Biochimica et Biophysica Acta, 1069 (1991) 99–109

ADONIS 000527369100369Q

BBAMEM 75384

Structural requirements of lysophospholipid-regulated mitochondrial Ca^{2+} transport

Ingo Rustenbeck [1], Hansjörg Eibl [2] and Sigurd Lenzen [1]

[1] *Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen (F.R.G.)* and [2] *Max-Planck-Institute of Biophysical Chemistry, Göttingen (F.R.G.)*

(Received 25 June 1991)

Key words: Phospholipid; Lysophospholipid; Calcium ion transport: Phospholipase A_2; Liver mitochondrion; (Rat)

Analogues of lysophosphatidylcholine, including PAF (platelet-activating-factor) and HePC (an experimental anticancer drug), were studied for their influence on mitochondrial Ca^{2+} transport and membrane potential. Lysophospholipids released Ca^{2+} from mitochondria and reduced the maximal Ca^{2+} uptake. The structure-activity relations indicate that deprotonated head groups like phosphocholines yield active compounds while partially protonated head groups like phosphoethanolamines are essentially inactive. Structural requirements for the apolar part of the molecules were acyl or alkyl chain lengths of less than 18 carbon atoms at the C_1-position of the glycerol backbone and residues of small size and/or low polarity at the C_2-position. Choline lysophospholipids, but not ethanolamine lysophospholipids, may therefore induce mitochondrial Ca^{2+} efflux and become mediators of ischaemic tissue damage where dysregulated phospholipase A_2 activity and an impairment of mitochondrial function are supposed to play a crucial role.

Introduction

Mitochondrial Ca^{2+} transport has been the subject of intense research during the last two decades. While it was originally supposed that mitochondria would act as intracellular Ca^{2+} buffers which can regulate the cytoplasmic Ca^{2+} concentration [1–3], the role of mitochondrial Ca^{2+} transport is currently often regarded as relaying changes in cytoplasmic Ca^{2+} concentrations to the velocity of oxidation via regulation of dehydrogenases in the Krebs cycle [4–6]. Nevertheless, an influence of mitochondrial Ca^{2+} transport on the cytoplasmic Ca^{2+} concentration cannot be ruled out [7].

Despite efforts to isolate transport proteins [8,9] the pathways of mitochondrial Ca^{2+} uptake and release are functionally defined only and exhibit a marked asymmetry between maximal uptake and release velocity. The uptake is mediated by a uniport mechanism (10), which is driven by the membrane potential and may reach very high transport rates at high extramitochondrial Ca^{2+} concentrations [11]. The Ca^{2+} efflux is brought about by a Na^+/Ca^{2+} exchange and in some tissues a H^+/Ca^{2+} exchange [12,13] is proposed to exist. There is, however another pathway for release of Ca^{2+} ions which depends on the Ca^{2+} loading state of the mitochondria and involves a Ca^{2+}-induced activation of mitochondrial phospholipase A_2 (PLA_2) and oxidation of pyridine nucleotide and glutathione pools [14]. This event bears similarity to, or may be identical with the Ca^{2+}-induced 'permeabilization' (and probably also the mitochondrial membrane 'transition' as

Abbreviations: LPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine = lysophosphatidylcholine; LPDME, 1-palmitoyl-*sn*-glycero-3-(*N*,*N*-dimethyl)ethanolamine, C_{16}-L-PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{16}-L-lysoPAF, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; C_{16}-D-PAF, 3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine; C_{16}-D-lysoPAF, 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine; C_{18}-L-PAF, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; TeMGPC, 1-*O*-tetradecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; HeMGPC, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; OMGPC, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; 1O3MG2PC, 1-*O*-octadecyl-3-*O*-methyl-*sn*-glycero-2-phosphocholine; HePC, hexadecyl-phospho-(*N*,*N*,*N*-trimethyl) ethanolamine = hexadecylphosphocholine; HeP_3C, hexadecyl-phospho-(*N*,*N*,*N*-trimethyl)propanolamine, HeP_4C, hexadecyl-phospho-(*N*,*N*,*N*-trimethyl)butanolamine; HeP_6C, hexadecyl-phospho-(*N*,*N*,*N*-trimethyl)hexanolamine; HePDME, hexadecyl-phospho-(*N*,*N*-dimethyl)ethanolamine; OleylPC, *cis*-octadec(9,10)enyl-phospho choline; HeP_6E, hexadecyl-phospho-hexanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine = phosphatidylcholine.

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

termed by Hunter and Haworth [15]) which was characterized by Crompton and collaborators as a loss of small solutes from mitochondria. Since the mitochondrial matrix proteins were retained and the permeability increase was reversible upon withdrawal of Ca^{2+}, this release was interpreted to be caused by a Ca^{2+}-activated pore rather than by a deleterious rupture of the inner mitochondrial membrane [16]. This release mechanism is of particular interest for studies on ischaemic cell injury which is usually accompanied by a cellular Ca^{2+} overload, leading to mitochondrial dysfunction and cessation of ATP supply which are critical steps towards irreversibility of the injury [17]. Regardless of whether mitochondrial dysfuncton may involve increased PLA_2 activity or decreased re-esterification as described by Parce et al. [18], it will result in the inner mitochondrial membrane containing an increased amount of lysophospholipids and unesterified fatty acids.

The lysophospholipid species responsible for changes in mitochondrial Ca^{2+} transport was identified to be lysophosphatidylethanolamine (LPE) [19–21]. Dalton et al. [21] regarded LPE not primarily as a potentially destructive agent but as a physiological mediator of Ca^{2+} release since they found no other effects of LPE on mitochondrial function. In studies on mitochondrial aging and phospholipase A_2 function [18,22] LPE was considered as representative for lysophospholipid properties. However, in a recent study on the effects of lysophospholipids on mitochondrial Ca^{2+} transport LPE was the least active naturally occurring lysophospholipid [23]. Since authors investigating ischaemic tissue damage usually analyze the phospholipid composition for the occurrence of LPC but not of LPE [24] or have found higher increases of LPC than of LPE [25] these conflicting results require clarification.

In order to elucidate the active lysophospholipid species and to further define the functional consequences – particularly the relation between Ca^{2+} transport and membrane potential – we used systematically varied analogues of LPC, some of which are naturally occurring like L-PAF (C_{16} and C_{18}), while others are non-natural lysophospholipid analogues with cytostatic properties. A further aim was to characterize effects of PAF not mediated by its action on the plasma membrane receptor, since it was recently suggested that intracellularly synthesized PAF could accumulate in high amounts where such effects are likely to occur [26].

