Biochimica et Biophysica Acta 982 (1989) 140–146
Elsevier

BBAMEM 74465

Regulation of transmembrane ion transport by reaction products of phospholipase A_2. I. Effects of lysophospholipids on mitochondrial Ca^{2+} transport

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(Received 23 December 1988)

Key words Lysophospholipid, Calcium ion transport, Phospholipase A_2, (Rat liver mitochondria)

Lysophospholipids inhibited mitochondrial Ca^{2+} uptake, induced a net Ca^{2+} efflux, and thereby increased the extramitochondrial Ca^{2+} concentration The inhibitory potency decreased in the order lysophosphatidylcholine (LPC) = lysophosphatidylglycerol (LPG) > lysophosphatidylinositol (LPI) > lysophosphatidylserine (LPS) >> lysophosphatidylethanolamine (LPE) This relative order is in inverse relation to the ability of the various phospholipid head-groups to build up intermolecular hydrogen bonds with neighbouring membrane lipids This indicates that changes in Ca^{2+} transport induced by lysophospholipids are mediated by the interaction of the lysophospholipids with the mitochondrial membrane bilayer structure. The mitochondrial membrane potential, which is the main driving force for mitochondrial Ca^{2+} uptake, was affected in the same order by the various lysophospholipids This reduction of the mitochondrial membrane potential may be the underlying cause for the inhibition of the mitochondrial Ca^{2+} uniport and the resulting release of Ca^{2+} from the mitochondria.

Introduction

The generation of phospholipid-dependent second messengers via activation of phospholipase C has proved to be a pivotal step in cellular signalling Inositol 1,4,5-trisphosphate (and other inositol phosphates) raises cytoplasmic Ca^{2+} levels by efflux from the endoplasmic reticulum [1,2], while diacylglycerol activates protein kinase C by decreasing its requirement for Ca^{2+} [3,4] However, receptor-mediated changes in phospholipid metabolism during cell stimulation do not always involve an activation of phospholipase C but, as has been recently shown for several tissues, an activation of phospholipase A_2 (PLA_2) [5–8], providing support for the existence of a PLA_2-mediated signalling pathway [9]

PLA_2 comprises a family of enzymes which are either secreted or resident in the cell [10] In liver, PLA_2 is ubiquitous in the membranes, mitochondria displaying a higher activity than microsomes, which have mainly a PLA_1 [11,12] Since by the action of PLA_2 lysophospholipids and fatty acids are generated in the inner mitochondrial membrane [13], it can be assumed that the membrane transport systems can be affected by the resulting changes in the lipid bilayer structure Microsomes have also been reported to release Ca^{2+} upon exposure to lysophospholipids [14] But apparently, microsomes contribute far less to the increase in the ambient free Ca^{2+} concentration than do mitochondria, as was recently shown in studies with permeabilized liver cells as well as isolated and concubated subcellular fractions from liver [15]

In the present study we investigated the effects of various lysophospholipids on mitochondrial Ca^{2+} transport in order to elucidate their mechanism of action and evaluate a possible second messenger role of these substances The effects of fatty acids are described in a second paper [28]

Materials and Methods

Chemicals All lysophospholipids (either palmitoyl or stearyl form) were obtained from Sigma $^{45}CaCl_2$ was from Amersham International All other chemicals of

Abbreviations LPC lysophosphatidylcholine, LPG, lysophosphatidylglycerol LPI lysophosphatidylinositol, LPS, lysophosphatidylserine, LPE, lysophosphatidylethanolamine, PLA_2, phospholipase A_2 (EC 3.1.1.4) TPP^+, tetraphenylphosphonium

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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 [16,17] 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 [16,17] 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) [17–19] Protein was determined according to McKnight [20] The protein content of 1 µl of the resuspended mitochondrial fractions was 36 ± 1 µg ($N = 82$)

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 [16] Addition of lysophospholipids 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 [21] 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 [21]

Measurement of mitochondrial Ca^{2+} content Mitochondrial Ca^{2+} content was measured by atomic absorption spectroscopy with a Massmann cuvette from Beckman Instruments [16]

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 [19] Measurement of the TPP^+ concentration in the incubation medium was performed in a microincubation chamber [16] using a TPP^+ ion-sensitive membrane [22] mounted on the tip of the exchangeable membrane support inset of the minielectrode which has also been used for free Ca^{2+} ion measurements [16] and calculated as recently described [17]

