Phospholipase A_2 as a Mechanosensor

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ABSTRACT Osmotic swelling of large unilamellar vesicles (LUVs) causes membrane stretching and thus reduces the lateral packing of lipids. This is demonstrated to modulate strongly the catalytic activity of phospholipase A_2 (PLA_2) toward a fluorescent phospholipid, 1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (PPDPC) residing in LUVs composed of different unsaturated and saturated phosphatidylcholines. The magnitude of the osmotic pressure gradient $\Delta\Omega$ required for maximal PLA_2 activity as well as the extent of activation depend on the degree of saturation of the membrane phospholipid acyl chains. More specifically, $\Delta\Omega$ needed for maximal hydrolytic activity increases in the sequence DOPC < SOPC < DMPC in accordance with the increment in the intensity of chain-chain van der Waals interactions. Previous studies on the hydrolysis of substrate monolayers by *C. adamanteus* and *N. naja* PLA_2 revealed maximal hydrolytic rates for these two enzymes to be achieved at lipid packing densities corresponding to surface pressures of 12 and 18 mN m^{-1}, respectively. In keeping with the above the magnitudes of $\Delta\Omega$ producing maximal activity of *Crotalus adamanteus* and *Naja naja* toward PPDPC/DMPC LUVs were 40 and 20 mOsm/kg, respectively. Our findings suggest a novel possibility of regulating the activity of PLA_2 and perhaps also other lipid packing density-dependent enzymes in vivo by osmotic forces applied on cellular membranes. Importantly, our results reveal serendipitously that the responsiveness of membranes to osmotic stress is modulated by the acyl chain composition of the lipids.

INTRODUCTION

Mechanosensitivity has been reported for both animal and plant cells (Sachs, 1990) and has been shown to be mediated by stress-sensitive membrane ion channels as well as by cytoskeleton (Wang et al., 1993). Evidence has also been presented for changes in the activity of different phospholipases by mechanical stimulus in vivo, but the underlying mechanisms have remained elusive (Brophy et al., 1993; Sadoshima and Izumo, 1993). Intracellular phospholipases A_2, C, and D have been shown to be centrally involved in mechanisms controlling cellular growth (Ferguson and Hanley, 1991; Shinomura et al., 1991; Nishizuka, 1992; Venable et al., 1994). Phospholipases A_2 (PLA_2s) have important functions in a wide variety of physiological and pathophysiological processes ranging from cellular signal transduction cascades to inflammation, ischemia, platelet aggregation, and acute hypersensitivity (Exton, 1990; Dennis et al., 1991; Mayer and Marshall, 1993). Liberation of arachidonic acid by PLA_2 is currently thought to represent the rate-limiting step in the formation of eicosanoids (Mayer and Marshall, 1993). The mechanisms regulating the activity of these enzymes have been intensively investigated.

Both intra- and extracellular forms of PLA_2 are known, and studies on this enzyme have been aided by its availability in several toxins and pancreatic tissue (Waite, 1987). The amino acid sequences of extracellular PLA_2s from mammalian pancreas, snake venom, and mammalian intracellular PLA_2s exhibit a high degree of structural homology and are highly conserved, thus suggesting common modes of, e.g., enzyme-substrate interaction (Waite, 1987; Wery et al., 1991). Accordingly, the readily available pancreatic and venom enzymes are considered to provide good models for the less easily accessible mammalian intracellular PLA_2s (Waite, 1987; Cordella-Miele et al., 1990).