Materials and Methods

Preparation of mitochondrial fractions. Liver was obtained from Wistar rats, homogenized in homogenization medium (210 mM mannitol, 70 mM sucrose, 20 mM Hepes and 0.5 mM EGTA, adjusted to pH 7.0 with KOH) and maintained on ice [27,28]. The sediment obtained after centrifugation for 15 min at $660 \times g$ was discarded. The mitochondrial pellet was obtained by centrifugation of the supernatant for 15 min at $4000 \times g$ at 4 °C [27,28]. After resuspension and recentrifugation the pellet was resuspended in test medium (125 mM KCl, 2 mM KH_2PO_4, 5 mM succinate, 25 mM Hepes, adjusted to pH 7.0 with KOH) [28–30]. As stated earlier, the Ca^{2+} load of mitochondria isolated this way is around 9.5 nmol/mg protein as measured by atomic absorption spectrometry [23]. Protein was determined according to McKnight [31]. The protein content of 1 μl of the mitochondrial fractions resuspended in test medium was 27 ± 1 μg ($n = 23$). 0.5–2.0 μl of these resuspended mitochondria were used in the experiments as described in the tables and legends to figures. The integrity of the mitochondrial fractions was checked by determination of the soluble mitochondrial matrix enzyme, glutamate dehydrogenase (E.C. 1.4.1.2) in the mitochondria and the incubation medium. After the incubations, control mitochondria retained 91% of the enzyme, while mitochondria exposed to a high concentration of LPC (100 μM) retained 86% ($n = 15$).

Measurement of free Ca^{2+} concentration. Ca^{2+} uptake and efflux by isolated mitochondria were measured in test medium at 25 °C with a Ca^{2+} ion-sensitive minielectrode and a microincubation chamber (40 μl vol.) as described in detail [27]. Addition of phospholipids 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 labeled test medium at 25 °C was measured at a free Ca^{2+} concentration of 10 μM as described [32]. 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 microfiltration device [32].

Measurement of mitochondrial membrane potential. Membrane potential of isolated mitochondria was determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) between the incubation medium and the mitochondrial matrix, using the same setup as for the Ca^{2+} measurements except that instead of a Ca^{2+} ion-sensitive membrane a TPP^+ ion-sensitive membrane [33] was mounted on the tip of the exchangeable membrane support inset of the minielectrode. Calculations were performed as described earlier [30].

Phospholipid analysis. To quantitate the amount of the added lysophospholipids and analogues actually present in the organelle membranes, mitochondrial fractions were incubated under the same conditions as for the Ca^{2+} uptake studies. At the end of the 30 s

incubation period the samples were cooled on ice and the mitochondria were pelleted by centrifugation for 5 min at 4 °C and at 100000 × *g* with an air-driven ultracentrifuge. The added phospholipids and the endogenous phosphatidylcholine and lysophosphatidylcholine were determined as described recently in detail [34,35]. Briefly, the lipids were extracted with hexane-isopropanol [36] and the resulting total lipid extract was separated by anion-exchange chromatography with subsequent desalting by gel filtration. The neutral fraction of the lipid extract, containing the zwitterionic phospholipids and the neutral lipids, was then chromatographed on silica gel HPTLC plates using modifications of the solvent systems published by Kolarovic and Traitler [37]. After development, the lipid bands on the plate were charred with a cupric sulphate reagent [35] and quantified by in situ densitometry in a Desaga CD60 densitometer interfaced with an IBM AT03 microcomputer.

Chemicals. LPC, C_{16}-L-PAF, and C_{16}-L-lysoPAF were obtained from Sigma (St. Louis, MO, U.S.A.). LPDME, C_{16}-D-PAF, C_{16}-D-lysoPAF, C_{18}-L-PAF, TeMGPC, HeMGPC, OMGPC, 1O3MG2PC, HePC, HeP_3C, HeP_4C, HeP_6C, HePDME, OleylPC, and HeP_6E were synthetized by H. Eibl [38–40].

$^{45}CaCl_2$ was from Amersham (Braunschweig, F.R.G.). All other chemicals of analytical grade were from Sigma, Boehringer (Mannheim, F.R.G.), Serva (Heidelberg, F.R.G.), or Merck (Darmstadt, F.R.G.). HPTLC 60 F_{254} plates were from Merck. The phospholipids used in this study can be assorted to four different groups: (A) phospholipids with an ester bond in the C_1-position of the glycerol backbone (lysophospholipids); (B) phospholipids with an ether bond in the C_1-position, (platelet-activating factors and alkyl-lysophospholipids); (C) phospholipids with ether bonds in both the C_1- and C_2 position (alkyl-methyl-glycerophosphocholines); and (D) phospholipids without a glycerol backbone, an alkyl chain being linked directly to the phosphate group (alkylphosphocholines and -homocholines). The structure of a species from each group is depicted in Fig. 1 A–D.

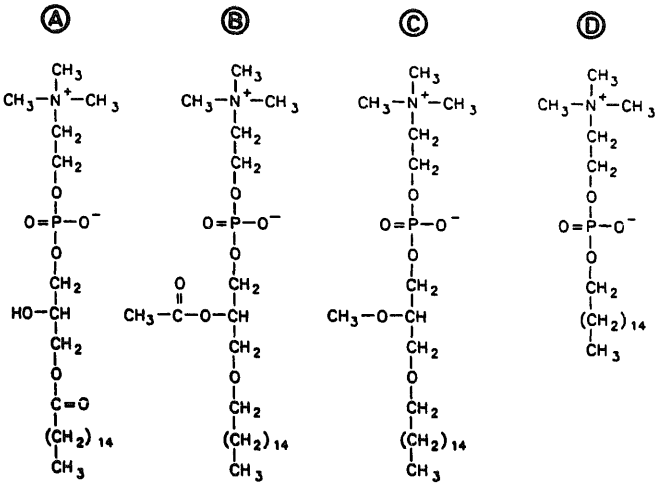

Fig. 1. Chemical formulas of LPC (A), C_{16}-L-PAF (B), HeMGPC (C) and HePC (D).

Results

Induction of mitochondrial Ca^{2+} efflux by HePC. Isolated liver mitochondria incubated in a test medium of an ionic composition simulating the composition of the cytosol [29,30] maintain a steady-state free Ca^{2+} concentration well below 1 μM (Fig. 2). Addition of HePC (hexadecylphosphocholine) (50 μM) to the incubation medium increased the free Ca^{2+} concentration to values around 2 μM. Nevertheless, addition of spermine (400 μM), a known activator of mitochondrial Ca^{2+} uptake [30,41,42], decreased the free Ca^{2+} concentration again well below 0.5 μM, apparently through stimulation of mitochondrial Ca^{2+} uptake.

The mitochondrial Ca^{2+} efflux induced by HePC was visible at 5–10 μM and was spontaneously reversible up to 100 μM. However, from 25 μM onwards, the spontaneous re-uptake of Ca^{2+} was incomplete, resulting in a higher steady state than before the addition of HePC. At all HePC concentrations up to 100 μM addition of spermine (400 μM) resulted in a lowering of the free extramitochondrial Ca^{2+} concentration (data not shown).

Effects of choline lysophospholipids and analogues on mitochondrial Ca^{2+} uptake and Ca^{2+} efflux. Ca^{2+} uptake was started by injection of mitochondria into the microincubation chamber. 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

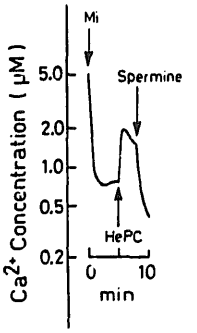

Fig. 2. Effect of HePC (50 μM) on the regulation of the free Ca^{2+} concentration by rat liver mitochondria and its modulation by spermine (400 μM). The test medium in the microincubation chamber was supplemented with 0.3 mM $MgATP^{2-}$. Mitochondria (suspended in 1–2 μl test medium) were added to the test medium with an initial Ca^{2+} concentration of 5 μM in the microincubation chamber at min 0. HePC (50 μM) and spermine (400 μM) were added after 5 and 7.5 min, respectively. The curve represents a typical recording of four experiments.

