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Calcium diphosphatidate membrane traversal is inhibited by common phospholipids and cholesterol but not by plasmalogen

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Phosphatidate-mediated Ca^{2+} membrane traversal is inhibited by phospholipids (PL) such a phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin and lysoPC, but not by PC-plasmalogen. Kinetics of Ca^{2+} traversal through a 'passive' bilayer consisting of OH-blocked cholesterol show competition between PC and phosphatidic acid (PA); it appears likely that a Ca(PA · PC) complex is formed which is not a transmembrane ionophore but will reduce the amount of phosphatidic acid available for the formation of the ionophore, $Ca(PA)_2$. PS and PI may inhibit Ca^{2+}-traversal in the same manner by forming Ca(PA · PL) complexes. We suggest that PC-plasmalogen, with one of the Ca^{2+}-chelating ester CO groups missing, cannot engage in calcium cages, i.e., Ca(PA · PL) complexes, and thus does not interfere with $Ca(PA)_2$ formation. Double-reciprocal plotting of Ca^{2+} traversal rates in cholesterol-containing liposomes vs. calcium concentration suggests that cholesterol inhibits Ca^{2+} traversal by competing with Ca^{2+} for PA. The inhibition does not seem to be caused by a restructuring or dehydration of the membrane 'hydrogen belts' affected by cholesterol; most probably, it is due to hydrogen bonding of the cholesterol-OH group to a CO group of PA; this reduces the amount of PA available for the calcium ferry. The inhibition by sphingomyelin and lysoPC may also be explained by their OH group interacting with PA via hydrogen bonding. The pH dependence of Ca^{2+} traversal suggests that $H[Ca(PA)_2]^-$ can serve as Ca^{2+} cross-membrane ferry but that at physiological pH, $[Ca(PA)_2]^{2-}$ is the predominant ionophore. In conclusion, the results indicate that Ca^{2+} traversal is strongly dependent on the structure of the hydrogen belts, i.e., the membrane strata occupied by hydrogen bond acceptors (CO of phospholipids) and donors (OH of cholesterol, sphingosine), and that lipid hydrogen belt structures may regulate storage and passage of Ca^{2+}.

Abbreviations: PA, phosphatidic acid or phosphatidate; PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipids other than phosphatidate; PS, phosphatidylserine; lysoPC, lysophosphatidylcholine; PC-plasmalogen, 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine; OH-blocked cholesterol, *O*-methoxyethoxyethoxyethylcholesterol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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Introduction

The function of the hydrogen belts of biological membranes [1] – those strata which are composed of hydrogen bond acceptors (CO groups of phospholipids) and donors (OH of cholesterol, sphingosine, α-hydroxy acids, proteins) – is still obscure; but evidence is accumulating to demonstrate the role of hydrogen bond composition in lipid–lipid [2] and protein–lipid interactions [3,4].

Phosphatidic acid (PA) can carry calcium across membranes [5–11]. It appears that the ionophore is $Ca(PA)_2$ in which the cation and the two PA headgroups form a dehydrated, lipid-soluble complex as an eight-cornered coordination cage [6,9]. The composition of hydrogen belts is also critical (certainly in vitro) for Ca^{2+} membrane traversal: formation of the dehydrated ionophore [$Ca(PA)_2$] is accelerated 100-fold if the conventional diester phosphatidylcholine bilayer is replaced by a 'passive bilayer' of lipids free of CO or OH groups; i.e., PC inhibits $Ca(PA)_2$ formation [12]. Free cholesterol in passive bilayer systems also depresses the traversal rates [12]. In order to understand the mechanism of inhibition of Ca^{2+} traversal by PC and cholesterol, kinetic studies were done in the present study. PC and cholesterol are shown to act as competitive inhibitors of PA-mediated Ca^{2+} traversal, and other phospholipids, i.e., phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylcholine (lysoPC), and sphingomyelin also inhibit calcium ferry formation. It is suggested that the common diester phospholipids (PL) may suppress [$Ca(PA)_2$] formation by engaging PA in [Ca(PA)(PL)] cages; these are no longer trans-membrane ionophores because of their hydrophilic anchoring group (e.g., choline). Phospholipids without CO groups, such as diether-PC, on the other hand, may not form such complexes, and for this reason may not interfere with [$Ca(PA)_2$] formation. Diether phospholipids are known in nature, but they are, in animals, very rare and very minor lipids. Phospholipids with only one CO group, however, rather than two, are widely distributed, as 1-etherphospholipids (1-*O*-alkyl) or, more often, as 1-vinylether phospholipids, or plasmalogens. The most commmon of these is PE-plasmalogen, which constitutes, for example, 35 mol% of the myelin phospholipid of the rat [13]. At present, no purpose can be ascribed to the ether groups in these lipids. We suggest a role of plasmalogens, and of phospholipids as a group, in calcium storage and transport.

