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Stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase activity in human erythrocyte membranes by synthetic lysophosphatidic acids and lysophosphatidylcholines. Effects of chain length and degree of unsaturation of the fatty acid groups

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Synthetic lysophosphatidic acids and lysophosphatidylcholines were examined for their effects on the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes. Addition of these compounds to erythrocyte ghosts caused significant changes in ATPase activity. The degree of unsaturation and the length of the *sn*-1 long chain hydrocarbon moiety were both contributing factors. All lysophosphatidic acids tested stimulated $(Ca^{2+} + Mg^{2+})$-ATPase activity. Of the species having a saturated acyl group, the most active was the myristoyl derivative. Linoleoyllysophosphatidic acid was the most potent of the unsaturated species. Saturated lysophosphatidylcholines with a short chain fatty acyl group (C_{10} to C_{14}) exhibited only a moderate stimulatory activity, whereas the longer chain homologues, i.e., C_{16} and C_{18} were inhibitory at high concentrations. On the other hand, unsaturated lysophosphatidylcholines had stimulatory activities comparable to the unsaturated lysophosphatidic acids. These results suggest that the acidic moiety of lysophosphatidic acid is not an important structural determinant for expressing ATPase stimulatory activity in ghosts. Rather the nature of the hydrocarbon chain as well as the lyso structure of these compounds appear most critical under these conditions. The stimulatory effects of lysophosphatidic acids or lysophosphatidylcholines were additive to that induced with calmodulin, suggesting that these lysophospholipids affect the $(Ca^{2+} + Mg^{2+})$-ATPase by a mechanism which is different from that seen with calmodulin.

Introduction

It is now well-accepted that the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes can be significantly influenced by the addition of various lipids to the assay medium [1]. As an example, long-chain fatty acids have been shown to mimic the stimulatory effect of calmodulin on this $(Ca^{2+} + Mg^{2+})$-ATPase [2–4]. Recently, this laboratory showed that egg yolk phosphatidylcholine-derived lysophosphatidic acid behaved like calmodulin on this $(Ca^{2+} + Mg^{2+})$-ATPase, whereas lysophosphatidylcholine (lysoPC) derived from egg yolk phosphatidylcholine exhibited inhibitory activity [5]. However, there were unanswered questions regarding the possible influence of the chain length and degree of unsaturation in the hydrocarbon moieties of these

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Abbreviation LysoPC, lysophosphatidylcholine.

lysophospholipids on the ATPase activity, since these lipids were really mixtures of lysophospholipids having various kinds of hydrocarbon chains. Hence, the current study was designed to explore the effect of several synthetic lysophosphatidic acids and lysoPCs, with well-defined chain lengths and degree of unsaturation upon the human erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPase. The data presented here would support the importance of the physical state of these lipids, rather than their charge, as a determinant of stimulatory activity.

Materials and Methods

Various synthetic lysoPCs (1-acyl-2-lyso-*sn*-glycero-3-phosphocholines) were obtained from the following sources: 1-decanoyl(10 : 0), lauroyl(12 : 0) and myristoyl(14 : 0)-lysoPC (Sigma, St. Louis, MO, U.S.A.); 1-palmitoyl(16 : 0), stearoyl(18 : 0) and oleoyl(18 : 1)-lysoPC (Avanti Polar Lipids, Birmingham, AL, U.S.A.) and 1-linoleoyl(18 : 2)-lysoPC (P-L Biochemicals, Inc., Milwaukee, WI, U.S.A.). 1-Linolenoyl(18 : 3) and arachidonyl(20 : 4)-lysoPC were prepared by the treatment of synthetic dilinolenoyl- and diarachidonylphosphatidylcholine (Avanti) with phospholipase A_2 as described earlier [6] with the exception that all organic solvents used in this study contained 0.001% 2,6-di-*tert*-butyl-4-methylphenol. 1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine was a product of Bachem, Bubendorf, Switzerland.