Importantly, the activity of PLA_2s depends on several parameters characterizing the physical properties of the substrate such as the phase state of the membrane (Op den Kamp et al., 1975), membrane lipid packing defects (Lichtenberg et al., 1986; Grainger et al., 1990; Sen et al., 1991; Burack et al., 1993; Maloney and Grainger, 1993), and substrate conformation (Thuren et al., 1984; 1987a; van den Berg et al., 1993). The activity of PLA_2 can be triggered by electric fields imposed across the substrate membrane (Thuren et al., 1987b), and a role for the substrate surface potential has also been suggested (Mustonen and Kinnunen, 1991; Mustonen et al., 1993). In addition, the activity of these enzymes toward substrate monolayers residing on air/water interface has been established to vary as a function of surface pressure (Verger et al., 1973; Demel et al., 1975; Pattus et al., 1979; Verheij et al., 1980). Dependency on lipid packing has been verified also for phospholipase C (Boguslavsky et al., 1994) as well as for protein kinase C (Souvignet et al., 1991). The possible physiological significance of the dependency of the activity of the above enzymes on lipid packing density has remained uncertain. This stems mostly from the fact that values for the equilibrium lateral pressure of the various cellular membranes cannot be measured. Furthermore, potential mechanisms by which this property could be controlled by cells have not been established. In this communication we demonstrate how osmotic stretching of large unilamellar liposomes can modulate the susceptibility of the bilayer phospholipids to the action of PLA_2s.

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MATERIALS AND METHODS

Reagents

1-palmitoyl-2-[(6-pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (PPDPC) was obtained from K&V Bioware (Espoo, Finland). The other lipids (dioleoyl phosphatidylcholine, DOPC; 1-stearoyl-2-oleoyl phosphatidylcholine, SOPC; and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, DMPC), as well as fatty acid depleted bovine serum albumin and both phospholipases (*Crotalus adamanteus* and *Naja naja*) were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) (PEG, approximate molecular weight of 6000) was from Fluka (Bubendorf, Switzerland) and was used without further purification (Yamazaki et al., 1989; Burgess et al., 1991). The rest of the chemicals were of reagent grade and were purchased from Sigma Chemical Co.

Preparation of liposomes

A low pressure homogenizer (LiposoFast, Avestin, Inc., Ottawa, Canada) with 100-nm pore size membranes (Nucleopore, Pleasanton, CA) was used to form large unilamellar vesicles (LUVs). This procedure has been shown to yield LUVs with a fairly narrow size distribution independent of the lipid composition. While the resulting liposome preparations do contain some small vesicles as well as multilamellar vesicles, their share of the total amount of vesicles is small (MacDonald et al., 1991). Lipids were hydrated in 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.4, containing 1 mg/ml of defatted bovine serum albumin and 1–10% (w/w) PEG. In all liposomes 5 mol % PPDPC was included. Osmotic swelling of liposomes was induced by transferring liposomes prepared in PEG-containing buffers into a buffer with no PEG, thus causing an osmotic water inflow and swelling of liposomes. In this procedure the LUVs become diluted 50-fold to a final total phospholipid concentration of 75 μM. The residual PEG outside of LUVs is maximally 0.2% by weight and can be considered negligible. The liposomes were allowed to equilibrate for 30 min before the addition of 5 μg of *C. adamanteus* or 0.25 μg of *N. naja* PLA_2. At 25°C the permeabilities of DMPC and DOPC are almost equal (Carruthers and Melchior, 1983). Accordingly, it can be assumed that swelling of LUVs was complete even if there were minor differences in the water permeabilities of liposomes composed of different phosphatidylcholines. PEG was used as an osmotically active substance, as it does not bind to phosphatidylcholine liposomes (Arnold et al., 1990). No aggregation of LUVs by PEG was observed, in keeping with previous studies under similar conditions (Tilcock and Fisher, 1982). Increase in the excimer-to-monomer fluorescence emission ratio (Kinnunen et al., 1993) of PPDPC in different matrices was observed upon osmotic swelling, in accordance with an increased lateral mobility of the probe (Lehtonen and Kinnunen, 1994). Shrinkage of LUVs by PEG added to the outside of liposomes so as to enhance lipid packing was not investigated because of the dehydrating effect of PEG on proteins (Arakawa and Timasheff, 1985) and increment in the viscosity of the medium. Osmolalities were measured using a freezing point osmometer (Model 3Mo, Advanced Microosmometer, Needham Heights, MA).