Materials and Methods

Egg phosphatidylcholine (PC) was isolated and purified [14]. Cholesterol was recrystallized three times from ethanol. 3-*O*[[(Methoxyethoxy)ethoxy]ethyl]cholesterol (OH-blocked cholesterol) was synthesized [15]. Cholesterol-*O*-methyl ether (*O*-methylcholesterol), lysophosphatidylcholine (lysoPC, palmitoyl), sphingomyelin, phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI) were purchased from Sigma. PC-plasmalogen from Serdary Research Lab, London, Ontario, Canada, was characterized after HCl hydrolysis by two-dimensional thin-layer chromatography [16] and the phosphorus content was estimated [17] in lysoPC (formed from PC-plasmalogen) and PC. PC-plasmalogen was found to be 84% pure; the rest was PC. Arsenazo-III dye purchased from Sigma was purified [18].

Chloroform/methanol (1 : 1, v/v) solutions containing 50 μmol of lipid were dried under vacuum at 70 °C for 1 h while being flushed with N_2 every 10 min. Small unilamellar liposomes were prepared in 2 ml of 72.5 mM NaCl/72.5 mM KCl/5 mM Hepes/Tris buffer (pH 7.45) containing 40 μmol of Arsenazo-III with sonication (4 h) under argon; they were then isolated by Sepharose 4B column chromatography, as described earlier [19]. Phospholipid phosphorus and cholesterol concentration in the liposomes were estimated according to Marinetti [17] and Zlatkis et al. [20]. Ca^{2+}-traversal rates were determined by adding 3 mM $CaCl_2$ to 3.0 μmol liposomal lipid with trapped dye in a total volume of 2.0 ml of buffer, and recording of the increase of absorbance at 650 nm. Control were performed with liposomes without dye to correct for Ca^{2+}-dependent aggregation; this was always found to be negligible. The rate of Ca^{2+}-Arsenazo-III complex formation was quantitated from the initial slope of absorbance with a molar absorption coefficient of $2.06 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [5]. Ca^{2+} transport rates were corrected for the surface concentration of phosphatidic acid because only the phosphatidic acid on the outside takes part in complexing calcium. A correction factor (surface factor) of 0.23 is valid for sonicated, all-cholesterol liposomes [12,19]. Controls were performed for leakage of Ca^{2+} in liposomes without phosphatidic acid, for leakage of dye, and for leakage of calcium-dye complex from liposomes with and without phosphatidic acid, by measuring the change of absorbance on addition of EDTA [10], and by isolation of the non-trapped liquid from the liposomes by Sep-

harose separation after 2 h incubation with Ca^{2+}. All these controls were negative.

Results

Kinetics of Ca^{2+} transport inhibition by PC were studied with different PA concentrations at 0, 30, 50 and 70 mol% of PC in *O*-methylcholesterol/ OH-blocked cholesterol liposomes (Fig. 1). Ca^{2+} transfer rates were found to be of second order relative to PA, i.e., the rates were proportional to the square of PA concentrations in *O*-methylcholesterol/ OH-blocked cholesterol liposomes (Fig. 1A), in confirmation of previous results in PC liposomes [6]. The double-reciprocal plot of $1/Ca^{2+}$-traversal rate ($1/v$) vs. $1/[PA]^2$ indicates a competition between PC and PA (Fig. 1B).

Fig. 2A shows the effects which the addition of 1, 2, 3, 5 or 7.5 mM Ca^{2+} to passive liposomes containing 0, 10, 20, 30 or 50 mol% of cholesterol has on PA-mediated Ca^{2+} transport rates. The double-reciprocal plot indicates that cholesterol is an inhibitor for Ca^{2+} transport competitive with Ca^{2+} (Fig. 2B).