The 18 : 1- and 18 : 2-lysophosphatidic acids were purchased from P-L Biochemicals. Other lysophosphatidic acids and 1-*O* – hexadecyl-2-lyso-*sn*-glycero-3-phosphate were prepared by hydrolysis of the corresponding lysoPCs and 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine, respectively, with phospholipase D (*Streptomyces chromofuscus*; Calbiochem-Behring, La Jolla, CA, U.S.A.) according to the procedures described by Imamura and Horiuti [7]. Organic solvents used for the preparation of polyunsaturated lysophosphatidic acids contained 0.001% 2,6-di-*tert*-butyl-4-methylphenol.

Each lysophospholipid used in this study migrated as a single spot on silica-gel 60 thin-layer chromatography plates in a solvent system of chloroform/methanol/water (65 : 35 : 6, v/v).

Erythrocyte membrane preparation. Blood was obtained fresh from healthy adult donors, or obtained as outdated units from the Medical Center Hospital blood bank. The plasma and buffy coat were removed by centrifugation at $1500 \times g$ for 8 min at 4°C and the erythrocyte pellet was washed with 0.155 M NaCl and then lysed with 0.1% saponin as previously described [8], with the exception that 2 mM EGTA was present during lysis. The lysed erythrocytes were washed free of hemoglobin with 0 155 M NaCl/1 mM histidine (pH 7.6) on a Millipore Pellicon Cassette System using HVLP microporous filters. The erythrocyte membranes were recovered by centrifugation at $27\,500 \times g$ at 4°C for 1 h. The pellet was resuspended in 0.155 M NaCl, to a concentration of 4 mg/ml membrane protein, and the suspension frozen at −80°C for later use. Protein was determined by the method of Lowry et al. [9] using sodium dodecyl sulfate to solubilize the membranes.

Assay of ($Ca^{2+} + Mg^{2+}$)-ATPase. The ATPase activity was assayed as described elsewhere [8]. Essentially, 50 μl of the suspension of erythrocyte membrane (2 mg protein/ml in 0.155 M NaCl) were added to glass tubes on ice. To these suspensions was added 0.5 ml of the ATPase cocktail in which various concentrations of the lysophospholipids were dispersed by a brief sonication with a Heat Systems-Ultrasonics sonicator (Model W = 375). The ATPase cocktail comprised 86 mM histidine/4.3 mM $MgCl_2$/39.6 mM KCl/60 μM $CaCl_2$/3 mM ATP (pH 7.6). Final concentration of Ca^{2+} in the incubation mixture was 54.5 μM. The reaction was started by placing the tubes in a water-bath at 44°C immediately after addition of the ATPase cocktail. After 1 h incubation, the reaction was stopped by addition of 1.4 ml 0.5% sodium dodecyl sulfate. Controls for calcium-independent ATP hydrolysis (Mg^{2+}-ATPase and ($Na^+ + K^+$)-ATPase) were included; these were assayed in the ATPase cocktail mixture in the presence of 2 mM EGTA instead of 54.5 μM Ca^{2+}. Phosphate was measured by the methods of Fiske and SubbaRow [10]. When human erythrocyte calmodulin was included in the assay, it was added as an aliquot of stock solution (2 μg/ml in 0.9% saline). Calmodulin was a gift from David Nelson.

Results

Nine species of synthetic lysophosphatidic acids were tested for their effects on ATPase activities in human erythrocyte membranes. All lysophosphatidic acids tested here activated ($Ca^{2+} + Mg^{2+}$)-ATPase in a dose-dependent manner, but to varying degrees as shown in Fig. 1. Among the saturated species, 14 : 0-lysophosphatidic acid was the most active; it evoked a nearly 2.5-fold increase in the ATPase activity at a concentration of $1.6 \cdot 10^{-4}$ M. Decreasing or increasing chain length in the fatty acyl group from 14 : 0 resulted in a progressive reduction of the stimulating activity, although the effect of 12 : 0-lysophosphatidic acid was more potent than that of 16 : 0-lysophosphatidic acid. In a series of lysophosphatidic acids bearing a C-18 fatty acyl group only, the order of potency in their stimulatory effect was as follows: 18 : 2 > 18 : 1 ≧ 18 : 3 > 18 : 0. Lysophosphatidic acid having an arachidonyl residue was also active, but its stimulatory activity was almost equal to the 18 : 3-analogue.