Assay of PLA_2

All experiments were carried out at a constant temperature of 25°C using magnetically stirred, thermostated cuvettes. PLA_2 activity was measured in a total volume of 2.0 ml (Radvanyi et al., 1989). This study was conducted solely with vesicles in the liquid crystalline state. Accordingly, complexities due to changes in lipid phase state influencing the action of PLA_2 (Burack and Biltonen, 1994) should not interfere with our measurements, and we can focus on the effects resulting from changes in lipid packing. While, e.g., Brockman and his coworkers (Tsujita et al., 1989; Cunningham et al., 1989) have shown that the presence of fatty acids and diacylglycerol may activate lipolytic enzymes, such effects need not to be considered here. This is due to the fact that the degree of hydrolysis is low and initial reaction rates were measured. Also, to avoid the accumulation of free fatty acids and lysoPC within the substrate membranes, the reactions were carried out in the presence of albumin, which scavenges the products. The method used for the quantitation of PLA_2 activity rests on the photophysics of pyrene fluorescence. In brief, irradiation of this aromatic hydrocarbon at ~345 nm generates the monomeric excited state, which relaxes back to ground state by emitting a photon with a maximum at ~395 nm, the peak energy depending on solvent polarity. If the local concentration of pyrene is high enough the excited monomer may collide with a ground state pyrene, forming an excited dimer complex (excimer), which dissociates back to two ground state pyrenes by emitting photons as a broad and structureless band centered at ~480 nm. Measuring the steady-state emission intensity ratio of the excimer and monomer fluorescence (I_E/I_M) for various lipid derivatives containing covalently bound pyrene allows the monitoring of a variety of membrane phenomena (for a brief recent review see Kinnunen et al., 1993). Measurement of PLA_2 is possible using intramolecular excimer forming lipids, which contain two pyrenes in the same molecule, e.g., 1,2-bis pyrene butanoyl phosphatidylglycerol (Thuren et al., 1984, 1987c) or 1,2-bis pyrenedecanoylphosphatidylcholine (bisPDPC) (Sunamoto et al., 1980; Burack et al., 1993). Cleavage of the *sn*-2 acyl chain by PLA_2 from these lipids results in an enhancement of the monomer emission, thus allowing for a homogenous real-time assay for this enzyme. Although it has been suggested that PLA_2 might to some extent prefer bisPDPC over DPPC the values measured were observed to correspond to those recorded by pH titration (Burack et al., 1993).

Sufficiently high concentrations of monopyrene derivatives such as PPDPC can also be used for the assessment of PLA_2 activity (Thuren et al., 1987c; Radvanyi et al., 1989). Whereas 5 mol % of PPDPC in LUVs yields strong excimer emission, the action of PLA_2 on this lipid liberates pyrene decanoid acid (PDA), which after being bound by albumin can only emit as a monomer (Radvanyi et al., 1989). Under the conditions employed liberation of PDA began immediately after the addition of the enzyme. Importantly, the hydrolytic rates are those measured for the fluorescent phospholipid probe. Although these values may differ from those for the hydrolysis of the different matrices this does not undermine the observation that the osmotic pressure gradient $\Delta\Omega$ strongly influences the action of PLA_2 on PPDPC in these matrices. Finally, it has been shown that PPDPC forms superlattices in liquid crystalline membranes (e.g., Somerharju et al., 1985; Tang and Chong, 1992). However, these structures are not static but probably rather short-lived, measured on a time scale related to the lifetime of the excited state of pyrene, i.e., a maximum of ~100 ns. Therefore, it is rather unlikely that these superlattices should interfere with the action of PLA_2 on such mixed membranes, as the hydrolytic event catalyzed by PLA_2 occurs in the millisecond range. This is also supported by the observation that the specific activities measured using PPDPC are of the same order of magnitude as those obtained with other assays (e.g., Deems and Dennis, 1975a,b; Kunze et al., 1976).

