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INVOLVEMENT OF PERIODIC DEACYLATION-ACYLATION CYCLES OF MITOCHONDRIAL PHOSPHOLIPIDS DURING Sr^{2+}-INDUCED OSCILLATORY ION TRANSPORT IN RAT LIVER MITOCHONDRIA

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Lysophosphatidylcholine and lysophosphatidylethanolamine levels were determined during Sr^{2+}-induced oscillating ion fluxes in mitochondria prelabelled in vivo with $^{32}P_i$. Periodic fluctuations of both lyso compounds were established with an increase at the stage of simultaneously monitored K^+ influx and a decrease at K^+ efflux. The periodic activations and inactivations of phospholipase were found to be associated with periodic changes in the incorporation rates of labelled polyunsaturated fatty acids with an apparent phase difference of 180°. Periodic deacylation-acylation cycles of phospholipids accompanying the periodic cycles of reversible ion accumulation and release are suggested to be involved in the trigger mechanism generating the permeability changes during oscillatory ion transport.

Introduction

Mitochondrial cation transport systems are involved in the maintenance of cellular Ca^{2+} levels [1]. A delicate balance between accumulation and release should be adjusted and the mechanism and regulation of these processes, especially the cation release, is actively investigated [2,3]. The involvement of mitochondrial phospholipids, in particular the liberation of fatty acids and lysophospholipids by mitochondrial phospholipase A_2, has been proposed to be associated with the regulation and dysregulation of mitochondrial functions [4,5]. For the recently described system of Sr^{2+}-induced oscillatory ion fluxes in rat liver mitochondria, the contribution of deacylation-acylation cycles of mitochondrial phospholipids to cyclic permeability changes of the mitochondrial membrane was considered [6]. Results on periodic fluctuations of lysophospholipids during phosphate-induced damped oscillations [7] were reported in a preliminary communication [8]. In the present study we have investigated sensitively the levels of lysophospholipids of phosphatidylcholine and phosphatidylethanolamine during Sr^{2+}-initiated cyclic ion fluxes by using mitochondria with ^{32}P-labelled phospholipids. As an index for the repair mechanism, to be assumed as acylation of mitochondrial lysophospholipids, incorporation of polyunsaturated fatty acids during periodic ion fluxes was measured. The phase relationships between phospholipids and ion fluxes are discussed in terms of the proposed mechanism of oscillatory ion transport and the regulation of cation release from mitochondria.

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Materials and Methods

Chemicals. [1-^{14}C]Linoleic acid (60 mCi/mmol) and [5,6,8,9,11,12,14,15(n)-^{3}H]arachidonic acid (135 Ci/mmol) were obtained from the Amersham International (U.K.) and $NaH_2{}^{32}PO_4$ from Isocommerz (Dresden, G.D.R.). Silicagel H for thin-layer chromatography was a product from E. Merck (Darmstadt, F.R.G.) and DEAE-cellulose for column chromatography came from Serva (Heidelberg, F.R.G.). Organic solvents were purified by standard procedures, dried and freshly redistilled. 50 mg butylated hydroxytoluene/litre solvent were added as antioxidant [9]. All other chemicals were of analytical grade.

In vivo labelling of phospholipids and isolation of mitochondria. Femal rats weighing 200–250 g were injected intraperitoneally with about 2 mCi $NaH_2{}^{32}PO_4$ dissolved in isotonic saline solution. After 20–30 h the fasted rats were killed and the liver mitochondria were isolated in a solution containing 300 mM sucrose/0.5 mM EDTA/5 mM Tris-HCl (pH 7.5). They were washed with the same medium omitting EDTA, suspended in sucrose/Tris-HCl and kept in ice.

Incubations and induction of oscillatory ion transport. Mitochondrial suspension corresponding to 5 mg protein/ml was transferred to 1 ml incubation mixture containing 20 mM sucrose/1 mM KCl/5 mM succinate-Tris (pH 7.4). K^+ and Sr^{2+} activities were measured with ion sensitive electrodes [10] and the oxygen concentration with a closed Clark-type electrode [11]. All measurements were done in an open, fast-stirred cell at room temperature (23°C). Oscillations were initiated by addition of valinomycin (6 ng/mg protein) and Sr^{2+} (320 μmol/l) to the incubation mixture. At selected time intervals, marked on the registration curve for K^+ activities, samples for phospholipid analysis were taken into acidified chloroform/methanol mixtures under intensive stirring.