Calcium-independent ATPase activity in the erythrocyte membrane was affected to a much lesser extent by the addition of various lysophosphatidic acids as compared with ($Ca^{2+} + Mg^{2+}$)-ATPase activity. the highest level of 16 : 0-lysophosphatidic acid produced a significant inhibition of the calcium-independent ATPase activity as did the 20 : 4 and 18 : 3-lysophosphatidic acids, while other lysophosphatidic acids had a weak stimulating effect (Fig. 2).

Next, a homologous series of lysoPCs were examined to assess the relative importance of the headgroup and the hydrophobic residue in modulating the ATPase activities. Figs. 3 and 4 show typical results for the effects of three different levels of synthetic lysoPCs on ($Ca^{2+} + Mg^{2+}$)-ATPase and calcium-independent ATPases, respectively. Unexpectedly, unsaturated species of lysoPCs stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase, with the 18 : 2-derivative exhibiting most activity. The dose-dependency of the stimulatory effect of the lysoPCs was more complex than observed with the lysophosphatidic acids. At low concentrations, unsaturated lysoPCs elicited a moderate activation of the ATPase, whereas high levels of the unsaturated lysoPCs, except for the 18 : 2 and 20 : 4-species gave no further activation of the enzyme; in some instances high concentrations of 18 : 1 and 18 : 3-lysoPCs caused less activation than lower concentrations (Fig. 3).

When the effect of lysoPCs with a saturated fatty acyl group was compared, a different but equally complex pattern was observed. Among the saturated lysoPCs, 14 : 0-lysoPC was the most potent stimulant at low concentrations. However, at high concentration, lysoPCs with a chain length longer than C_{12} suppressed the activity of the ($Ca^{2+} + Mg^{2+}$)-ATPase in the following order: 18 : 0 > 16 : 0 > 14 : 0. Such biphasic action was not

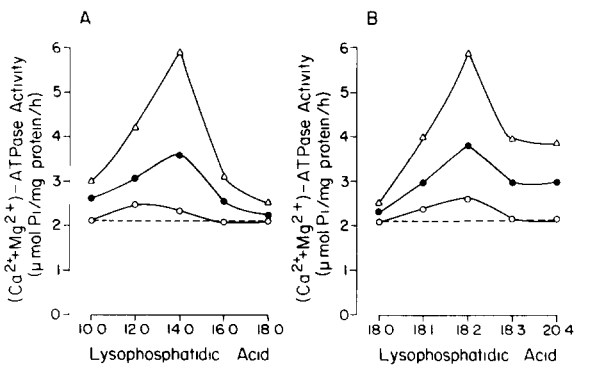

Fig. 1 Effects of various lysophosphatidic acids on ($Ca^{2+} + Mg^{2+}$)-ATPase activity in human erythrocyte ghosts. Influence of chain length (A) and degree of unsaturation (B) on the activity. Values are the means of three determinations. Standard deviations are below 4%. The doses of lysophosphatidic acids are as follows: ○———○, $6.6 \cdot 10^{-6}$ M; ●———●, $3.3 \cdot 10^{-5}$ M and △———△, $1.6 \cdot 10^{-4}$ M. The dotted line shows the control level of the activity.

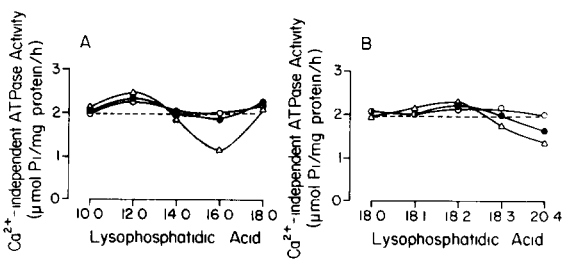

Fig. 2 Effects of various lysophosphatidic acids on calcium-independent ATPase activity in human erythrocyte ghosts. Influence of chain length (A) and degree of unsaturation (B) on the activity. Values are the means of three determinations. Standard deviations are below 3%. See legend to Fig. 1 for symbols.