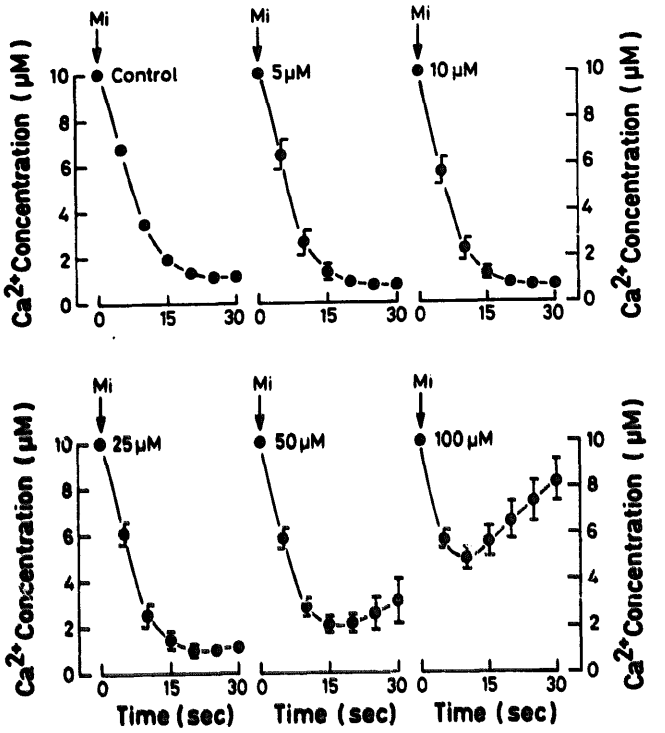

Fig. 3. Concentration-dependent effects of C_{16}-L-lysoPAF on the kinetics of Ca^{2+} uptake and Ca^{2+} efflux by isolated rat liver mitochondria. The test medium in the microincubation chamber contained spermine (100 μM) and increasing C_{16}-L-lysoPAF concentrations (5, 10, 25, 50 or 100 μM). The initial Ca^{2+} concentration in the incubation medium was adjusted to 10 μM at the beginning of the experiment. At min 0 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 six experiments.

after 15–20 s even in the presence of the highest initial free Ca^{2+} concentration of 10 μM (Fig. 3). C_{16}-L-lysoPAF affected the kinetics of Ca^{2+} uptake and Ca^{2+} efflux by isolated rat liver mitochondria in a concentration-dependent manner (Fig. 3). Up to concentrations of 25 μM, C_{16}-L-lysoPAF slightly reduced the amount of Ca^{2+} taken up by mitochondria during a 30-s incubation period. At 50 μM C_{16}-L-lysoPAF this resulted in a higher steady state Ca^{2+} concentration of 2–3 μM. At 100 μM C_{16}-L-lysoPAF, the ability of the mitochondria to take up Ca^{2+} deteriorated further. After uptake of a reduced amount of Ca^{2+} with a nadir after 15 s, mitochondria released Ca^{2+}, so that the initial Ca^{2+} concentrations of 10 μM were almost reached again within less than 30 s of incubation (Fig. 3).

LPC, HePC and C_{16}-L-PAF also induced a potent release of Ca^{2+} from mitochondria as measured according to the protocol described in Fig. 3 for C_{16}-L-lysoPAF. The amount of Ca^{2+} released again during the 30-s incubation period by the isolated liver mitochondria under the influence of increasing phospholipid concentrations is expressed in % of the maximal amount of Ca^{2+} taken up during the first 10–20 s of the incubation period (Fig. 4).

The increased rate of mitochondrial Ca^{2+} release induced by LPC, HePC, C_{16}-L-PAF and C_{16}-L-lysoPAF also resulted in a significant concentration-dependent decrease of mitochondrial Ca^{2+} uptake as is depicted in Fig. 3 for C_{16}-L-lysoPAF. The amount of Ca^{2+} uptake which in control liver mitochondria increased linearily in dependence on the free Ca^{2+} concentration in the incubation medium was decreased by these choline phospholipids (100 μM) to a variable degree. The relative inhibitory potency of LPC, HePC, C_{16}-L-lysoPAF and C_{16}-L-PAF remained the same (75, 70, 40 and 32% reduction of control uptake, respectively) at all initial extramitochondrial free Ca^{2+} concentrations in the incubation medium which were 0.5, 1, 2.5, 5, 7.5 and 10 μM, equivalent to 1.1, 2.2, 5.4, 10.8, 16.1, or 21.5 nmol Ca^{2+} per mg mitochondrial protein. The reduction of Ca^{2+} uptake was not determined by the Ca^{2+} loading state of the mitochondria and its possible complicating consequences and was therefore a useful measure of phospholipid activity.

Reduction of mitochondrial $^{45}Ca^{2+}$ uptake by choline phospholipids and analogues. The different phospholipids (100 μM) reduced the amount of $^{45}Ca^{2+}$ accumulated by isolated control liver mitochondria during a 30 s incubation (1.29 nmol/mg protein) to a variable degree. The inhibitory potency was similar for LPC,

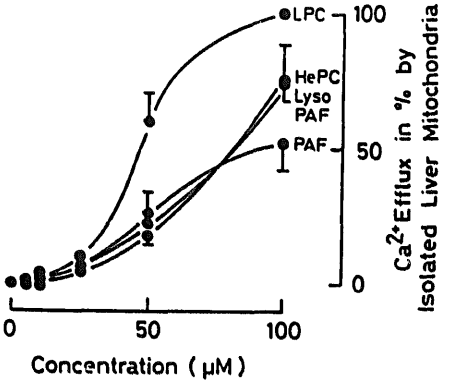

Fig. 4. Comparison of the concentration-dependent effects of various choline lysophospholipids and analogues on Ca^{2+} efflux by isolated rat liver mitochondria. The test substances shown in this figure are LPC, HePC, C_{16}-L-PAF and C_{16}-L-lysoPAF (5, 10, 25, 50 or 100 μM). The test medium in the 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 mitochondria (0.5 μl = 19.1 μg protein) in the incubation chamber decreased the Ca^{2+} concentration within 30 s below 0.5 μM, resulting in an accumulation of 19.9 nmol Ca^{2+} per mg protein. In control experiments 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 0% and compared with the amount of Ca^{2+} released by the mitochondria at the increasing test substance concentrations. The values represent means ± S.E. from 12–17 experiments. All test substances significantly increased Ca^{2+} efflux ($P < 0.001$) (analysis of variance).