Results

Effects of lysophosphatidylcholine 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 ionic composition simulating the composition of the cytosol [17–19] are able to maintain a steady-state free Ca^{2+} concentration well below 1 µM (Fig 1A) Addition of lysophosphatidylcholine (50 µM) to the incubation medium increased the free Ca^{2+} concentration to values above 1 µM The effect of LPC was spontaneously reversible (Fig 1a) Subsequent addition of spermine (400 µM) an activator of the mitochondrial Ca^{2+} uptake [19], further decreased the ambient free Ca^{2+} concentration well below 0 5 µM through stimulation o. mitochondrial Ca^{2+} uptake

Addition of Na^+ (5 mM), an activator of mitochondrial Ca^{2+} efflux via Na^+–Ca^{2+} exchange [23] (Fig. 1B), as well as Ruthenium red (250 nM) an inhibitor of the mitochondrial Ca^{2+} uniporter [24], to the incubation

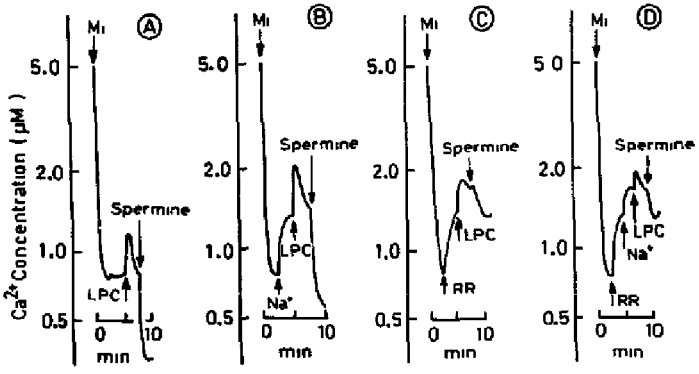

Fig. 1 Effect of LPC (50 µM) on the regulation of the free Ca^{2+} concentration by rat liver mitochondria and its modulation by Ruthenium red (RR) (250 nM), Na^+ (5 mM), and spermine (400 µM) Mitochondria (suspended in 1–2 µl test medium) were added to the test medium with an initial Ca^{2+} concentration of 5 µM in the microincubation chamber at min 0 LPC 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

medium induced the typical Ca^{2+} efflux from liver mitochondria (Fig 1C) The latter effect of Ruthenium red (250 nM) on Ca^{2+} efflux was potentiated by Na^+ (5 mM) (Fig 1D) Addition of lysophosphatidylcholine (50 μM) to the incubation medium further increased the free Ca^{2+} concentration in all three situations (Fig 1B–D) However, the effect of LPC (50 μM) was attenuated when both Ruthenium red and Na^+ had been added earlier to the mitochondrial incubation medium (Fig 1D)

When spermine (400 μM) was added, its effect was attenuated not so much after previous addition of Na^+ (5 mM) plus LPC (50 μM) (Fig 1B), but rather after previous addition of Ruthenium red (250 nM) plus LPC (50 μM) (Fig 1C) with or without concomitant presence of Na^+ (5 mM)

Effects of lysophospholipids 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 in the incubation medium were decreased by the different lysophospholipids (100 μM) to a variable degree (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+} Uptake of Ca^{2+} by the mitochondria was initiated by injection of mitochondria into the microincubation chamber The percentage reduction of Ca^{2+} uptake remained constant for each lysophospholipid at all Ca^{2+} concentrations tested The inhibitory potency decreased in the order LPC = LPG > LPI > LPS ≫ LPE This corresponds to reductions to 47, 49, 64, 76 and 94% of control uptake, respectively (Fig 2) Control mitochondria rapidly decreased the free Ca^{2+} concentration in the incubation medium, achieving a steady state in the range well below 0 5 μM, which was reached after 10–20 s irrespective of the initial free Ca^{2+} concentration