RESULTS AND DISCUSSION

The activity of *C. adamanteus* PLA_2 toward PPDPC residing in unilamellar liposomes composed of a diunsaturated phospholipid, DOPC, is shown in Fig. 1 *A*. The lateral packing of nonstressed PPDPC/DOPC liposomes readily allows for nearly maximal hydrolysis by PLA_2. Yet, osmotic swelling of LUVs due to encapsulated PEG results in the stretching of the bilayers, and an osmotic gradient of $\Delta\Omega$ = 3.6 mOsm/kg induced a 10% activation of PLA_2. At higher values of $\Delta\Omega$, causing further decrease in the lateral packing density of the membrane lipids, the enzyme activity declined to ≈17% of the initial value measured for the nonstretched PPDPC/DOPC liposomes. In subsequent experiments LUVs composed of PPDPC and SOPC, with a saturated and an unsaturated acyl chain, were used. The activity of PLA_2 toward nonstressed PPDPC/SOPC liposomes was about 60% of that measured with DOPC (Fig. 1 *B*). Interestingly, maximally 50% activation was achieved by osmotic stretching of PPDPC/SOPC membranes. However, compared with

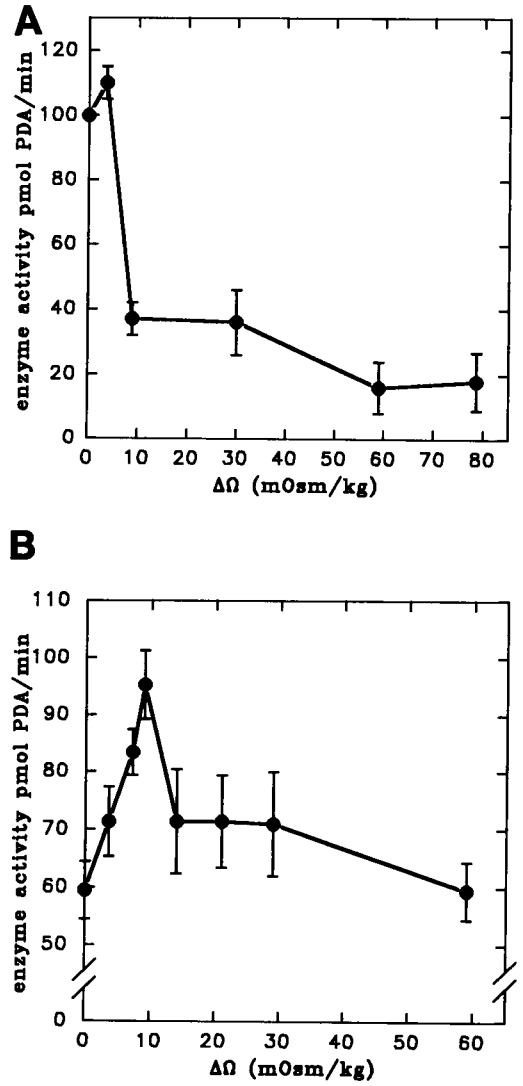

FIGURE 1 Activity of *C. adamanteus* PLA_2 toward PPDPC residing in LUVs subjected to an increasing extent of membrane stretching due to an osmotic pressure gradient $\Delta\Omega$ across the bilayer. Liposomes were composed of DOPC (*A*), SOPC (*B*), and DMPC (*C*). Values are means from four measurements, and the error bars indicate the range of measurements.

PPDPC/DOPC LUVs, a larger osmotic pressure gradient of $\Delta\Omega \approx 10$ mOsm/kg was needed to reach lateral packing, which produced maximal PLA_2 activity. Higher values of $\Delta\Omega$ attenuated the rate of pyrenyl fatty acid release by PLA_2 to the same level as measured for nonstressed PPDPC/SOPC liposomes (Fig. 1). Compared with DOPC and SOPC, saturated phosphatidylcholines such as DMPC form more rigid liposomes, as the lack of double bonds allows for more efficient intermolecular van der Waals interactions between the acyl chains. Notably, *C. adamanteus* PLA_2 had no measurable activity toward PPDPC in nonstressed DMPC liposomes (Fig. 1 *C*), whereas upon osmotic swelling a dramatic activation of PLA_2 was observed at $\Delta\Omega \approx 40$ mOsm/kg, with higher values of $\Delta\Omega$ again reducing the enzyme activity.