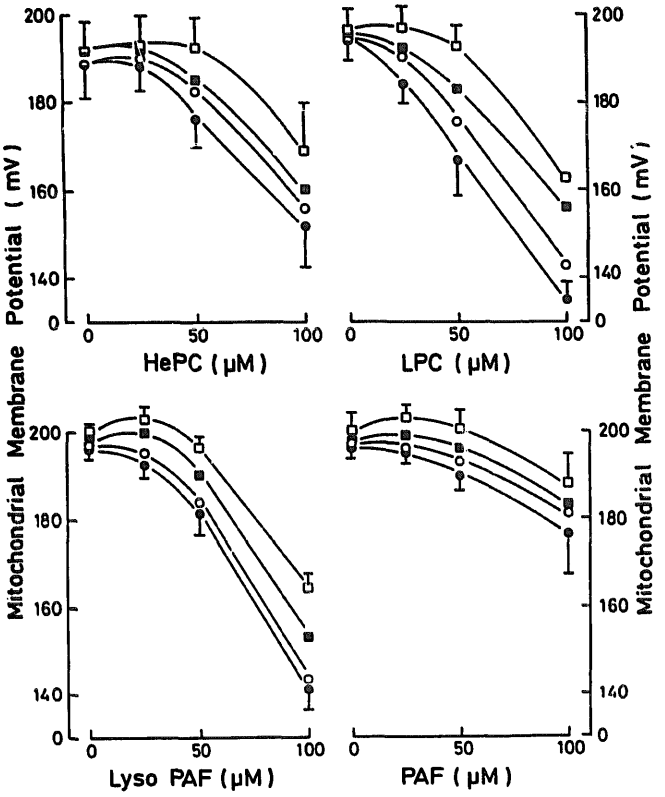

Fig. 5. Concentration-dependent effects of various choline lysophospholipids and analogues on the mitochondrial membrane potential of isolated rat liver mitochondria at different initial Ca^{2+} concentrations (□, 0.01 μM; ■, 1 μM; ○, 5 μM; ●, 10 μM). The test medium in the microincubation chamber was supplemented with 0.3 mM K_2ATP^{2-}, 100 μM spermine, 8 μM tetraphenylphosphonium (TPP^+) and LPC, HePC, C_{16}-L-PAF and C_{16}-L-lysoPAF 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 four experiments. All test substances significantly ($P < 0.01$) decreased the mitochondrial membrane potential at all initial Ca^{2+} concentrations (analysis of variance). When applying a correction procedure for internal membrane binding of TPP^+ [67], the membrane potentials ranged between 147 and 67 mV.

HePC and C_{16}-L-lysoPAF (0.39, 0.32 and 0.36 nmol/mg protein, respectively), but lower for C_{16}-L-PAF (0.59 nmol/mg protein).

Effects of choline phospholipids on mitochondrial membrane potential. The phospholipids reduced the membrane potential of isolated liver mitochondria in a concentration-dependent manner as determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) (Fig. 5). The reduction of the mitochondrial membrane potential by the phospholipids was accentuated by high initial free Ca^{2+} concentrations in the incubation medium (Fig. 5). Thus the reduction of the membrane potential in the presence of 50 μM LPC was already achieved by 25 μM LPC when the initial extramitochondrial Ca^{2+} concentration was 10 μM instead of 1 μM. The membrane potential lowering potency decreased in the order LPC ≥ C_{16}-L-lysoPAF > HePC > C_{16}-L-PAF (Fig. 5). C_{16}-L-lysoPAF was virtually equipotent to LPC in lowering the membrane potential, in contrast to its comparatively weaker influence on Ca^{2+} transport (Fig. 4).

Structure-activity-relationship of choline phospholipids and analogues on mitochondrial Ca^{2+} transport and mitochondrial membrane potential. The hitherto described results permit the conclusion that mobilization of Ca^{2+} from mitochondria by the ether phospholipids PAF and lysoPAF and the phospholipid analogue HePC is brought about by the same basic mechanisms as the Ca^{2+} mobilization induced by the ester lysophospholipids such as LPC, as described in a recent publication [23]. To gain further insight into the structural requirements synthetic, naturally and non-naturally occurring phospholipids were used, which can be assorted to four groups (Table I). A typical specimen of each group showing the main structural characterics is depicted in Fig. 1A–D. The structure-activity relations will be presented following two fundamental types of modification in the phospholipid structure: (1) changes in the polar head group region; and (2) changes in the apolar region comprising the substituents at the C_1 and C_2 atoms of the glycerol backbone and the glycerol itself (Fig. 1).

(A) Structural changes in the head group region. By modification of the structure of the polar head group we investigated if the inability of lysophosphatidylethanolamine (LPE) to influence mitochondrial Ca^{2+} transport as compared to LPC [23] may be due to its property to build up stabilizing intermolecular hydrogen bonds between the lipid head groups [46–48]. This was done by reducing the degree of methylation of the choline group (= *N,N,N*-trimethylethanolamine) by one methyl substituent. Such a head group structure led to a 50% loss of activity when present in a lysophospholipid (LPDME vs. LPC) and to a nearly complete loss of activity when present in an alkylphosphocholine (HePDME vs. HePC). The fact that identical changes in the polar head group structure did not result in the same loss of activity indicates that there are interactions between the head group and parts of the phospholipid molecule where lysophospholipids and alkylphosphocholines are different. Such an interaction could be the dipole–dipole interaction between the phosphorous-nitrogen dipole of the head group and the dipole moment generated by the carbonyl groups of the glyceride part [49] which is present only in the lysophospholipids. Analogous observations were made by Vaughan and Keough [50] on the changes in lipid fluidity of model membranes following the insertion of di- and tri-methylated ester and ether phosphoethanolamines.

Enlargement of the phosphorus-nitrogen distance in the choline headgroup had only modest functional

consequences at the first glance, since the loss of activity from LPC to LPE [23] was reflected by a comparable loss of activity from HeP_6C to HeP_6E. An elongation of the natural phosphorus-nitrogen distance from two carbon atoms, as present in HePC, to three carbon atoms was accompanied by a decrease in inhibitory potency on mitochondrial Ca^{2+} uptake followed by a regain with a C_4 and a C_6 distance (HeP_3C, HeP_4C and HeP_6C). This preserved activity with a highly deformed head group structure supports the assumption that not binding of the choline phospholipid to a neighbouring membrane lipid or an integral membrane protein is responsible for its effects on membrane transport but rather that the inability to build up bonds with neighbouring lipids is a prerequisite for its effectiveness.

(B) Structural changes in the apolar region. In the apolar region, a fundamental requirement for naturally occurring phospholipids to interfere with mitochondrial Ca^{2+} transport and membrane potential is the

TABLE I

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

All substances 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. 3 and 4 and for their ability to inhibit mitochondrial TPP^+ (tetraphenylphosphonium) uptake as a measure of the mitochondrial membrane potential according to the protocol described in the legend to Fig. 5. The degree of inhibition of uptake was expressed as % reduction of the control rate of uptake. Mitochondria not exposed to a phospholipid served as controls. The mitochondria decreased the free Ca^{2+} concentration in the incubation medium from 10 μM to 0.55 ± 0.04 μM. This decrease of the free Ca^{2+} concentration in the incubation medium of 9.45 ± 0.04 μM corresponded to a mitochondrial Ca^{2+} uptake of 28.9 ± 1.5 nmol of Ca^{2+} per mg protein ($n = 28$). The mitochondria decreased the TPP^+ concentration in the incubation medium from 8 μM to 2.3 ± 0.1 μM. This decrease of the TPP^+ concentration in the incubation medium of 5.7 ± 0.1 μM corresponded to a mitochondrial membrane potential of 206 mV in controls ($n = 16$). Applying a correction procedure for internal binding of this lipophilic cation [67], this would correspond to a value of 152 mV. Half-maximal inhibitory concentrations of the various test substances expressed in μM for mitochondrial Ca^{2+} uptake and mitochondrial TPP^+ uptake as a measure of the mitochondrial membrane potential were calculated for all test substances which reduced uptake by more than 50% at concentrations up to 250 μM and in one case up to 1000 μM. These values were obtained in experiments where the concentration-dependent effects of the test substances were determined for Ca^{2+} uptake according to the protocol described in the legends to Figs. 3 and 4 and for TPP^+ uptake according to the protocol described in legend to Fig. 5. All values represent means ± S.E. of 4–12 experiments. All concentration-dependent inhibitory effects of the test substances on mitochondrial Ca^{2+} uptake and membrane potential greater than 5% were significant ($P < 0.01$) (analysis of variance).