LPC affected the kinetics of Ca^{2+} uptake and Ca^{2+} efflux by isolated rat liver mitochondria in a concentration-dependent manner Under control conditions mitochondria decreased the initial free Ca^{2+} concentration of 10 μM within less than 30 s to values well below 1 μM, thereby increasing the mitochondrial Ca^{2+} content from $9\,6 \pm 1\,6$ nmol/mg protein to $29\,2 \pm 4\,1$ nmol/mg protein ($n = 7$) Up to concentrations of 25 μM, LPC slightly reduced the amount of Ca^{2+} taken up by mitochondria during a 30 s incubation period Thus resulted in a higher steady-state Ca^{2+} concentration, which approached 2 μM at 25 μM LPC At higher concentrations of LPC (50 and 100 μM), the ability of the mitochondria to take up Ca^{2+} gradually deteriorated further 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+} concentrations of 10 μM were

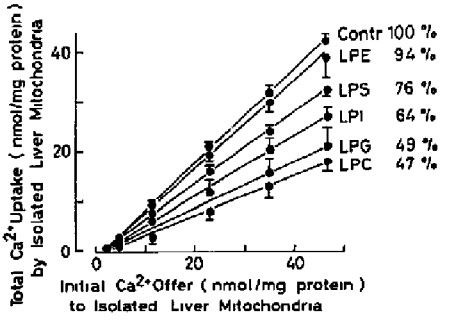

Fig 2 Effects of a single concentration (100 μM) of various lysophospholipids on Ca^{2+} uptake by isolated rat liver mitochondria at different initial Ca^{2+} concentrations The test medium in the microincubation chamber contained spermine (100 μM) and LPC LPG, LPI, LPS or LPE at a concentration of 100 μM Before 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 the various lysophospholipids The values represent means ± S E from four or five experiments The significant ($P < 0.001$) (analysis of variance) increase in mitochondrial Ca^{2+} uptake in dependence on the Ca^{2+} concentration was reduced by 6% by LPE, 24% by LPS, 36% by LPI 51% by LPG and 53% by LPC when compared with the control

achieved within less than 30 s of incubation with 100 μM LPC again (Fig 3).

Rates of mitochondrial Ca^{2+} uptake (Fig 4A) and Ca^{2+} efflux (Fig 4B) were measured in dependence on the concentration of the different lysophospholipids according to the protocol described in Fig 3 for lysophosphatidylcholine In Fig. 4A, the amount of Ca^{2+} maximally taken up by the isolated liver mitochondria during a 30 s incubation period (i e, at the minimum of the curves) under the influence of increasing lysophospholipid concentrations is expressed in percentage of the Ca^{2+} uptake value obtained in the absence of any lysophospholipid. All lysophospholipids, with the exception of LPE, showed a significant concentration-dependent decrease in mitochondrial Ca^{2+} uptake The concentrations at which mitochondrial Ca^{2+} uptake was inhibited by 50% were 76 ± 3 μM ($n = 12$) for LPC, 73 ± 4 μM ($n = 4$) for LPG, 97 ± 4 μM ($n = 13$) for LPI and above 100 μM ($n = 5$) for LPS In Fig 4B the amount of Ca^{2+} released again during the 30 s

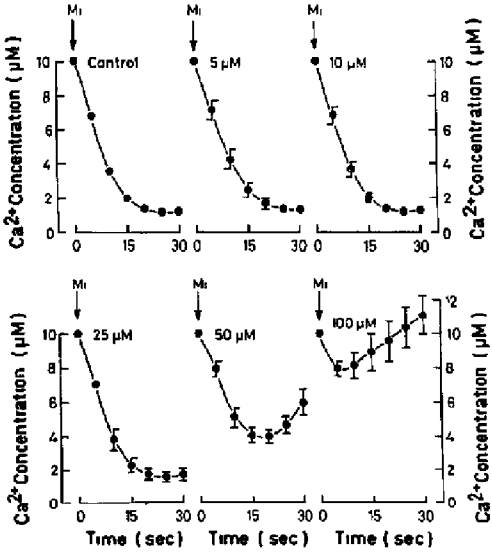

Fig 3 Concentration-dependent effects of LPC on the kinetics of Ca^{2+} uptake and Ca^{2+} efflux b, isolated rat liver mitochondria The test medium in the microincubation chamber contained spermine (100 μM) and increasing LPC concentrations (5, 10, 25, 50 or 100 μM) The initial Ca^{2+} concentration in the incubation medium was adjusted to 10 μM at the beginning of the experiment At time zero the experiments were started 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 eight to ten experiments

incubation period by the isolated liver mitochondria under the influence of increasing lysophospholipid 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 lysophospholipids, with the exception of LPE, also induced a potent release of Ca^{2+} from mitochondria, so that the initial Ca^{2+} concentrations of 10 μM were reached again within less than 30 s, as shown in Fig. 4B