In contrast to PLA_2 from *C. adamanteus*, the *N. naja* enzyme showed hydrolytic activity also toward PPDPC in nonstressed DMPC liposomes (Fig. 2), and maximally a threefold activation was evident due to swelling at $\Delta\Omega \approx 20$ mOsm/kg. This observation is concordant with studies using phospholipid monolayers residing on an air/water interface as substrates, which have revealed PLA_2s from different sources to exhibit markedly different dependencies on lateral lipid packing with characteristic surface pressure values π_0 allowing for their maximal activity. Thus, the enzyme from *N. naja* is most active at $\approx$18 mN m^{-1}, whereas for *C. adamanteus* PLA_2 the optimum is $\approx$12 mN m^{-1} (Demel et al., 1975). Likewise the critical surface pressure abolishing hydrolytic activity is ~35 mN m^{-1} for *N. naja* and 23 mN m^{-1} for *C. adamanteus* PLA_2 (Demel et al., 1975). This difference between the two enzymes in their action on phospholipid monolayers further suggests that the equilibrium lateral pressure in the outer monolayer of nonstretched PPDPC/DMPC membranes is in the range of $35 > \pi > 23$ mN m^{-1}, where π is surface pressure. Similarly to *C. adamanteus* PLA_2 the

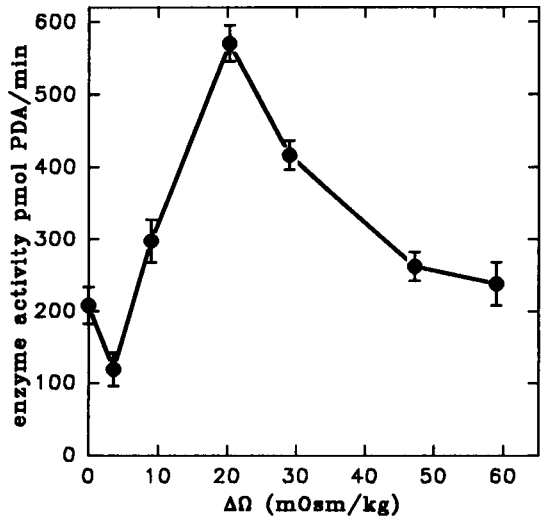

FIGURE 2 Enhancement of the hydrolytic activity of *N. naja* PLA_2 by osmotic stretching of DMPC LUVs. Experimental conditions were essentially similar to those described in the legend for Fig. 1, except that 0.25 μg of enzyme was used.

hydrolytic activity of *N. naja* enzyme declined at higher values of $\Delta\Omega$, producing extensive stretching of the membranes.

Because of the impermeability of the phospholipid bilayer to PEG, water will flow into the vesicles with encapsulated polymer. Vesicles behave as osmometers, and their internal volume increases in response to osmotic gradients; the influx of water tends to reduce the osmotic gradient to 0. Osmotic pressure thus becomes counterbalanced by the elastic properties of the bilayer, and the size of the vesicles increases until these forces are at equilibrium. The extent of membrane swelling has been found to be linearly proportional to the osmotic difference across the bilayer (Sun et al., 1986). Because of the discrepancies in the elastic constants measured for liposomes composed of different phospholipids (Rutkowski et al., 1991), it is difficult to estimate the dependency of bilayer elastic modulus on the structure of the phospholipid in question. Yet, a fairly narrow range of Young's modulus for both cell membranes and liposomes composed of synthetic phospholipids has been measured (Haines et al., 1987; Rutkowski et al., 1991). The fact that a vesicle expands upon osmotic swelling may be described at molecular level as lateral stretching of the membrane. Although membranes are highly resistant to such area/molecule dilatation, increases in area up to 25% have been measured (Hantz et al., 1986). Accordingly, upon increase in the area/molecule ratio of phospholipids, the extent of *trans* → *gauche* isomerization of the acyl chains should increase. This is consistent with the observed increment in membrane fluidity and free volume in membranes subjected to osmotic swelling (Boronchov and Boronchov, 1979; Lehtonen and Kinnunen, 1994).