Fig. 3 shows the effect of cholesterol on the rate of Ca^{2+}-membrane traversal; a previous graph showing the influence of cholesterol on the aggregation of liposomes by Ca^{2+} is included for comparison. Aggregation is not affected by cholesterol up to a concentration of 30 mol%; at higher concentrations of cholesterol, aggregation rates in-

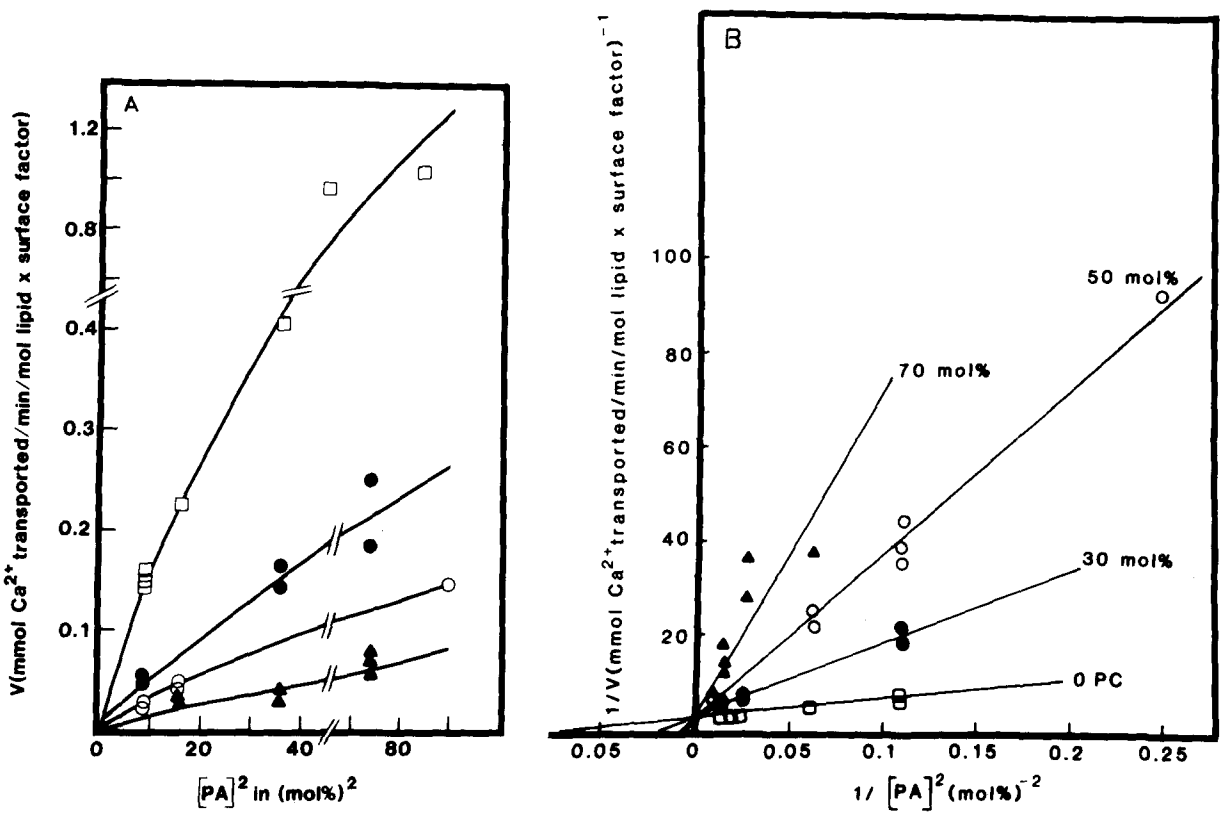

Fig. 1. Ca^{2+}-transport rates versus $[PA]^2$ at different fixed concentrations of PC in *O*-methylcholesterol/OH-blocked cholesterol liposomes (Panel A). Sonicated liposomes with and without PC and at different PA concentrations were prepared in buffer containing 20 mM Arsenazo III, 72.5 mM NaCl, 72.5 mM KCl, and 5 mM Hepes, adjusted to pH 7.45 with Tris. Ca^{2+}-traversal rates (v) upon addition of 3 mM $CaCl_2$ were measured at 650 nm from the initial slope of change in absorbance. Panel B shows a double-reciprocal plot ($1/v$ vs. $1/[PA]^2$). PC concentrations in the liposomes were ▲, 70 mol%; ○, 50 mol%; ●, 30 mol% and □, 0 PC.