When the palmitic acid in the LPC molecule was replaced by other fatty acids, the inhibitory potency decreased in the following order: palmitic acid ≥ stearic acid > lauric acid = oleic acid Replacement by caproic acid caused a complete loss of activity (data not shown)

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

The different lysophospholipids (100 μM) also reduced the rate of $^{45}Ca^{2+}$ accumulation by isolated liver mitochondria to a variable degree (Table I) This indicates that the lysophospholipids inhibited Ca^{2+} uptake rather than that they induced Ca^{2+} efflux by unspecific leakage from mitochondria If stimulation of Ca^{2+} efflux were the primary effect of lysophospholipids, an increased cycling of $^{45}Ca^{2+}$ would result in an increased $^{45}Ca^{2+}$ content of the mitochondria due to fast equilibration of labelled Ca^{2+} with unlabelled intramitochondrial Ca^{2+} The degree of inhibitory potency also decreased in the order LPC = LPG > LPI > LPS ≫ LPE (Table I)

Effects of lysophospholipids on mitochondrial membrane potential

The lysophospholipids reduced the membrane potential of isolated liver mitochondria in a concentration-dependent manner as determined from the distribution of the lipophilic cation TPP^+ (Fig 5) The reduction of the mitochondrial membrane potential by the lysophospholipids was accentuated by increasing the initial free Ca^{2+} concentration in the incubation medium (Fig 5), but was still clearly visible with the lowest initial free Ca^{2+} concentration of 0 01 μM The lowest value to which mitochondrial membrane potential was decreased by the action of lysophospholipids was 150 mV (Fig 5), whereas uncoupling by dinitrophenol caused a complete breakdown of the membrane potential (less than 40 mV, determination limit, unpublished observation)

The degree of membrane potential reducing potency decreased in the order LPC = LPG > LPI > LPS LPE did not significantly reduce the mitochondrial membrane potential at any of the free Ca^{2+} concentrations studied (not shown in Fig 5)

The concentrations at which 50% of the TPP^+ uptake by the mitochondria at an initial Ca^{2+} concentration of 10 μM was inhibited were 74 ± 4 μM ($n = 6$) for LPC,

TABLE I

Effects of various lysophospholipids on $^{45}Ca^{2+}$ accumulation by isolated rat liver mitochondria

The test medium contained spermine (100 μM) and 100 μM of LPC, LPG, LPI, LPS or LPE. 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 rates of $^{45}Ca^{2+}$ accumulation by the mitochondria are expressed as nmol $^{45}Ca^{2+}$ per mg protein and are means ± S E. with the number of experiments given in parentheses. * $P < 0.05$, ** $P < 0.01$ compared with control (Student's t-test)

Lysophospholipid (100 μM)	$^{45}Ca^{2+}$ accumulation nmol/mg protein		% inhibition
Control	2 11 ± 0 37	(28)	
LPE	2 02 ± 0 37	(11)	4
LPS	1 12 ± 0 21	(12)	47
LPI	0 87 ± 0 20 *	(15)	59
LPG	0 69 ± 0 18 **	(24)	67
LPC	0 53 ± 0 19 **	(16)	75