For *C. adamanteus* PLA_2, the activity toward nonstretched as well as optimally stretched vesicles (allowing maximal hydrolytic rates) decreased in the sequence DOPC > SOPC > DMPC. This could reflect augmenting intensity of conformational dynamics of the lipids due to an increase in the acyl chain unsaturation, which would in turn allow for faster diffusion of the reactants in the substrate film (Baenziger et al., 1992). The exact molecular mechanism responsible for enzyme activation at optimal lateral packing of the substrate remains elusive, however. Because osmotically swollen liposomes should remain intact up to an osmotic gradient of <1000 mOsm/kg with practically no leakage of the encapsulated material (Mui et al., 1993, 1994), formation of surface defects does not explain the present results. PLA_2 activity has been inferred also to depend on the conformation of the substrate phospholipids (Thuren et al., 1984, 1987b). Such conformational changes in the substrate are highly unlikely to underlie the changes in enzyme activity due to osmotic swelling, as the latter leads to looser lateral packing of phospholipids in the membrane. This should, in turn, favor the orientation of the glycerol backbone more or less parallel to the surface (Eklund et al., 1992), which again can be anticipated to make the lipid less susceptible to hydrolysis by PLA_2 (Thuren et al., 1984; 1987b). Quantitative analysis of the PLA_2 action on lipid films suggests that it is the apparent specific activity of the enzyme that is increased at π_0 (the lateral pressure allowing maximal activity of PLA_2) and not the amount of enzyme present in the monolayers (Pattus et al., 1979). Decline in lipolytic activity at low lateral pressures could result from surface denaturation of the enzyme (Momsen and Brockman, 1976). It is also possible that the active conformation of the enzyme depends on lipid packing density in a manner specific for PLA_2 from different sources. Interestingly, the conformation of the 25-residue membrane-penetrating signal peptide of lamB protein has been shown to be influenced by the lipid monolayer surface pressure (Cornell et al., 1989).

Only indirect estimates are available on the equilibrium lateral pressure π_B of liposomes (Gruen and Wolfe, 1982; Konttila et al., 1988). Accordingly, the values for π_B in nonstressed DOPC, SOPC, and DMPC LUVs remain essentially unknown. In a bilayer membrane under no tension, the forces between phospholipids are at equilibrium, and the area/molecule has a value at which the free energy of the system is at a minimum. Because of van der Waals interactions, which increase with acyl chain saturation, we may assume π_B to increase in the sequence DOPC < SOPC < DMPC. This dependency on acyl chain saturation is also in keeping with the magnitudes of osmotic pressures required for the phase separation of PPDPC in these matrices (Lehtonen and Kinnunen, 1995). While also taking into account that the elastic moduli for the membranes of these lipids are likely to differ, if the lipid packing at the lateral pressure π_0 allowing maximal activity of PLA_2 is independent from lipid unsaturation, then the differences in $\Delta\Omega$ required for optimal stretching would reflect the magnitude of the difference $\Delta\pi = \pi_B - \pi_0$ for the different lipids. Accordingly, higher osmotic pressure should be required for saturated lipids to counteract van der Waals forces so as to bring the membrane lipid packing density within the range allowing maximal PLA_2 activity. In keeping with this, stretching of the substrate bilayers enhanced their susceptibility to the action of PLA_2; yet the osmotic pressure gradient $\Delta\Omega$ required for maximal hydrolytic activity for the matrices of the different lipids increased in the sequence DOPC < SOPC < DMPC.