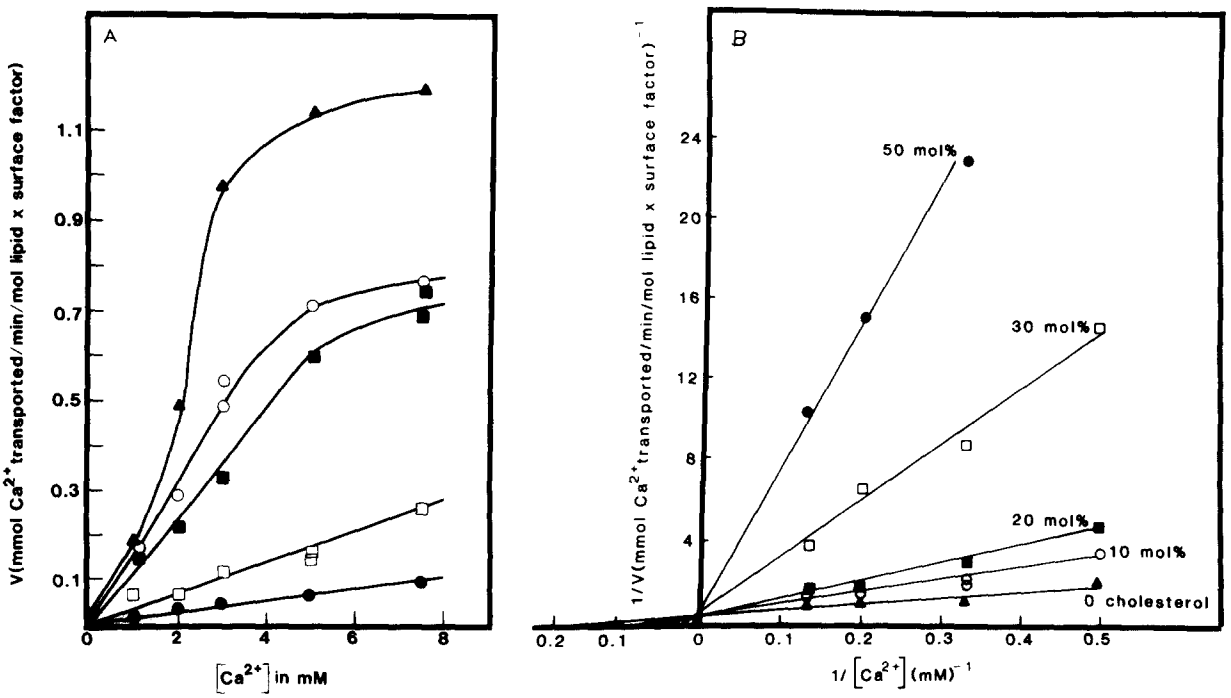

Fig. 2. Effect of Ca^{2+} concentration on PA-mediated Ca^{2+}-traversal rate in the presence and absence of cholesterol in liposomes (Panel A). Liposomes contained 7 mol% PA, 50 mol% cholesterol/*O*-methylcholesterol and 43 mol% OH-blocked cholesterol. Ca^{2+}-traversal rates (v) were measured upon addition of 2, 3, 5 and 7.5 mM $CaCl_2$ [S] to the liposomal solution in buffer, pH 7.45. Panel B shows a double-reciprocal plot ($1/v$ versus $1/[S]$) in the absence and at various concentrations of cholesterol. The cholesterol concentrations employed were ●, 50 mol%; □, 30 mol%; ■, 20 mol%; ○, 10 mol%; and ▲, 0 cholesterol.

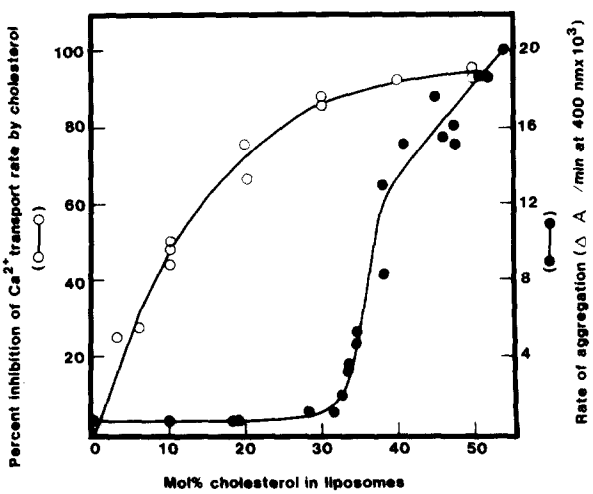

Fig. 3. Inhibition of $Ca(PA)_2$ membrane traversal by cholesterol (○———○) in comparison to the aggregation of acidic liposomes by cholesterol (●———●). Liposomes were suspended in buffer containing 72.5 mM NaCl, 72.5 mM KCl and 5 mM Hepes/Tris (pH 7.45). For Ca^{2+} traversal experiments, liposomes contained 7 mol% PA, 43 mol% OH-blocked cholesterol and 50 mol% cholesterol/*O*-methylcholesterol. Ca^{2+}-traversal rates were measured at 650 nM after addition of 3 mM $CaCl_2$ to liposomal solution with trapped dye. The data for aggregation (●———●) are reproduced from Ref. 19.