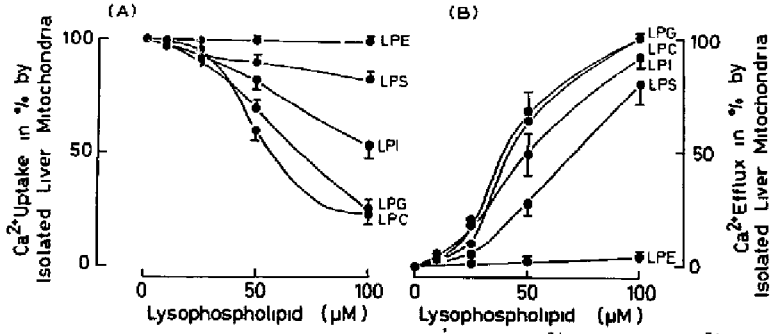

Fig 4 Comparison of the concentration-dependent effects of various lysophospholipids on Ca^{2+} uptake (A) and Ca^{2+} efflux (B) by isolated rat liver mitochondria. The lysophospholipids shown in this figure are LPC, LPG, LPI, LPS and LPE In (A), mitochondrial Ca^{2+} uptake is presented in dependence on the lysophospholipid 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 39 6 nmol Ca^{2+} per mg protein This amount was set 100% and compared with the amount of Ca^{2+} taken up by the mitochondria at increasing lysophospholipid concentrations In (B) mitochondrial Ca^{2+} efflux is presented in dependence on the lysophospholipid 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 lysophospholipid concentrations The values represent means ± S E. from 4–12 experiments All lysophospholipids with the exception of LPE significantly decreased mitochondrial Ca^{2+} uptake ($P < 0.001$) and significantly increased Ca^{2+} efflux ($P < 0.001$) (analysis of variance)

84 ± 4 μM ($n = 4$) for LPG, 97 ± 5 μM ($n = 7$) for LPI, and > 100 μM ($n = 4$) for LPS

Discussion

Addition of lysophospholipids to isolated liver mitochondria leads to a reduction in the initial velocity of uptake and to a diminished accumulation of Ca^{2+} in the mitochondria resulting in a net Ca^{2+} efflux from the mitochondria The efficiency of the lysophospholipids to induce these effects depends on the structure of the polar head-group The relative potency decreased in the order LPC = LPG > LPI > LPS, while LPE was virtually ineffective This relative order is in inverse correlation with the ability of the various head-groups to build up intermolecular hydrogen bonds [25,26] For example, both LPE and LPC are zwitterionic phospholipids, but the ethanolamine head-group can participate in hydrogen bonds with neighbouring membrane lipids, while the choline head-group cannot participate [25,26] Furthermore the glycerol head-group which could theoretically build up such bonds does not do so in membranes, according to differential scanning calorimetric data, where it behaves like a choline head-group [25,26] This fits again into the structure–activity relationship which we established for the influence of lysophospholipids on mitochondrial Ca^{2+} transport Thus, it is not surprising that we could not confirm in our experiments a potent effect of LPE on mitochondrial Ca^{2+} transport as reported by Dalton et al [27] In view of this good correlation with the biophysical properties of the various lysophospholipids, the alternative explanation, that the potency is dependent primarily on the extent of incorporation into the mitochondrial membranes, is less likely.

Whether a disturbance of the membrane polar head-group region and the attached Chapman Stern layer is causative to the effects on mitochondrial Ca^{2+} transport or only a prerequisite to changes in the hydrophobic membrane core which in turn could influence the activity of an integral membrane protein cannot be decided as yet. The latter assumption is supported by the observation that the activity of LPC depends on the type of fatty acid esterified to the C_1-atom of the glycerol backbone This latter hypothesis would infer a similar mechanism of action for lysophospholipids as for the unsaturated fatty acids [28] to explain the influence on mitochondrial membrane potential

In fact, a reduction of mitochondrial membrane potential is induced by all lysophospholipids which have an impact on mitochondrial Ca^{2+} transport The close correlation illustrates that only one transport system may be directly affected by the changes in the lipid membrane and the other effect may be secondary As the membrane potential is the main driving force for mitochondrial Ca^{2+} uptake [23,29,30] a reduction in the membrane potential to such a degree as seen in our experiments is sufficient to explain the rises in the ambient free Ca^{2+} concentration Mitochondrial Ca^{2+} efflux seen with the higher concentrations of lysophos-