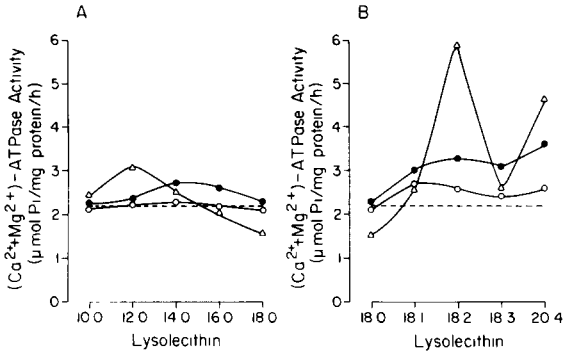

Fig 3 Effects of various lysoPCs on $(Ca^{+} + Mg^{2+})$-ATPase activity in human erythrocyte ghosts. Influence of chain length (A) and degree of unsaturation (B) on the activity Values are the means of three determinations Standard deviations are below 5%. The doses of lysoPCs are as follows· ○——○, $6.6 \cdot 10^{-6}$ M; ●——●, $3.3 \cdot 10^{-5}$ M and △——△, $1.6 \cdot 10^{-4}$ M The dotted line shows the control level of the activity

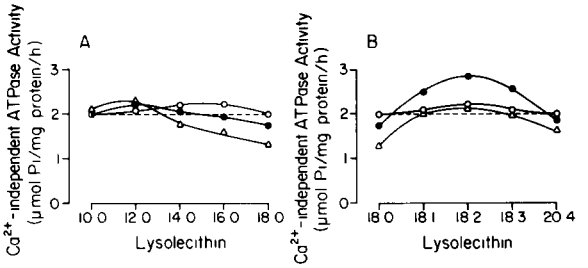

Fig. 4 Effects of various lysoPCs on calcium-independent ATPase activity in human erythrocyte ghosts. Influence of chain length (A) and degree of unsaturation (B) on the activity Values are the means of three determinations. Standard deviations are below 3%. See legend to Fig 3 for symbols

seen in the case of the lysophosphatidic species.

The calcium-independent ATPase activity was not significantly reduced at concentrations less than $1 \cdot 10^{-4}$ M. however, higher levels of 14 : 0, 16 : 0 and 18 : 0-lysoPCs inhibited this ATPase activity; the order of their potencies was the same as that for their inhibitory effects toward the $(Ca^{2+} + Mg^{2+})$-ATPase.

A lysophosphatidic acid with an alkyl ether in place of a carboxylic acid ester, that is, a 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphate, also stimulated the $(Ca^{2+} + Mg^{2+})$-ATPase to 167% of the control value at $1.6 \cdot 10^{-4}$ M. The corresponding alkyl ether lysoPC (1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine) behaved as a weak stimulant of the $(Ca^{2+} + Mg^{2+})$-ATPase at low levels, whereas it had an inhibitory effect on the $(Ca^{2+} + Mg^{2+})$-ATPase at high concentration (Table I).

The stimulatory activities of the lysophosphatidic acids and lysoPCs were additive to that elicited with calmodulin. Table II shows a typical example of experiments performed with 18 : 2-lysophosphatidic acid, 18 : 2-lysoPC and calmodu-

TABLE I

EFFECTS OF ALKYL ETHER TYPE LYSOPHOSPHOLIPIDS ON THE ATPase ACTIVITIES IN HUMAN ERYTHROCYTE GHOSTS

The assays were done as described in Materials and Methods Values are the means ± S.D of three determinations

Phospholipid added	Concn (M)	$(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol P_i/mg protein per h)	Calcium-independent activity (μmol P_i/mg protein per h)
Control	–	2.23 ± 0.06	1.96 ± 0 03
1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phosphate	$6.6\ 10^{-6}$	2 29 ± 0.04	2.00 ± 0 02
	$3.3 \cdot 10^{-5}$	2 54 ± 0 04	2 05 ± 0.03
	$1.6 \cdot 10^{-4}$	3 73 ± 0 08	1 78 ± 0.05
1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine	$6.6\ 10^{-6}$	2.14 ± 0.06	2.02 ± 0.04
	$3.3\ 10^{-5}$	1.90 ± 0.05	1 79 ± 0.04
	$1\ 6 \cdot 10^{-4}$	1.52 ± 0.04	1 56 ± 0.09