Incorporation of labelled fatty acids. 2.5 μCi of both labelled fatty acids, dissolved in a small volume of freshly distilled ethanol, were added to the complete incubation medium immediately before oscillations were induced. Samples for the measurements of fatty acid incorporation in mitochondrial phospholipids (not prelabelled by $^{32}P_i$) were quenched as above.

Extraction, separation and identification of lipids. Lipids were extracted essentially according to the Folch procedure [12], but using acid $CHCl_3$/CH_3OH mixtures (21 ml $CHCl_3/CH_3OH$ (2:1) and 50 μl 1 M HCl per ml sample) and 0.2 vol. 10 mM HCl to obtain phase separation. HCl was necessary in order to avoid selective loss of lysophospholipids [13]. After washing the $CHCl_3$ phase with a mixture containing $CHCl_3$/CH_3OH/0.01 M HCl (3:48:47, v/v) [14], it was filtered through a small plug of cotton wool to remove proteins and taken to dryness in a rotary evaporator under a stream of nitrogen. In the experiments with ^{32}P-labelled rat liver mitochondria, phospholipids were first separated by DEAE-cellulose column chromatography in non-acidic and acidic fractions [15]. The non-acidic phospholipids, consisting of phosphatidylcholine, phosphatidylethanolamine, their corresponding lyso compounds and sphingomyelin, were completely eluted with $CHCl_3/CH_3OH$ (2:1), while the acidic ones (phosphatidylinositol, cardiolipin, phosphatidylglycerol and phosphatidic acid) were recovered in a second fraction. Further separation and identification was done by two-dimensional TLC [16]. The individual phospholipids were localized by iodine vapour and identified with suitable standards and with specific staining [45]. Radioactivity was detected on plates using a thin-layer scanner (Professor Berthold, Wildbad, F.R.G.). The respective spots were scraped off, extracted repeatedly with a mixture of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2) [17] and counted for ^{32}P by Čerenkov counting in a scintillation spectrometer (Intertechnique, France).

The incorporation of labelled fatty acids in mitochondrial phospholipids was followed by the same extraction procedure and by thin-layer chromatography in the system of Wagner et al. [18] using microplates. For good separation of highly labelled fatty acids from phospholipids, repeated washing of plates with acetone and chloroform before the main run was inevitable. After chromatographic separation and identification, the individual fractions were scraped off, transferred to the scintillation fluid [19] and counted in ^{3}H- and ^{14}C-channels for radioactivity.

Protein and phosphate determination. Protein was determined by the biuret method [20] and phosphorus according to Rouser et al. [16].

Results

Sr^{2+}-induced oscillatory ion fluxes and periodic fluctuations of mitochondrial lysophosphatidylcholine and lysophosphatidylethanolamine

Under appropriate conditions, by the addition of Sr^{2+} (or Ca^{2+}), rat liver mitochondria can be induced to undergo practically undamped periodic changes of ion fluxes, volumes, redox ratios, respiratory rates and membrane potential [6,21, 22,23]. The Sr^{2+}-initiated self-oscillations are governed mainly by the electrochemical potentials and the membrane permeability as the basic control parameters. Phase relationships between Sr^{2+} (Ca^{2+}), K^+ and H^+ were reported earlier [21]. In the following, for simplicity only, the K^+ activities are used as reference for the phospholipid changes.

Since mitochondrial lysophospholipid contents are rather low [24], an assessment of their fluctuations by the usual analysis of phospholipid-phosphorus proved unsuccessfully. Therefore levels of individual phospholipid classes were determined with high sensitivity using mitochondria prelabelled in vivo with [^{32}P]phosphate. No fluctuations in the sum of non-acidic phospholipids as in phosphatidylcholine, phosphatidylethanolamine and the constantly present sphingomyelin contamination [25] exceeding methodical errors could be detected during three or four oscillation periods or after incubation for the same time in a non-oscillatory state (Table I). After initiation of oscillatory ion fluxes, only lyso forms of phosphatidylcholine and phosphatidylethanolamine exhibit characteristic variations under oscillating conditions (Table I). The results depicted in Fig. 1 prove the periodic nature of these lysophospholipid changes. If each oscillatory cycle is devided according to the simultaneously registered extramitochondrial K^+ activity into the phases of K^+ influx and K^+ efflux, both lyso compounds show increases during the influx and decreases during the efflux. To account for the lysophospholipid accumulation, a mitochondrial phospholipase must be activated during ion influx. To explain the dissipation of lysophospholipids during ion efflux a deactivation of phospholipase and an activation of the pathway for lysocompound removal must be included. According to its known properties [26], mitochondrial phospholipase should be activated by Sr^{2+} (Ca^{2+}) and by the prevailing