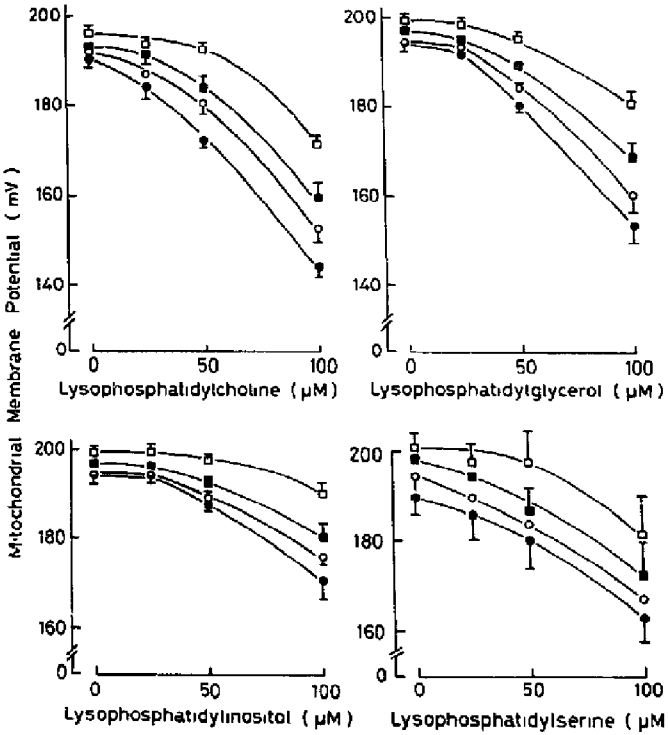

Fig. 5 Concentration-dependent effect of various lysophospholipids on the mitochondrial membrane potential of isolated rat liver mitochondria at different initial Ca^{2+} concentrations (□, 0.01 ■ 1 ○ 5 ● 10 μM) The test medium in the microincubation chamber was supplemented with 100 μM spermine, 8 μM TPP^+, and LPC, LPG, LPI, or LPS in increasing concentrations (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 4–6 experiments LPC LPG, LPI and LPS significantly ($P < 0.01$) decreased the mitochondrial membrane potential at all initial Ca^{2+} concentrations (analysis of variance)

pholipids in the present study was considerably faster than reported for the sodium-dependent and sodium-independent Ca^{2+} efflux pathways [31] Lysophospholipid-induced Ca^{2+} release may thus involve a reverse Ca^{2+} unport, as suggested for uncoupler-induced Ca^{2+} release [32] Inhibition of $^{45}Ca^{2+}$ uptake through the lysophospholipids also supports this conclusion However, lysophospholipids do not exert deleterious or irreversible effects on mitochondria in our experiments The lowering of the mitochondrial membrane potential is limited (Fig. 5) and the effects on Ca^{2+} transport are spontaneously reversible, probably in parallel to metabolic degradation in particular during prolonged incubation (Fig 1b) The unchanged ability of spermine to decrease the free Ca^{2+} concentration by activation of Ca^{2+} uptake (Fig 1b) indicates that the mitochondria return to a fully energized state

With regard to the physiological relevance, lysophospholipids may be generated by intracellular PLA_2 in sufficient amounts to elicit effects such as described above, when assuming that during a short incubation period of 30 s only a fraction of these agents is incorporated into the mitochondrial membranes The effects of lysophospholipids are seen in freshly isolated mitochondria without additional Ca^{2+} loading (Fig. 2) The Ca^{2+} content of our freshly isolated mitochondria is well within the range reported for in situ mitochondria by other investigators [33,34] In conjunction with the hypothesis that activity of mitochondrial PLA_2 on the phospholipids of the inner mitochondrial membrane is dependent on the free intramitochondrial Ca^{2+} concentration [35], our data indicate the existence of a feedback loop by which mitochondria could be enabled to inhibit further Ca^{2+} uptake or release Ca^{2+} if free

intramitochondrial Ca^{2+} concentrations were to reach critical levels Changes in the Ca^{2+} concentration which can affect PLA_2 activity may be brought about by initiation of signal pathways such as the phosphoinositide cascade [1,2], resulting in the mobilization of Ca^{2+} from the endoplasmic reticulum

The amounts of Ca^{2+} released from the mitochondria in our experiments are compatible with a physiologically relevant role for mitochondria in the regulation of the cytoplasmic Ca^{2+} concentration [19,23,34,36], in addition to its ability to control the mitochondrial matrix Ca^{2+} concentration [30,33,37] rather than just being a sink for Ca^{2+} [38]

Acknowledgments

The skilful technical assistance of Ms D Lischke and Mr R Andag is gratefully acknowledged Some of the results of this study were obtained during medical thesis work by J -K G I R was recipient of a postdoctoral research grant by the Deutsche Forschungsgemeinschaft This study was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, F R G

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