TABLE II

ADDITIVE STIMULATION OF $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY IN HUMAN ERYTHROCYTE GHOSTS BY THE ADDITION OF CALMODULIN AND 18 2 LYSOPHOSPHATIDIC ACID (lysoPC) or 18 2-lysoPC

Dose of calmodulin (39 ng/ml) which induced a half-maximal stimulation of the ATPase was used in these experiments Values are the means ± S.D of three determinations

Phospholipid added	Concn (M)	Calmodulin	$(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol P_i/mg protein per h)
Control	–	–	2 24 ± 0 02
	–	+	4 00 ± 0 06
18 2-LysoPA	3 3 10^{-5}	–	4 22 ± 0 02
	3 3 10^{-5}	+	6 15 ± 0 06
18 2-LysoPC	3 3 10^{-5}	–	3 66 ± 0 02
	3 3 10^{-5}	+	5 62 ± 0 03

lin in the presence of 54.5 μM Ca^{2+}. At a concentration of $6.5 \cdot 10^{-5}$ M, these lysophospholipids expressed approximately half-maximal activation toward the $(Ca^{2+} + Mg^{2+})$-ATPase. When calmodulin was also added, additional stimulation was observed in both cases.

Discussion

Previous reports from other laboratories have noted the activation of $(Ca^{2+} + Mg^{2+})$-ATPase by addition of lysoPC to phospholipase C-treated [11,12] and normal human erythrocyte ghosts [11,13]. In contrast, another group of investigators have shown inhibition of the ATPase or no effect after addition of lysoPC to ghosts that have been treated in various ways [2,4]. Recently, we observed that lysoPC inhibited the ATPase in normal ghosts, whereas lysophosphatidic acid activated the ATPase [5]. In certain of the above studies, the chemical structure and purity of the lysophospholipids had not been well documented, while the other experiments had been performed with only one species of lysoPC. On the basis of studies reported here, using different types of synthetic lysophospholipids, it is suggested that the activation or inhibition of the ATPase by addition of lysophospholipids is highly concentration-dependent as well as sensitive to the chemical nature of the hydrophobic components These observations may account for the conflicting results described above and are discussed further below.

The addition of lysophosphatidic acids to human erythrocyte membranes can stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase activity. The degree of activation induced by the individual lysophosphatidic acids was dependent upon the nature of their hydrophobic fatty acyl groups. Among the saturated lysophosphatidic acids, a favorable range of chain length for stimulation was 12–14 carbons, the latter of which is less than the chain length of the fatty acids normally encountered in the lipids of the human erythrocyte membrane. In addition to chain length as a factor, the degree of unsaturation in the fatty acyl groups was also important in expressing an activating effect. The lysophosphatidic acid with an 18 : 2 fatty acyl moiety was the most active. Earlier studies using free fatty acids to stimulate ATPase activity had revealed a similar, though not identical dependence on chain length and degree of unsaturation [2–4]. These observations suggest that the chemical nature of the hydrophobic component of lysophosphatidic acids is critical to their ability to activate the $(Ca^{2+} + Mg^{2+})$-ATPase.

The purified $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes is more active in phospholipid vesicles consisting of acidic phospholipids such as phosphatidylserine than in phosphatidylcholine vesicles [14,15]. Thus, one might suppose that the acidic portion of the lysophosphatidic acids was a contributing factor to the activating effects of these lipids on membrane $(Ca^{2+} + Mg^{2+})$-ATPase. However, this was not the case in ghosts where