TABLE I

DISTRIBUTION OF NON-ACIDIC PHOSPHOLIPIDS IN ^{32}P-LABELLED RAT LIVER MITOCHONDRIA

Lipid phosphorus determinations were from three different mitochondrial preparations without incubation. Total contents of phospholipids were 187.6, 191.1 and 184.1 nmol lipid phosphorus/mg protein, respectively. Calculations from phosphate determinations. Means ± S.D. ($n = 9$). Specific activities were determined from one experiment under oscillating conditions from one mitochondrial preparation. Calculations from phosphate determinations subsequent to ^{32}P counting. The standard error ($n = 9$) represents incubation of labelled mitochondria as described in methods without induction of oscillations by addition of valinomycin and Sr^{2+}. Calculations from radioactivities; standard error is expressed as percentage of the arithmetic mean. The standard error ($n = 21$) represents incubation of labelled mitochondria under oscillating conditions as described in methods. Calculations and standard error as in the $n = 9$ case.

Phospholipid	Lipid phosphorus		Specific activities (cpm/nmol lipid phosphorus) ($n = 12$)	Standard error ($n = 9$)	Standard error ($n = 21$)
	(% of total)	(nmol/mg protein)			
Phosphatidylcholine	39.6 ± 2.3	74.3 ± 4.3	135 ± 4	3.7	4.4
Phosphatidylethanolamine	30.7 ± 2.1	57.6 ± 4.0	79 ± 5	4.6	5.4
Lysophosphatidylcholine	1.0 ± 0.3	1.9 ± 0.6	138 ± 13	6.9	(23.3)
Lysophosphatidylethanolamine	0.7 ± 0.3	1.3 ± 0.5	81 ± 11	7.5	(27.0)
Sphingomyelin	1.9 ± 0.3	3.6 ± 0.5	150 ± 6	6.1	5.8

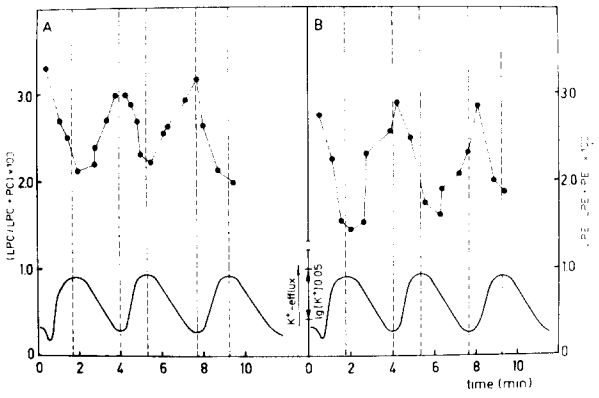

Fig. 1. Periodic changes of mitochondrial lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) levels during oscillatory ion transport. Conditions as reported under Materials and Methods. Addition of valinomycin (6 ng/mg protein) at zero time and addition of Sr^{2+} (320 μmol/l) after 30 s. Phospholipids were determined by their radioactivities.

alkaline matrix pH at the stage of ion influx and deactivated during the stage of ion efflux. Both, phosphatidylethanolamine and phosphatidylcholine were attacked similarly and small changes in the acid phospholipid classes (diphosphatidylglycerol, phosphatidic acid, phosphatidylinositol) were observed, too (data not shown).

Although most of the mitochondrial phospholipase is associated with the outer membrane [27], a smaller, but significant portion localized in the inner membrane [28] is compatible with the suggested mode of action.

The data given in Table II document that rates and directons of lysophospholipid changes are different between the two phases of K^+ fluxes at high levels of probability. The estimates of the periodic lysophospholipid fluctuations yield an apparent lysophospholipid turnover of 1.5 nmol/mg protein during one cycle of ion movement. Since calculations were based on linear extrapolations and imply a mutual exclusion of phospholipase action and lysophospholipid dissipation, this value represents only the lower limit for the magnitude of lysophospholipid fluctuations [27].