	Half-maximal inhibitory concentration of test agent (A) and % uptake inhibiton at 100 μM of test agent (B) for			
	Mitochondrial Ca^{2+} uptake		Mitochondrial TPP^+ uptake	
	A	B	A	B
Group I (lysophospholipids):				
LPC	88 ± 4 μM	62 ± 4%	70 ± 5 μM	75 ± 3%
LPDME	115 ± 8 μM	42 ± 6%	124 ± 4 μM	37 ± 4%
Group II (platelet activating factors, alkyl-lysophospholipids and alkyl-acetyl-glycerophosphocholines):				
C_{16}-L-PAF	153 ± 11 μM	25 ± 3%	175 ± 3 μM	17 ± 5%
C_{16}-D-PAF	123 ± 11 μM	40 ± 4%	106 ± 4 μM	47 ± 2%
C_{16}-L-lysoPAF	115 ± 6 μM	47 ± 4%	100 ± 10 μM	53 ± 6%
C_{16}-D-lysoPAF	128 ± 16 μM	37 ± 10%	132 ± 9 μM	34 ± 4%
C_{18}-L-PAF		2 ± 0 %		3 ± 3%
Group III (alkyl-methyl-glycerophosphocholines):				
TeMGPC	86 ± 2 μM	70 ± 1%	86 ± 2 μM	69 ± 5%
HeMGPC	94 ± 3 μM	59 ± 5%	87 ± 1 μM	66 ± 2%
OMGPC	> 1000 (16 ± 1% at 1000 μM)	8 ± 1%	493 ± 18 μM	13 ± 1%
1O3MG2PC		5 ± 1%		5 ± 2%
Group IVa (alkyl-phosphocholines and -homophosphocholines):				
HePC	87 ± 3 μM	74 ± 4%	123 ± 6 μM	39 ± 3%
HeP_3C	100 ± 8 μM	49 ± 7%	139 ± 2 μM	30 ± 2%
HeP_4C	80 ± 5 μM	72 ± 5%	126 ± 5 μM	42 ± 2%
HeP_6C	75 ± 5 μM	78 ± 5%	99 ± 4 μM	57 ± 3%
Group IVb (alkyl-phosphocholines and homophosphocholines):				
HePDME		7 ± 2%		7 ± 3%
OleylPC	147 ± 6 μM	16 ± 4%	163 ± 1 μM	16 ± 1%
HeP_6E		1 ± 0 %		3 ± 3%

absence of the fatty acid substituent at the C_2 atom of the glycerol backbone. In addition, the length of the acyl chain esterified at the C_1 position was found to be of influence, chain lengths shorter than C_{12} and longer than C_{18} resulted in inactive compounds [23]. This relation was also observed with the alkyl-methyl-glycerophosphocholines (TeMGPC, HeMGPC and OMGPC) used in this study. Tetradecyl and hexadecyl side chains gave highly active compounds, while the presence of an octadecyl side chain resulted in a sharp decline in potency. A nearly total loss of activity was also registered when in group II (PAFs and lysoPAFs) the C_{16} side chain was replaced by a C_{18} side chain. These two compounds (C_{16}-L-PAF and C_{18}-L-PAF) are identical to the biologically active lipid PAF (platelet-activating factor), which is a mixture of both lipid species. Generally, the hydrocarbon chain could be linked by an ester or ether bond to the C_1 position of the glycerol backbone to give an active compound, although an ester bond resulted in a higher activity in the lower concentration range (LPC vs. C_{16}-L-lysoPAF in Fig. 4). The substituents at the C_2 position which favored the inhibitory action of the phospholipids on mitochondrial Ca^{2+} uptake had the following order of potency: *O*-methyl > hydroxyl > acetyl (HeMGPC, C_{16}-L-lysoPAF and C_{16}-L-PAF). The requirements for substituents at this site can therefore be described as small size and/or low polarity.

The most substantial modification in the apolar region was the removal of the glycerol backbone and linkage of an alkyl chain directly to the phosphocholine head group (group IVa and IVb). The resulting group of substances, the alkylphosphocholines, showed effects of roughly the same potency on mitochondrial Ca^{2+} transport as lysophosphatidylcholine and C_{14}- and C_{16}-alkyl-methyl-glycerophosphocholines. The alkyl chain of the alkylphosphocholines has probably a position within the membrane similar to the C_1-acyl side chain of the glycerophospholipids, since the presence of an unsaturation, in the form of an octadecenyl (OleylPC) as well as an octadecenoyl chain (oleoyl-LPC) [23] led to a marked loss of activity. Yet there was one remarkable difference between these two groups: The membrane potential was less decreased by the alkylphosphocholines at equal potency on mitochondrial Ca^{2+} uptake. This feature was most conspicuous

TABLE II

Incorporation of various choline lysophospholipids and analogues into a rat liver mitochondrial fraction

Rat liver mitochondria (1 μl) were added to the incubation medium containing 100 μM phospholipid. After incubation for 30 s at 25 °C and centrifugation the mitochondrial pellet was analyzed for the lysophospholipid and phosphatidylcholine content as described in the methods section. The data are means ± S.E. of four experiments, except for LPC and OMGPC or HePC, which are means ± S.E. of eight and six experiments, respectively.

Lysophospholipid	Amount of lysophospholipid as pmol	Lysophospholipid content as percentage of endogenous PC	Lysophospholipid content as nmol per mg protein
Group I (lysophospholipids):			
LPC	1114 ± 132	61 ± 6	59 ± 7
LPDME	600 ± 31	25 ± 3	34 ± 2
Group II (platelet activating factors, alkyl-lysophospholipids, and alkyl-acetyl-glycerophosphocholines):			
C_{16}-L-PAF	1143 ± 190	69 ± 9	86 ± 17
C_{16}-D-PAF	1137 ± 171	62 ± 4	92 ± 9
C_{16}-L-lysoPAF	1200 ± 217	67 ± 7	86 ± 14
C_{16}-D-lysoPAF	874 ± 171	48 ± 4	70 ± 11
C_{18}-L-PAF	963 ± 104	60 ± 8	70 ± 6
Group III (alkyl-methyl-glycerophosphocholines):			
TeMGPC	768 ± 143	43 ± 6	54 ± 7
HeMGPC	1404 ± 224	83 ± 11	104 ± 18
OMGPC	1221 ± 223	73 ± 13	60 ± 14
1O3MG2PC	1578 ± 178	96 ± 4	114 ± 10
Group IVa (alkyl-phosphocholines and -homophosphocholines):			
HePC	1067 ± 167	50 ± 4	48 ± 10
HeP_3C	1072 ± 108	52 ± 3	62 ± 6
HeP_3C	907 ± 131	39 ± 4	52 ± 8
HeP_6C	1041 ± 129	48 ± 4	60 ± 8
Group IVb (alkyl-phosphocholines and -homophosphocholines):			
HePDME	1110 ± 232	53 ± 10	62 ± 11
OleylPC	1671 ± 37	76 ± 4	96 ± 2
HeP_6E	984 ± 264	41 ± 9	56 ± 13

with HePC (TPP^+ uptake at 100 or 50 μM of HePC vs. TeMGPC: $P < 0.001$ or $P < 0.01$ respectively; Student's paired t-test).