creased rapidly, with an inflection point at approx. 35 mol% (data reproduced from Ref. 19). On the other hand, the inhibition of Ca^{2+} transport by cholesterol takes effect even at low concentrations and increases steadily with increasing cholesterol concentration, following a monotone course (Fig. 3). The dependence on PC of the inhibition of Ca^{2+}-traversal is shown in Fig. 4. The course of inhibition following increasing concentration of PC is nearly identical to that obtained for cholesterol.

Table I gives the effect of plasmalogen and other phospholipids on PA-mediated Ca^{2+} traversal rates in passive liposomes of *O*-methylcholesterol and OH-blocked cholesterol. For our experiments we could not use PE-plasmalogen

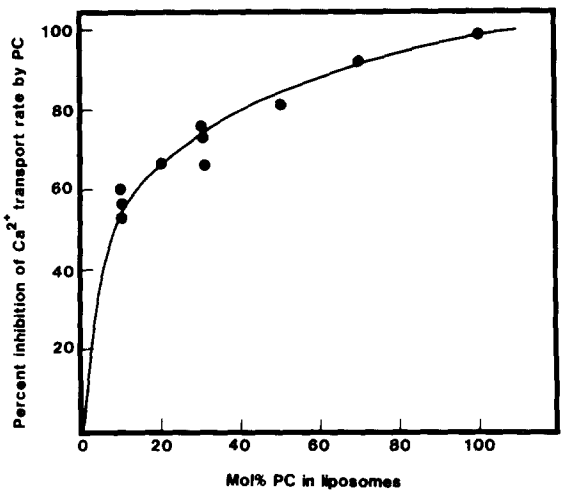

Fig. 4. Effect of phosphatidylcholine (PC) concentration on PA-mediated calcium transport in *O*-methylcholesterol/OH-blocked cholesterol liposomes. Ca^{2+}-traversal rates were measured at 650 nm in liposomes containing 6% PA and various concentrations of PC after addition of 3 mM $CaCl_2$. The percent inhibition of Ca^{2+} transport was calculated with respect to Ca^{2+}-transport rate in liposomes without PC, and plotted against PC concentration in liposomes.

since this lipid caused extensive aggregation of liposomes during the assay; this made Ca^{2+}-traversal rate measurements impossible. PC-plasmalogen (from bovine heart) was manageable. When PC-plasmalogen was included in the liposomes (double molar to the pro-ionophore, PA), there was only insignificant inhibition of Ca^{2+} traversal; the small degree of inhibition that did show, if real, may have been due to the contaminating 16 percent PC (see Materials and Methods). This indicates that plasmalogen does not interfere in the formation of $Ca(PA)_2$. On the other hand, if PC was added to the liposomes at 2 : 1 molar ratio of PC : PA, inhibition of 57 percent resulted. There was no change in this inhibition by PC when plasmalogen was also included in the liposomes at equal molarity with PC (Table I). When PS, PI, sphingomyelin or lysophosphatidylcholine were included in *O*-methylcholesterol/OH-blocked cholesterol vesicles at 10 mol%, they were all found to inhibit Ca^{2+} traversal rates, like PC and cholesterol and to similar degrees (Table I).

Since two PA molecules possess four acidic protons, of which only two are neutralized by

TABLE I

EFFECT OF PHOSPHATIDYLCHOLINE (PC), PHOSPHATIDYLSERINE (PS), PHOSPHATIDYLINOSITOL (PI), LYSOPHOSPHATIDYLCHOLINE (lysoPC), PC-PLASMALOGEN AND SPHINGOMYELIN ON PA-MEDIATED Ca^{2+}-TRAVERSAL RATES IN LIPOSOMES