Osmotic forces and other mechanical phenomena have currently attracted wide interest along with the demonstration of osmotic effects on mechanically activated ion channels (Martinac et al., 1987; Morris and Sigurdson, 1989; Morris, 1990; Oliet and Bourque, 1993), cell metabolism (Häussinger and Lang, 1991), gene expression (Izumo et al., 1988; Poulin and Pegg, 1990; Douzou, 1994), diffusion of quinones in membranes (Mathai et al., 1993), and on a receptor tyrosine kinase (Brewster et al., 1993; Tilly et al., 1993). There is evidence indicating that in addition to cytoskeleton (Watson, 1991) membranes could also act as a receptor for mechanical stimuli (Martinac et al., 1990). Analogously to liposomes equilibrium pressure values for various cellular membranes remain to be elucidated. Employing phospholipases with different optimal lipid packing densities and comparing their hydrolytic rates toward erythrocyte plasma membrane lipids allowed the deduction of a range of 31–35 mN m^{-1} (Demel et al., 1975). Importantly, in the absence of gross changes in the amount of lipids in the plasma

membrane, osmotic swelling and shrinkage should readily decrease and increase, respectively, the lateral packing pressure of lipids in the bilayer. In spite of its inherent limitations the simple model system utilized in the present study clearly demonstrates that 1) a membrane bilayer can act as mechanosensor; 2) changing the lateral lipid packing by osmotic swelling may strongly influence PLA_2 activity; and 3) the osmotic sensitivity of the membrane depends on lipid composition, i.e., on the degree of acyl chain saturation. Unfortunately, optimal lipid packing densities for mammalian intracellular PLA_2s have not been determined. Accordingly, the present investigation had to be conducted with two snake venom PLA_2s for which these data were available (Demel et al., 1975). The elucidation of the in vivo significance of our results must therefore await the characterization of intracellular PLA_2s in this respect. Yet, it is possible that, in addition to PLA_2, similar mechanisms could also be important in the regulation of phospholipases C and D. Sensitivity of these enzymes to changes in the lateral packing of substrate lipids has been reported (Rao and Sundaram, 1993; Boguslavsky et al., 1994). Interestingly, the stereoselectivity of several lipases has been found to be surface pressure dependent (Rogalska et al., 1993). Likewise, in lipid monolayers, the activity of protein kinase C is critically dependent on lipid packing and does not correlate to the membrane penetration of the protein (Souvignet et al., 1991). Protein kinase C has also been shown to be activated by mechanical stretching of cells (Komuro et al., 1991). The physiological significance of the apparent surface pressure dependency of all the above enzymes has remained uncertain, however. In light of our data it is tempting to speculate that osmotic forces could be utilized by cells in vivo to control the lateral packing of membranes and that such a regulatory mechanism may well be involved in the control of the action of lipid packing density-dependent enzymes in general.

CONCLUSIONS

Notably, because of the well-known apparent dependency of the catalytic rates of PLA_2s on surface pressure (further correlating with lipid packing), effects due to osmotic stretching of LUVs can be readily anticipated. However, because of the fact that 1) equilibrium pressure of LUVs and 2) dependency of surface pressure on acyl chain saturation is not known, the present results could not be predicted either qualitatively or quantitatively. It is also important to emphasize that direct extrapolation of results obtained with lipid monolayers to liposomes warrants caution. For instance, both pancreatic lipase and lipoprotein lipase do hydrolyze phosphatidylcholine liposomes, whereas for unknown reasons monolayers of this lipid are not being hydrolyzed by these lipolytic enzymes (Pieroni and Verger, 1979; Vainio et al., 1983). Our data demonstrate for the first time that the response of membranes to osmotic stress can be controlled by lipid acyl chain unsaturation. Finally, it is tempting to hypothesize that cells could utilize osmotic forces to control the lateral packing of the constituents in their membranes and by these means control the activities of lipolytic enzymes as well as other membrane proteins.

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