Phospholipid uptake. The differences in potency of the phospholipids with respect to their influence on mitochondrial Ca^{2+} transport or membrane potential could be due either to interactions within the inner mitochondrial membrane or to different degrees of incorporation into the membrane. We therefore simulated the short term incubation conditions and analyzed the incubated mitochondrial fraction for the content of the added phospholipid and of endogenous phosphatidylcholine (PC) (Table II). The phospholipids were incorporated to different degrees, ranging from 25 to 96% of the endogenous PC content. Expressed as nmol/mg mitochondrial protein, the range was from 34 nmol/mg to 114 nmol/mg. Assuming a mitochondrial phospholipid content of 230 nmol/mg and a PC content of around 40 mol% of the phospholipid mass [51], the overall mean of lysophospholipids taken up corresponds to 24 mol% and 32 mol%, respectively, when based on PC or protein content. This is most likely due to the fact, that the PC content was determined from the individual sample after incubation, while the protein content was determined from the batch of mitochondria. Thus the values based on the PC content are probably the more accurate.

No correlation could be found between the extent to which an added phospholipid was taken up by the mitochondrial fraction and its ability to inhibit Ca^{2+} uptake at 100 μM. The linear regression through the origin resulted in $r = 0$ in both cases (i.e. values based on protein or PC content, respectively). The endogenous LPC content, which was determined as a measure of PLA_2 activity in incubations with HePC, OMGPC and LPE amounted to less than 7% of the added phospholipids.

Discussion

Choline lysophospholipids as modulators of membrane-bound enzymes and transport systems. One reason for this study was to clarify the role of lysophosphatidylethanolamine (LPE) which was proposed to be a mediator of mitochondrial Ca^{2+} release [19–21]. In experiments with naturally occurring lysophospholipids we had found LPE to have only minimal activity, while LPC and lysophosphatidylglycerol were the most potent substances, lysophosphatidylinositol and lysophosphatidylserine having intermediate activity [23]. The phospholipids with systematically varied head groups in the present study consistently showed a loss of activity when methylation of choline (= *N,N,N*-trimethylethanolamine) was reduced. Even highly deformed head groups with a phosphorous-nitrogen distance of six carbon atoms did reflect the loss of activity from LPC to LPE. These results support our conclusion, drawn from the structure-activity relation of the naturally occurring phospholipids, that in the head group region the inability to build up stabilizing hydrogen bonds with neighboring lipid head groups [47] is a prerequisite for a lysophospholipid or analogue to affect the mitochondrial capability to take up and retain Ca^{2+}. Further support comes from analogous findings in other biological systems, namely that LPC (C_{16} and C_{18} compounds) can partially mimick effects of PAF in the perfused liver while LPE cannot [52], that LPC is an activator of plasma membrane protein kinase C [53], whereas LPE is not and that the ethanolamine analogue of PAF is virtually ineffective in eliciting the typical cellular responses to PAF [54]. Apparently the requirement for such a head group structure is not limited to effects on mitochondrial membranes, but may represent a common biophysical prequisite for a lysophospholipid to interact with membrane-bound enzymes and transport systems. On the other hand, a consistent feature, for which we found no analogy, neither in studies with model membranes nor with biological membranes, was the reduction in potency following the elongation of the alkyl or acyl side chain from 16 to 18 carbon atoms. Since the amount of the C_{18}-compounds incorporated into the mitochondrial fraction tended to be even higher than that of the C_{16}-compounds this appears to be a unique feature of the mitochondrial membrane.

C_{16}-L-PAF appears to act here as a membrane perturbant and not on a receptor site, as indicated by the following facts: (a) the concentration of PAF to interact with the receptor is 10^5-fold lower than the concentrations required for mitochondrial effects [54,55]; (b) C_{16}-L-lysoPAF and C_{16}-D-PAF are virtually inert in studies on cells and whole organs [54], while they were more effective than C_{16}-L-PAF in influencing the mitochondrial Ca^{2+} transport (see above).

Nevertheless the effects reported here cannot be regarded as representing merely a destruction of the inner mitochondrial membrane bilayer integrity. Since the lipid bilayer has a zero permeability for Ca^{2+} ions but permits the passage of some protons via transient 'hydrogen-bonded chains of water' [56] or weak acid protonophores [57], a generalized disordering of the bilayer structure would primarily increase the proton permeability, resulting in the collapse of the membrane potential. Actually, however, the lowering of the membrane potential was limited, particularly when compared to the action of a classical uncoupler, dinitrophenol [23].

It has been proposed that on the level of the single mitochondrion the Ca^{2+} efflux during the membrane 'permeabilization' by the action of PLA_2 would be caused by a complete breakdown of the membrane potential, an event which would not not become visible

with a mitochondrial population due to the different energization states of the single mitochondria and the resulting different time courses of their permeabilization [58]. According to this hypothesis which explains the permeabilization as an energy-saving means of mitochondrial Ca^{2+} release, one would have to expect constant ratios of lysophospholipid-induced reductions in Ca^{2+} and TPP^+ uptake by the mitochondria in our experimental setting. This was not the case, as shown for the effects of TeMGPC and HePC.

However, it is quite possible that exogenous provision of lysophospholipids does not mimic the complete course of PLA_2 associated membrane permeabilization, particularly in the light of the constantly low endogenous LPC content of the mitochondria and the independence on the Ca^{2+} load, which makes an activation of the endogenous PLA_2 in our system unlikely. The underlying effect of the reduced Ca^{2+} accumulation capacity of the mitochondria could be due to an influence of the phospholipids either on the influx or efflux pathways. With a computerized data acquisition and evaluation system allowing a higher time resolution we found that the initial velocity of Ca^{2+} uptake was the same at 100 μM HePC as compared to controls (S. Lenzen, unpublished observation). A stimulation of the effux must therefore be assumed as the primary effect, while in an earlier study [23] we had concluded that the net efflux was due to a slower re-uptake in the course of mitochondrial Ca^{2+} cycling.