Liposomes contained *O*-methylcholesterol/OH-blocked cholesterol (1 : 1) and 5 mol% PA. PS, PC, PI, lysoPC, PC-plasmalogen, and sphingomyelin, when present in the liposomes, were substituted for *O*-methylcholesterol/OH-blocked cholesterol at a concentration of 10 mol%. The liposomes with trapped dye were suspended in buffer containing 72.5 mM NaCl, 72.5 mM KCl and 5 mM Hepes, adjusted to pH 7.45 with Tris. Ca^{2+}-traversal rates were quantitated from the initial slope of *A* at 650 nm after addition of 3 mM $CaCl_2$ to 3.0 μmol liposomal lipid solution and are expressed as mmol Ca^{2+} transported/min per (mol of PA) $\times$ *F*, where *F* represents a correction factor for the surface concentration of PA. *F* is 0.23 for these liposomes [12,19].

Phospholipids other than PA in liposomes (mol%)	mmol Ca^{2+} transported · min^{-1} · (mol of PA)$^{-1}$ · surface factor	% inhibition
–	9.7 ± 1.2 (5)	–
PC (10%)	4.2 ± 0.3 (3)	56.7
LysoPC (10%)	3.3 ± 0.7 (4)	60.0
PS (10%)	3.9 ± 0.6 (3)	59.8
PI (10%)	3.2 ± 0.7 (3)	67.0
Sphingomyelin (10%)	1.8 ± 0.8 (4)	81.4
PC-plasmalogen (10%)	9.3 ± 0.9 (5)	4.1
PC (10%) + PC-plasmalogen (10%)	4.0 ± 0.4 (3)	58.7

Ca^{2+}, it is of interest to know whether the diprotonated, monoprotonated, or dianionic form of the $Ca(PA)_2$ complex is the ionophore translocating calcium. Therefore, Ca^{2+}-transport rates were determined as a function of pH (Fig. 5). Furthermore, since $Ca(PA)_2$ can be expected to translocate calcium, if at all, only from the outside of a cell into the cytosol (following the normal Ca^{2+}-concentration gradient), and since the pH is not necessarily the same outside and inside, we also measured traversal rates at different intraliposomal pH. This pH_i is the pH of the buffer that was used during the dispersion of the lipid film, sonication, and isolation of the liposomes. These liposomes were resuspended in different buffers for Ca^{2+} transport experiments at different outside pH_o. The pH_o did not affect pH_i; this was checked by incubating liposomes of same pH_i, with buffers of different pH_o, and then eluting