Incorporation of polyunsaturated fatty acids into mitochondrial phospholipids during oscillatory ion fluxes

In view of the virtual absence of lysophospholipase in rat liver mitochondria [4] and the constancy of the fraction of non-acidic phospholipids during 3–5 cycles of oscillation, any contribution of this pathway in the dissipation of lysophospholipids appears unlikely. To account for the reversible fluctuations of lysophospholipids, the acylation pathway, well established in different mitochondrial systems [24], seems much more probable. In respect to the established posi-

TABLE II

RATES AND AMPLITUDES OF LYSOPHOSPHOLIPID FLUCTUATIONS DURING OSCILLATORY ION TRANSPORT

Data were calculated from three different experiments. Standard errors for LPC and LPE are adopted from sphingomyelin, determined in the corresponding samples. Calculations of lysophospholipids are based on the data shown in Table I. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

Phase and period of K^+ fluxes	Rate and direction of change (nmol/mg protein per min)		
	LPC	LPE	Sum (LPC + LPE)
Efflux 1	−0.57 ± 0.04	−0.52 ± 0.04	−1.09 ± 0.08
Influx 1	+0.31 ± 0.02	+0.44 ± 0.04	+0.75 ± 0.06
Efflux 2	−0.42 ± 0.03	−0.49 ± 0.02	−0.91 ± 0.05
Influx 2	+0.33 ± 0.02	+0.30 ± 0.03	+0.63 ± 0.05
Efflux 3	−0.57 ± 0.05	−0.53 ± 0.05	−1.10 ± 0.10
Influx 3	+0.37 ± 0.03	+0.32 ± 0.03	+0.69 ± 0.06
Means of efflux	−0.52 ± 0.04	−0.51 ± 0.05	−1.03 ± 0.09
Means of influx	+0.33 ± 0.03	+0.35 ± 0.04	+0.68 ± 0.07
Total change per cycle mean efflux	−0.73 ± 0.06	−0.71 ± 0.07	−1.44 ± 0.13
Mean influx	+0.78 ± 0.07	+0.77 ± 0.08	+1.55 ± 0.15

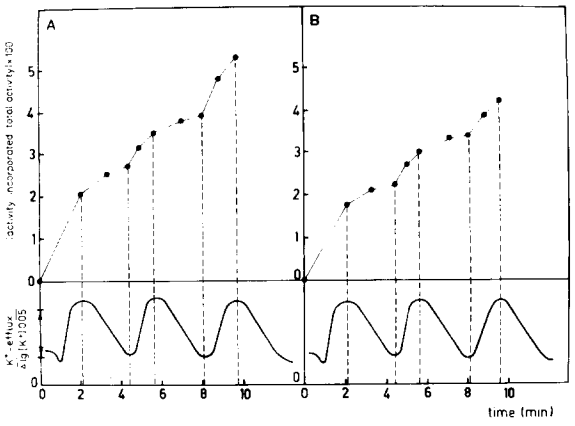

Fig. 2. Incorporation of polyunsaturated fatty acids into mitochondrial phospholipids during oscillatory ion transport. Conditions as described under Materials and Methods. Data from one representive experiment. Calculations were done referring the incorporated activities to the total activities (incorporated + free fatty acids). Zero values, obtained by taking samples from the incubation mixture immediately after fatty acid addition, were substracted. They correspond to apparent incorporations between 0.3–0.7%, probably due to unspecific fatty acid binding to the phospholipids. A, arachidonic acid; B, linoleic acid.

tional specificity of phospholipase A_2 [24] and the fatty acid distribution in mitochondrial phospholipids [29], the incorporation of polyunsaturated fatty acids rather than oleic acid or saturated ones should give a reliable index for the acylation pathway. The data depicted in Fig. 2 show that both linoleic and arachidonic acid were incorporated into mitochondrial phospholipids to increasing extents throughout all phases of the oscillatory cycle. Superficially, the progress of incorporation might be fitted by a linear function. The periodic unsteadyness within the curve is, however, a strong argument for a fluctuating incorporation rate. Upon applying the same approach as used for the evaluation of lysophospholipids, an about 3-fold higher incorporation rate during ion efflux compared to the influx phase can be stated, as shown in Table IIIA. The higher rate of incorporation during ion efflux is in good correspondence to the decline of lysophospholipids observed at the same interval of the cycle. The incorporation of both fatty acids (18:2 and 20:4) into total phospholipids was very similar. Table IIIB shows that the greater part of the incorporated fatty acids was recovered in the

TABLE III

RATES AND DISTRIBUTION OF POLYUNSATURATED FATTY ACID INCORPORATION INTO MITOCHONDRIAL PHOSPHOLIPIDS DURING OSCILLATING ION TRANSPORT

Calculations from four different experiments. 'Rest' denotes to activities recovered in all other fractions, in part identified as cardiolipin and phosphatidic acid.