Differential effects of structural modifications on mitochondrial Ca^{2+} transport and membrane potential. In an attempt to find out whether the different activities of the phospholipids on Ca^{2+} transport and membrane potential followed a regularity corresponding to structural modifications or merely represent a random scattering of the data, we plotted the quotient (Ca^{2+} values divided by TPP^+ values) of the half-maximal effective concentrations (Q_1) against the quotient of the percent inhibition of uptake at 100 μM (Q_2), as obtained from the data in Table I. The data points follow roughly a hyperbola (solid line in Fig. 6) defined by $Q_1 \cdot Q_2 = 1$, a deviation from unity indicating a dissociation between the effect on Ca^{2+} uptake from the one on TPP^+ uptake. In the homologous series from HePC to HeP_6C there is a continuous decrease of $Q_1 \cdot Q_2$ from 1.25 to 1.04, indicating a non random distribution of the data in Table I. In conclusion, the mitochondrial membrane system responds to small changes in the molecular structure of the phospholipids, which precludes the assumption of a destructive mode of action.

The substances which show a remarkable deviation from the ideal curve (Fig. 6) are HePC, OMGPC and C_{16}-L-PAF, while, among other substances, LPC and HeP_6C and Zwittergent are very close to it. Incidentally, in tissue culture the latter show only acute cyto-

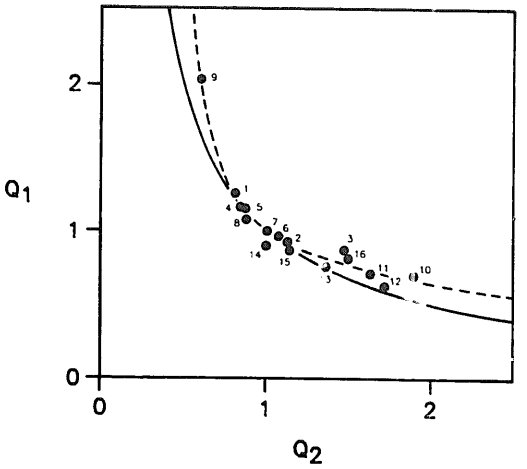

Fig. 6. Relation between the effects of choline lysophospholipids and analogues on mitochondrial Ca^{2+} uptake and on TPP^+ uptake. The quotients of the half-maximal effective concentrations of the substances (Q_1) as a measure of potency were plotted against the quotients of the percent reduction of uptake at 100 μM (Q_2; Ca^{2+} values divided by TPP^+ values for both quotients) as a measure of efficacy. An increasing potency for influence on Ca^{2+} transport ($Q_1 < 1$) was usually accompanied by an increasing inhibitory efficacy at 100 μM ($Q_2 > 1$), resulting in $Q_1 \cdot Q_2 = 1$ (solid line hyperbola branch) for most substances. The deviation of the curve fitted to all data points (dotted line) is mainly due to the following substances: HePC, HeP_3C, C_{16}-L-PAF, OMGPC and D,L-LPC. These substances therefore seem to have a more complex relation between their influence on Ca^{2+} uptake and TPP^+ uptake, irrespective of a preferential effect on TPP^+ uptake (e.g., OMGPC) or on Ca^{2+} uptake (e.g., HePC). The symbols are numbered as follows: 1, LPC; 2, LPDME; 3, C_{16}-L-PAF; 4, C_{16}-D-PAF; 5, C_{16}-L-lysoPAF; 6, C_{16}-D-lysoPAF; 7, TeMGPC; 8, HeMGPC; 9, OMGPC; 10, HePC; 11, HeP_3C; 12, HeP_4C; 13, HeP_6C; 14, OleylPC; 15, 3-(*N*,*N*-dimethyl, *N*-hexadecyl) ammoniopropanesulfonate (= Zwittergent 3-16); and 16, D,L-LPC.

toxicity (H. Eibl, unpublished data), while the former possess antineoplastic effects [59–61], which involve the induction of differentiation [61,62]. In the case of PAF this effect was observed at concentrations above those sufficient to saturate the high-affinity binding site (= PAF receptor) maximally [61] and will be due to a protein–lipid interaction in the plane of the membrane rather than to a receptor–ligand interaction. In a recent study with model membranes it was concluded that the antineoplastic effect of PAF was due to the same membrane perturbing effect as exerted by LPC [63]. Our results, however, indicate, that structural variations in lysophospholipid analogues do not only result in varying degrees of lytic potency, but can yield a more complex influence on biological membrane systems.

Physiological or pharmacological relevance in relation to membrane lysophospholipid content. A model for modulation of transmembrane ion transport by lysophospholipids without destructive effects on the lipid bilayer is the modulation of gramicidin channel conductance in artificial membranes in the presence of LPC [64]. A similar interaction between choline lyso-

phospholipids and the protein(s) constituting the Ca^{2+} uniport mechanism or Ca^{2+} releasing pore could be imagined. The molecular percentage of 5–10% (1 molecule LPC per 10 nm^2) in this study [64] is in the range of the 25 mol% as measured in the mitochondrial fractions at 100 μM of added lysophospholipid; in particular when keeping in mind that the half-maximal effective concentrations for most of the active compounds were less than 100 μM. In addition, when comparing these concentrations to those used by other authors, the supplementation of the test medium with spermine in the present experiments should be drawn into consideration. Spermine, which was included to mimic physiological conditions [30,42] antagonized lysophospholipid-induced Ca^{2+} release (see Fig. 2). Since the lysophospholipid content of natural membranes is usually around or less than 1 mol%, a physiological relevance of comparatively high amounts of lysophospholipids in membranes may be questioned. However, Upreti et al. [65] found in an analysis of the subcellular phospholipid composition of hepatoma cells that the mitochondria contained 9.6 mol% of LPC; an amount which in light of our results should have an impact on mitochondrial Ca^{2+} transport and energetics. When slowly metabolizable lysophospholipid analogues like HePC are administered as anticancer agents over periods of weeks, comparable molecular percentages are also not inconceivable. Lenting et al. [66] calculated the initial velocity of mitochondrial PLA_2-mediated hydrolysis of PC to be around 250 nmol/min per mg. Considering the values of our phospholipid uptake studies, the generation of an amount of LPC which would induce an efflux of Ca^{2+} from rat liver mitochondria would take 5–10 s. The generation of LPE by the mitochondrial PLA_2 which proceeds at a rate 2.5-fold that of LPC [66] would in our opinion not elicit functional consequences, if not through the concomitant generation of unsaturated fatty acids, some of which release Ca^{2+} from mitochondria [45].

Acknowledgements

The skilful technical assistance of R. Andag and D. Lischke is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, F.R.G.. I.R. was recipient of a Postdoctoral Research Fellowship from the Deutsche Forschungsgemeinschaft.