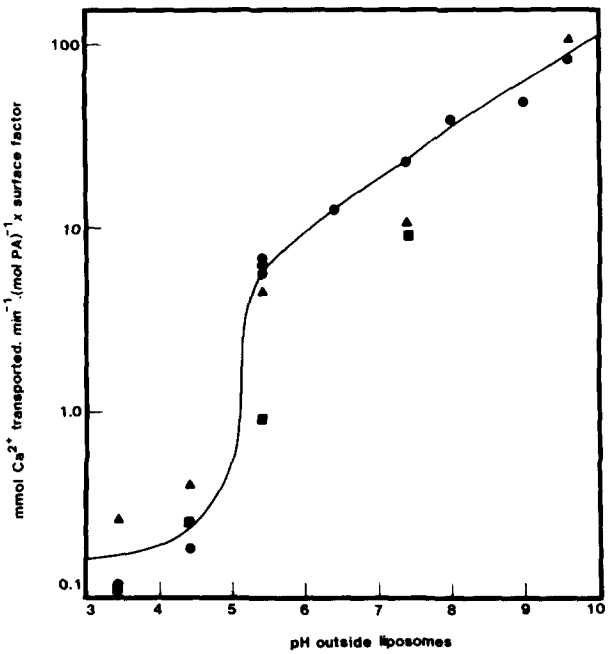

Fig. 5. Dependence on pH of PA-mediated Ca^{2+} traversal rates in *O*-methylcholesterol/OH-blocked cholesterol liposomes containing 6% PA. Buffers of 5 mM acetate (pH 3.4, 4.4, 5.4), 5 mM Hepes/Tris (pH 6.4, 7.4, 8.0, 9.0) and 5 mM glycine/NaOH (pH 9.6), containing 72.5 mM NaCl/72.5 mM KCl were prepared. The lipid film of 50 μmol lipid was dispersed and sonicated in buffer containing Arsenazo III, and the liposomes were collected by Sepharose 4B column chromatography with the same buffer. The intraliposomal pH_i was shown to be identical to that of the buffer in which the liposomes had been prepared; this was shown by pH measurement after disruption of the liposomes with 0.05% Triton X-100. These liposomes (3.0 μmol lipid) were then suspended in outside buffer, pH_o. Repetition of the collection of liposomes by Sepharose chromatography followed by rupture and pH measurement showed that the inside pH_i remained unchanged for at least 30 min. Ca^{2+}-traversal rates were determined after adding 3 mM $CaCl_2$ to the liposomal suspension, and expressed as mmol Ca^{2+} transported·min^{-1}·(mol PA)$^{-1}$·(surface factor F), where F represents a correction factor (in this case, 0.23) for the asymmetry of liposome surface concentration of PA [12,19]. Three sets of experiments were done for Ca^{2+} transport: (1) intraliposomal pH_i was 7.4 in all liposomes, pH outside (pH_o) was varied (●); (2) pH_i = 4.4, pH_o varied (▲); and (3) both pH_i and pH_o varied, but $pH_i = pH_o$ (■).

them with isotonic NaCl/KCl solution; the pH of the eluted liposomes, determined after sonication or lysis with Triton, was the original pH_i in all liposomes tested, irrespective of pH_o. Ca^{2+} transport was studied with liposomes having $pH_i = 4.4$, $pH_i = 7.4$ or variable pH_i but $pH_i = pH_o$ (Fig. 5).

It appears that the pH inside the liposomes did not influence the Ca^{2+} transport rate, while the extraliposomal pH did. Fig. 5 shows that Ca^{2+} is not transported until the pH exceeds 4.4; then there is a sharp increase in Ca^{2+} traversal rate, with an inflection point at about pH 5, followed by a gradual increase in Ca^{2+} transport with pH from 5.4 to 9.6.

Discussion

PA can transport Ca^{2+} across the bilayer, the vehicle being $Ca(PA)_2$ [6,9]. It was reported earlier that replacement of the membrane matrix phospholipid, PC, with diether-PC (without CO groups) increased Ca^{2+} traversal rates 100-fold [12]. The results here show that incorporation of other phospholipids (PS, PI, lysoPC, sphingomyelin) in a passive bilayer (*O*-methylcholesterol/OH-blocked cholesterol) also inhibit transport. Phosphatidylethanolamine could not be tested because it destabilized the liposomal bilayer; this effect of PE is well-known [21,22]. The double-reciprocal plot of Ca^{2+} transport rates vs. the square of PA concentration at different fixed concentrations of PC (Fig. 1) shows that PC inhibits Ca^{2+} traversal in competition with PA. The traversal rates are a measure of the formation of the ionophore, $Ca(PA)_2$, and so it appears that PC by virtue of its CO groups competes with PA in the formation of this calcium cage. In a passive bilayer without any CO or OH group, all PA can engage in $Ca(PA)_2$ formation, but in a bilayer containing PC the species Ca(PA · PC) will also be present. This species, because of the hydrophilic choline head group, is not a membrane-crossing ionophore; thus, Ca^{2+} traversal is inhibited because of the reduction of the amount of PA available for $Ca(PA)_2$ formation. It can then be expected that PS, PE, PI, lysoPC, and sphingomyelin would also compete with PA in Ca(PA) · (PL) complex formation; and they do (but we could not measure the inhibition by PE). In a plasmalogen, one of the chelating phospholipid CO groups is missing. The molecule can, therefore, not engage in a Ca · PA · PL complex (Fig. 6), and consequently does not reduce the concentration of PA available for the Ca · $(PA)_2$ complex; i.e., plasmalogens do not impede the PA-mediated Ca^{2+}-membrane traversal.

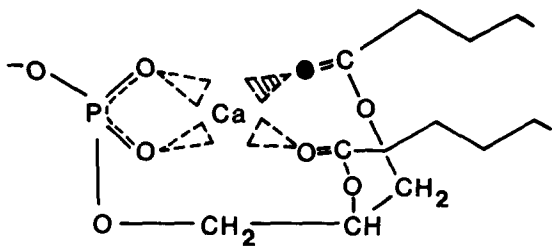

Fig. 6. Schematic model of one half of a calcium cage showing four out of the eight coordination bonds from PO and CO to the cation. (A space-filling model can be found in Ref. 6). Circles are oxygens, triangles are coordination bonds, the filled circle is the oxygen missing in plasmalogens, the hatched triangle the missing coordination bond.

They act, in this respect, like passive membrane lipids. It would appear that membranes which are rich in plasmalogens, and thus lacking in calcium cages, might therefore be poor reservoirs of calcium.