A Phase and period	Rate of incorporation (activity incorporated/activity total per min) (×100)	
	[^{3}H]Arachidonic acid	[^{14}C]Linoleic acid
Efflux 1	1.02±0.04	0.86±0.10
Influx 1	0.38±0.09	0.33±0.20
Efflux 2	1.10±0.08	0.77±0.09
Influx 2	0.27±0.07	0.26±0.10
Efflux 3	1.04±0.10	1.03±0.07
Influx 3	0.31±0.12	0.35±0.08
Mean of efflux	1.05±0.10	0.89±0.09
Mean of influx	0.32±0.11	0.31±0.13
B	Distribution between phospholipids means and range of total incorporation after three periods (%)	
	[^{3}H]Arachidonic acid	[^{14}C]Linoleic acid
Phosphatidylcholine fraction	52 (41–63)	56 (47–64)
Phosphatidylethanolamine fraction	20 (13–28)	21 (12–25)
'Rest'	20 (9–36)	23 (11–42)

phosphatidylcholine fraction and smaller amounts in the phosphatidylethanolamine and 'rest' fractions, as well. It should be mentioned, however, that phosphatidylcholine in the incorporation experiments may be contaminated to some extent by phosphatidylinositol due to the separation procedure used in this case.

An evaluation of the incorporation in terms of concentrations is, however, not possible, because no data on pool sizes and distributions of the label are available. As the pools of both fatty acids may be assumed to change nearly parallel to lysophospholipids above some basal level, it seems reasonable to conclude that the incorporation data reflect, at least qualitatively, different rates of the

acylation pathway. Recent data, showing increased mitochondrial content of polyunsaturated fatty acids under conditions promoting Ca^{2+} efflux, seem to support this suggestion [4]. While for phospholipase A_2 and lysophospholipid acyltransferase, a partial association with the inner mitochondrial membrane is well documented [27,30], differently localized systems performing fatty acid activation have to be considered [31]. Under certain conditions the intramitochondrial medium-chain acyl-CoA-synthetase may be assumed to participate in the activation of long-chain fatty acids, too [32]. The observed fluctuating accumulation of water-soluble products from labelled fatty acids, which proceeds in the absence of extramitochondrial cofactors such as ATP, CoASH and carnitine in the above experiments (data not shown), provides additional arguments for the presumed pathway.

Phase relationships between lysophospholipids, fatty acid incorporation and ion fluxes

More information on phase relationships should be obtained by resolving influx and efflux stages into smaller time intervals. As may be visualized from Fig. 3A, higher incorporation rates are observed at higher rates of K^+ efflux and lower incorporation rates at higher rates of K^+ influx, whereas medium incorporation rates seem to be connected with lower rates of K^+ fluxes in both directions. From Fig. 3B it appears that elevated rates of lysophospholipid formation correspond to high K^+ influxes and high rates of their reduction to high K^+ effluxes, while both changes seem to be in the middle range at lower rates of K^+ movements. Thus the following qualitative conclusions can be drawn:

(i) Phospholipase is most active at maximum K^+ influx and has its smallest activity at maximum K^+ efflux.

(ii) The reverse applies for the acylation with minimum rate at maximum influx and highest rate at maximum efflux.

(iii) Phospholipase and acylation activities exhibit a phase shift of about 180° relative to each other.

Discussion

Mitochondrial oscillatory systems of ion transport are interesting, not only in respect to the generation and maintenance of the oscillatory state, but also more generally as tools for the elucidation of the mechanism and the regulation of ion efflux. The obvious advantage of the systems of oscillatory ion fluxes lies in its inherent reversible nature, allowing the investigation of periodically repeated transport cycles of ion uptake and release. Periodic reversible changes of lysophospholipid levels occurring concomitantly with the periodic cation fluxes were proved in the present investigations to