References

1 Carafoli, E. and Crompton M. (1978) in Calcium Transport and Cell Function (Scarpa, A. and Carafoli, E., eds.), pp. 269–284, New York Academy of Sciences, New York.
2 Bygrave, F.L. (1978) Biol. Rev. 53, 43–79.
3 Nicholls, D. and Akerman, K. (1982) Biochim. Biophys. Acta 683, 57–88.
4 Denton, R.M., McCormack, J.G. and Edgell, N.J. (1980) Biochem. J. 190, 107–117.
5 Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharmacol. 102, 1–72.
6 McCormack, J.G. and Denton, R.M. (1988) Biochem. Soc. Trans. 16, 523–527.
7 Brand, M.D. (1988) ISI Atlas of Science; Biochemistry 350–354.
8 Panfili, E., Sandri, G., Sottocasa, G.L., Lunazzi, G. and Liut, G. (1976) Nature 264, 185–186.
9 Jeng, A. and Shamoo, A. (1980) J. Biol. Chem. 255, 6897–6903.
10 Rottenberg, H. and Scarpa, A. (1974) Biochemistry 13, 4811–4817.
11 Crompton, M. (1985) Curr. Top. Membr. Transp. 25, 231–276.
12 Nedergaard, J.and Cannon, B. (1980) Acta Chem. Scand. B34, 149–151.
13 Rizzuto, R., Bernardi, P., Favaron, M. and Azzone, G.F. (1987) Biochem. J. 246, 271–277.
14 Beatrice, M., Stiers, D.L. and Pfeiffer, D.R. (1984) J. Biol. Chem. 259, 1279–1287.
15 Hunter, D. and Haworth, R. (1979) Arch. Biochem. Biophys. 195, 453–459.
16 Al-Nasser, I. and Crompton, M. (1986) Biochem. J. 329, 19–29.
17 Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. (1989) Trends Pharmacol. Sci. 10, 281–285.
18 Parce, J.W., Cunningham, C.C. and Waite, M. (1978) Biochemistry 17, 1634–1639.
19 Pfeiffer, D.R., Schmid, P.C., Beatrice M. and Schmid, H.H.O. (1979) J. Biol. Chem. 254, 11485–11494.
20 Beatrice, M., M.C., Palmer, J.W. and Pfeiffer D.R. (1980) J. Biol. Chem. 255, 8663–8671.
21 Dalton, S., Hughes, B.P. and Barritt, G.J. (1984) Biochem. J. 224, 423–430.
22 Waite, M., Van Deenen, L.L.M., Ruigrok, T.J.C. and Elbers, P.F. (1969) J. Lipid Res. 10, 599–608.
23 Lenzen, S., Görlich J.-K. and Rustenbeck, I. (1989) Biochim. Biophys. Acta 982, 140–146.
24 Kinnaird, A., Choy P.C. and Man, R. (1988) Lipids 23, 32–35.
25 Okayasu, T., Curtis, M.T. and Farber, J.L. (1985) Arch. Biochem. Biophys. 236, 638–645.
26 Henson, P.M. (1987) in New Horizons in Platelet-Activating Factor Research (Winslow, C.L. and Lee, M.L., eds.), pp. 3–10, John Wiley and Sons, Chichester.
27 Lenzen, S. and Panten, U. (1985) Anal. Biochem. 149, 301–308.
28 Lenzen, S., Schmidt, W. and Panten, U. (1985) J. Biol. Chem. 260, 12629–12634.
29 Becker, G.L., Fiskum, G. and Lehninger, A. (1980) J. Biol. Chem. 255, 9009–9012.
30 Lenzen, S., Hickethier, R. and Panten, U. (1986) J. Biol. Chem. 261, 16478–16483.
31 McKnight, G.S. (1977) Anal. Biochem. 78, 86–92.
32 Lenzen, S. and Panten, U. (1983) Anal. Biochem. 134, 56–59.
33 Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.
34 Rustenbeck, I., Trumner, A. and Lenzen, S. (1989) J. Planar Chromatogr. 2, 207–210.
35 Rustenbeck, I. and Lenzen, S. (1990) J. Chromatogr. 525, 85–91.
36 Hara, A. and Radin, N.S. (1978) Anal. Biochem. 90, 420–426.
37 Kolarovic, L. and Traitler, H. (1985) J. High Resolut. Chromatogr. Chromatogr. Commun. 8, 341–346.
38 Eibl, H. and Woolley, P. (1986) Chem. Phys. Lipids 41, 53–63.
39 Eibl, H. and Woolley, P. (1988) Chem. Phys. Lipids 47, 47–53.
40 Eibl, H. and Woolley, P. (1988) Chem. Phys. Lipids 47, 63–68.
41 Nicchitta, C.V. and Williamson, J.R. (1984) J. Biol. Chem. 259, 12978–12983.
42 Rottenberg, H. and Marbach, M. (1990) Biochim. Biophys. Acta 1016, 77–86.
43 Crompton, M., Capano, M. and Carafoli, E. (1976) Eur. J. Biochem. 69, 453–462.

44 Moore, C.L. (1971) Biochem. Biophys. Res. Commun. 42, 298–305.
45 Rustenbeck, I. and Lenzen, S. (1989) Biochim. Biophys. Acta 982, 147–155.
46 Eibl, H. (1983) in Membrane Fluidity in Biology (Aloia, R.C., ed.), pp. 217–236, Academic Press, New York.
47 Boggs, J.M. (1984) in Membrane Fluidity (Kates, M. and Manson, L.A., eds.), pp. 3–53, Plenum Press, New York.
48 Boggs, J.M. (1987) Biochim. Biophys. Acta 906, 353–404.
49 Tocanne, J.-F. and Teissié, J. (1990) Biochim. Biophys. Acta 1031, 111–142.
50 Vaughan, D.J. and Keough, K.M. (1974) FEBS Lett. 47, 158–161.
51 Hovius, R., Lambrechts, H., Nicolay, K. and de Kruiff, B. (1990) Biochim. Biophys. Acta 1021, 217–226.
52 Altin, J.G., Dieter, P. and Bygrave, F. (1987) Biochem. J. 245, 145–150.
53 Oishi, K., Raynor, R.L., Charp, P. and Kuo, J.F. (1988) J. Biol. Chem. 263, 6865–6871.
54 Hanahan, D.J. and Kumar, R. (1987) Prog. Lipid Res. 26, 1–28.
55 Godfroid, J.J. and Braquet, P. (1986) Trends Pharmacol. Sci. 7, 368–373.
56 Deamer, D.W. and Nichols, J.W. (1989) J. Membrane Biol. 107, 91–103.
57 Gutknecht, J. (1987) J. Bioenerg. Biomembr. 19, 427–442.
58 Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
59 Herrmann, D. and Neumann, H. (1986) J. Biol. Chem. 261, 7742–7747.
60 Unger, C., Damenz, W., Fleer, E.A.M., Kim, D., Breiser, P., Hilgard, P., Engel, J., Nagel, G. and Eibl, H. (1989) Acta Oncol 28, 213–217.
61 Kornecki, E. and Ehrlich, Y. (1988) Science 240, 1792–1794.
62 Hilgard, P., Harlemann, J.H., Voegeli, R., Maurer, H.R., Echarti, C. and Unger, C. (1989) J. Cancer Res. Clin. Oncol. 115, S 54.
63 Sawyer, D.B. and Andersen, O. (1989) Biochim. Biophys Acta 987, 129–132.
64 Sawyer, D.B., Koeppe, R.E. and Andersen, O. (1989) Biochemistry 28, 6571–6583.
65 Upreti, G., DeAntueno, R. and Wood, R. (1983) J. Natl. Cancer Inst. 70, 567–573.
66 Lenting, H., Nicolay, K. and Van den Bosch, H. (1988) Biochim. Biophys. Acta 958, 405–415.
67 Rottenberg, H. (1984) J. Membrane Biol. 81, 127–138.