The effect of cholesterol on phospholipid bilayers has been widely investigated [23–28]. Many, though not all, structural membrane parameters change slowly with increasing cholesterol content up to about 32 mol%; then, an abrupt structural alteration takes place, as revealed by a change in hydrodynamic parameters above this cholesterol content [29–31]. Among other effects, liposome aggregation via inter-vesicle $Ca(PA)_2$ bridge formation (with its prerequisite, the dehydration of the membrane-water-membrane system) also displays a dramatic rate increase at about 33 mol% cholesterol (Fig. 3) [19]. It might then be speculated that the inhibition of $Ca(PA)_2$ formation by cholesterol might follow a similar course; that is, the inhibitive action of cholesterol would be delayed at low concentrations but take off at 33 mol%. This is, however, not the case: inhibition starts at low cholesterol level and increases gradually as the concentration of the sterol increases. Fig. 3 shows graphically the difference between the two effects. The inhibition is not likely to be the consequence of a restructuring of the membrane such as a change in the hydration of the 'hydration belt' region. The near-identity of the inhibition curves of PC and cholesterol make it likely that the mechanisms of inhibition are similar, namely, that both rest on the interference by the inhibitor in the formation of the calcium cage, $Ca(PA)_2$. In the inhibition by cholesterol, it is clearly the OH group which is the actual inhibitor (OH-blocked cholesterol, it may be recalled, is the inert matrix). This implies hydrogen bonding, and, given the lack of other possibilities, we may conclude that cholesterol and PA engage in OH · · · OC bonding. Thus, the pool of PA available for $Ca(PA)_2$ formation is again diminished, the result being the inhibition of Ca^{2+} traversal.

Sphingomyelin and lysoPC both have a CO as well as an OH group. They might engage in Ca-(PA · PL) formation or in (lipid)O-H · · · OC(PA) hydrogen bonding, or in both; a decision must remain postponed for the present.

The course of the pH dependence of Ca^{2+} traversal (Fig. 5) is best explained through reference to the ionic properties of phosphatidic acid. Both PA^{2-} and $Ca(PA)_2^{2-}$ can bind two PO-bound acidic protons, and the similarity of their structures makes it likely that the respective first dissociation constants, and also the second constants, will not be far apart. The pK_1 of PA has been reported as 3.8 in water, 3.0 in 0.1 M NaCl; pK_2 is 8.6 in water, 8.0 in 0.1 M NaCl [32]. Comparison of these values with Fig. 5 does not reveal a straightforward analogy, but a reasonably plausible interpretation can be suggested. The apparent change in protonation at pH 5 must correspond to a first dissociation constant, $pK_1 \approx 5$. The diprotonated complex below pH 4, $H_2[Ca(PA)_2]$, is either unstable (i.e., the two PA come apart) or it cannot cross the bilayer, for reasons yet unknown. The monoprotonated complex, pH > 5, is obviously a calcium ionophore. This complex is possibly stabilized by hydrogen bond sharing, PO-H · · · OP $\rightleftharpoons$ PO · · · H-OP, as previously suggested [3]. Surprisingly, further deprotonation leads to further increase in the rate of Ca^{2+} traversal, up to a pH of 9.6, where the complex must be entirely in its dianionic state. At physiological pH, this species seems to be the major calcium ionophore. Reusch [33] came to the same conclusion. At this time, we cannot offer an explanation for this effect.

These results demonstrate how profoundly a membrane-associated mechanism – calcium traversal – may be influenced by the composition of the hydrogen belts of the membrane. They hint at a rationale for the diversity of phospholipid structures in these regions: these may have the

function of controlling the passage and the storage of Ca^{2+} in the membrane. Our experiments leave many questions unanswered. For example, 1-ether phospholipids (non-vinyl) are also, presumably, passive in regard to calcium; why, then, don't organisms rest at the 1-ether level instead of proceeding to synthesize 1-vinylether phospholipids? Why do membranes with much plasmalogen, e.g., myelin, also contain much cholesterol and sphingomyelin, lipids which strongly interfere with $[Ca(PA)_2]$ formation? And the most significant question: can there be enough PA available in some biological membranes to make calcium ferrying feasible? Where will ferrying occur, and under what conditions? However, it is perhaps not so much Ca^{2+} transport but Ca^{2+} storage which is modulated by the composition of the hydrogen belt of the membrane.

Acknowledgement

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