids liberated in other cellular locations can reach the mitochondrial membrane is not settled at present, although there have been several arguments raised in favour of such a possibility [42] In this case, a PLA_2 signalling pathway would be conceivable consisting of a plasma membrane PLA_2, the reaction products of which could activate or inhibit protein kinase C [43,44] and influence cytoplasmic Ca^{2+} concentrations via interference with mitochondrial Ca^{2+} transport

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