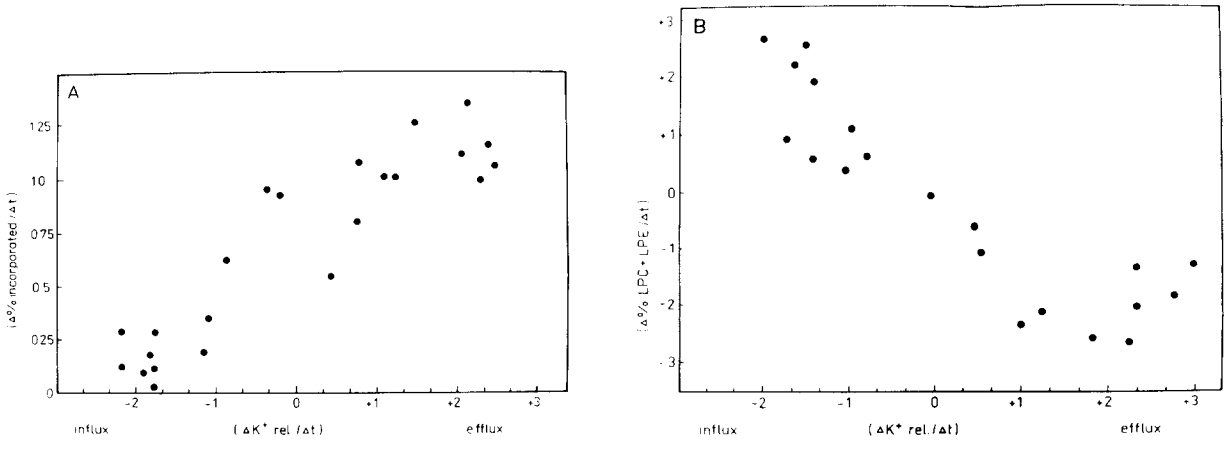

Fig. 3. Phase relations between lysophospholipids, fatty acid incorporation and K^+ fluxes. A: Difference quotients of arachidonic acid incorporation (results from four different experiments). B: Difference quotients of lysophosphatidylcholine and lysophosphatidylethanolamine changes (results from three different experiments).

be mediated by periodic activations of mitochondrial phospholipase on the one and different activities of the lysophospholipid reacylation system on the other hand. The simplified hypothesis on the involvement of deacylation-acylation reactions in cyclic ion transports durig self-oscillations may be summarized as follows:

(i) Ca^{2+} (Sr^{2+})-accumulation itself leads to an increase in phospholipid splitting by phospholipase activation with a concurrent suppression of the repair process.

(ii) The membrane permeability increases parallel to the accumulation of lysophospholipids and fatty acids, until some drop in the membrane potential cation efflux has been initiated.

(iii) Phospholipase is deactivated and the activation of the repair process by the reacylation pathway takes place, until an almost intact membrane is re-established. After restoration of the membrane potential, the next period of ion acccumulation starts (i).

While the trigger mechanism for phospholipase appears quite obvious, the regulation of the acylation pathway is hardly understood. Possible masking of phospholipase by the prevalence of reacylation reactions has been discussed in the past [5,33]. Fatty acid incorporation does apparently not become zero, even at the phase of most activated phospholipase in our system. Because lysophospholipids and fatty acids may be presumed to change in a parallel manner, they do not represent suitable candidates for the differential control of acylation at ion influx and efflux. Intramitochondrial ATP and/or its replenishment via substrate phosphorylation, which must proceed faster at higher respiration rates during the phase of ion efflux, may be discussed in this context, although corresponding fluctuations of ATP acting as an inhibitor of cation efflux could not be detected until now [34]. An explanation may be further attempted by invoking changes of mitochondrial Mg^{2+}, which is essential for both processes, the activation of fatty acids and the acylation of lysophospholipids [30,31]. Preliminary observations pointing to periodic Mg^{2+} movements during oscillations (Markefski, M., unpublished data) are in line with the suggestion of Siliprandi et al. [33,35] that Mg^{2+} losses may precede Ca^{2+} losses from mitochondria.

The mechanism of the permeability changes, presumably connected with different levels of lysophospholipids and/or fatty acids, cannot be explained on the basis of the present data. Both lysophospholipids and fatty acids may themselves act in the modulation of the membrane permeability, as suggested earlier [36,37]. The observed concentration dependencies might be accomplished by cooperativities creating specific threshold phenomena either in the whole plane or at restricted arrays of the membrane [36,38]. Interactions between lysophospholipids or fatty acids and membrane carrier proteins [36] may change the permeability properties of the membrane, too. It must be stressed, however, that the available results do not permit the conclusion of a direct involvement of phospholipase products in the generation of permeability changes.

Recent investigations point to an involvement of lipid peroxidations in the mechanism of oscillating ion fluxes [39]. During the process of peroxidation, ionophoretically active species may be generated [41], which are removed by the phospholipase reacylation pathway. On the other hand, the possibility of mutual activation of phospholipase by the peroxidation reactions also remains open [40]. In the light of recent findings, higher rates of oxygen radical formation [42,43] at a higher degree of reduction of certain mitochondrial redox couples may also provide a rationale in connecting the influence of hydrogen pressure and the ability of mitochondria to retain or release Ca^{2+} [44].

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