natural phospholipids already surrounded the ATPase. In the latter membrane system, several lysoPCs also activated the $(Ca^{2+} + Mg^{2+})$-ATPase, and as with the lysophosphatidic acids, the optimal activity level was achieved with the derivative containing an 18 : 2 fatty acyl group. This activity was nearly the same as the 18 : 2-containing lysophosphatidic acid. The similar response to acidic or zwitterionic lysophospholipids indicates that the charge on the hydrophilic part is not important under these conditions. Though the unsaturated lysoPCs and lysophosphatidic acids affected the ATPase in a similar way, there were some differences in the structure-activity relationships of their saturated analogues. Although an optimal activity range in the saturated fatty acyl chain length was 12–14 with both phospholipids, the maximal activation induced by a 14 : 0-lysoPC, the most active one among saturated species, was much lower than that produced by a 14 : 0-lysophosphatidic acid. Furthermore, the 16 : 0 and 18 : 0-lysoPCs actually exhibited a significant inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase at a concentration of $1.6 \cdot 10^{-4}$ M. A possible explanation for these differences might be that some saturated lysoPCs have both stimulating and inhibiting properties, with inhibition becoming dominant at high concentrations and at longer chain lengths. This idea may be supported by the finding that some of the saturated lysoPCs significantly reduced the calcium-independent ATPase in the following order at above $1 \cdot 10^{-4}$ M: 18 : 0 > 16 : 0 > 14 : 0. It is very probable that the inhibition of calcium-independent ATPase activity by the exogenous lipids is due to nonspecific membrane disordering effects as described previously [4]. In fact, the order of the potencies for the inhibitory effect noted with saturated lysoPCs is comparable with the reported order of hemolytic activity of lysoPCs [16,17]. Because none of the lysophosphatidic acids or lysoPCs tested activated the calcium-independent ATPase activity to a significant extent, the inhibitory activities of these lysophospholipids would be more clearly defined. On the other hand, the nonspecific membrane disordering effects of lysoPCs might mask their own, specific stimulatory effects on $(Ca^{2+} + Mg^{2+})$-ATPase, resulting in the observed low level of the maximal stimulation induced with 14 : 0-lysoPC and other saturated lysoPCs.

Lysophosphatidic acid has been known to have a variety of biological activities in vivo and in vitro. Recently, it has been found that alkyl ether type derivatives of lysophosphatidic acids, have much stronger biological activities than lysophosphatidic acids in producing hypotension in cats [18] and activating human platelets [19]. These findings suggest that the chemical nature of the linkage of a long-chain hydrocarbon (whether ether or acyl) to the *sn*-1 position of the glycerol moiety is a very critical structural factor for expressing the biological activities described above. It was not the case for the activating effect toward $(Ca^{2+} + Mg^{2+})$-ATPase activity in human erythrocyte membranes; the potency of 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphate in activating the ATPase was almost similar to that of 16 : 0-lysophosphatidic acid. In addition, 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine behaved similarly to the corresponding acyl type derivative toward the ATPase activities.

It is evident that both the lysophosphatidic acids and lysoPCs activate the $(Ca^{2+} + Mg^{2+})$-ATPase by a mechanism which is different from that of calmodulin, since the effects induced by the lysophospholipids are additive to that observed with calmodulin. The lysophospholipids probably affect the ATPase through interactions with lipid components surrounding the enzyme or hydrophobic parts of the enzyme embedded in the lipid bilayer. This is likely for two reasons: (1) The active lipids including free fatty acids have a common structural feature which is favorable for penetration into a lipid bilayer as a monomer. (2) The nature of the hydrophilic portion of the active lipids is not as important in expressing the stimulatory activity in the ghost. Rather, the potencies of the activating effects are highly dependent on their hydrophobic component. Possibly, the $(Ca^{2+} + Mg^{2+})$-ATPase can be activated either when the enzyme is surrounded by unusual lipids such as lysophosphatidic acids, lysoPCs and free fatty acids or when the membrane fluidity in the bulk phase of lipid bilayer is changed by the penetration of exogenously added lipids. A limited degree of perturbation of membrane structure may facilitate activation of the enzyme. However, a high degree of perturbation by the addition of high levels of some saturated lysoPCs can result in a nonspecific

reduction of membrane-bound enzyme activities such as the ATPases. Perhaps this is due to the displacement of required boundary lipids by the lysophospholipids.

Acknowledgements

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