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Calcium translocation in liposome systems modeled on the mitochondrial inner membrane

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Ca^{2+} uptake by liposomes consisting of phosphatidylcholine (PC) and cardiolipin (CL) has recently been reported (Smaal, E.B. et al. (1987) Biochim. Biophys. Acta 897, 191–196). In eukaryotic cells, CL is localized exclusively in the inner mitochondrial membrane, where it occurs in the presence of equimolar amounts of PC and phosphatidylethanolamine (PE). We have therefore re-examined CL-mediated Ca^{2+} translocation in liposomes of more nearly physiological composition, i.e., PC/PE/CL (2:2:1 and 4:4:1, mol/mol). In addition, the effect on Ca^{2+} uptake of plasmalogens of PE, which may account for up to 50% of mitochondrial PE, was determined. Our findings can be summarized as follows. (1) Ca^{2+} uptake into CL-containing liposomes was increased dramatically by PE. (2) Ca^{2+} entry into PC/CL liposomes was biphasic; in the presence of PE, uptake was dominated by a slow process. (3) Ca^{2+} uptake by PC/CL liposomes saturated at ≤ 2 mM external Ca^{2+}, whereas uptake into PC/PE/CL liposomes increased with increasing Ca^{2+} concentration up to 10 mM or until Ca^{2+} release ensued. (4) Ca^{2+} translocation by PE-containing liposomes and the slow phase of Ca^{2+} uptake into PC/CL liposomes were similarly and highly dependent on temperature. It can therefore be proposed that PE amplifies the slow component of CL-mediated Ca^{2+} translocation. This process is characterized by a requirement for high external Ca^{2+} concentrations and a large apparent activation energy. Ca^{2+} uptake was not significantly modified by plasmalogens of PE.

Introduction

The role of lipids in biological membranes is, at present, incompletely understood. Even the simplest membrane contains well over a hundred distinct lipid species. It would appear, however, that the best documented lipid function, namely bilayer formation with the resultant generation of both a support matrix for membrane proteins and a permeability barrier between cellular compartments, would require only one or a few lipid types.

One function that has been proposed for membrane lipids, particularly the acidic forms, is Ca^{2+} transport [1,2]. In the presence of Ca^{2+}, cardiolipin (CL) and phosphatidic acid (PA) have the ability to adopt inverted, non-bilayer configurations [3–5]. One of these structures, the inverted micelle, would provide an ideal vehicle for the sequestration of Ca^{2+} and its transport across the hydrophobic core of a lipid bilayer. Recently, several laboratories have reported the accumulation of Ca^{2+} by liposomes containing CL [6] or PA [7–11]. In particular, Smaal and co-workers [6] have demonstrated the entry of substantial amounts of Ca^{2+} into Arsenazo III-loaded liposomes composed of phosphatidylcholine (PC) and CL (1:1 and 4:1, mol/mol).

Cardiolipin in eukaryotic cells is found exclusively in the inner mitochondrial membrane. The inner mitochondrial membrane contains, in addition to CL, PC and phosphatidylethanolamine (PE) in almost equimolar amounts. Compositions ranging from 2:3:1 to 4:3.5:1 (PC/PE/CL) have been reported [12,13]. We have therefore examined Ca^{2+} translocation into CL-containing liposomes of more nearly physiological composition, namely PC/PE/CL (2:2:1) and PC/PE/CL (4:4:1). The former contain the same overall amount of CL as the PC/CL (4:1) liposomes investigated by Smaal et al. [6].

Abbreviations: CL, cardiolipin; Mops, 4-morpholinepropanesulphonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE_{pl}, PE-plasmalogen; PL, phospholipid.

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A substantial fraction of inner membrane PE (46% in pig heart [12], 20% of total mitochondrial PE in rat heart [14]) is present as the plasmalogen. Replacement of diacyl-PC with diether-PC markedly enhances Ca^{2+} uptake by PA-containing liposomes [15]. Furthermore, PC inhibits PA-mediated Ca^{2+} translocation whereas PC-plasmalogen does not [16]. Ca^{2+} uptake by PC/PE/CL liposomes containing physiological PE-plasmalogen (PE_{pl}) levels (PC/PE/PE_{pl}/CL, 4 : 3 : 1 : 1) has therefore also been measured.

Materials and Methods

Egg phosphatidylcholine, bovine heart phosphatidylethanolamine, bovine brain phosphatidylethanolamine (50% PE-plasmalogen), and bovine heart cardiolipin were purchased from Avanti Polar Lipids (Pelham, AL). Cardiolipin (disodium salt) was also obtained from Sigma Chemical (St. Louis, MO) as were the sodium salt of Arsenazo III and all other biochemicals. Other reagents were of the highest quality available.

Liposome preparation. CL was tested routinely for Ca^{2+} contamination using Arsenazo III and for lipid peroxidation by monitoring conjugated diene absorbance (233 nm) of cyclohexane solutions [17]. CL preparations from Avanti were highly variable, containing up to 10 mmol Ca^{2+} and 0.05 to 1.9 mol diene per mol lipid. Sigma CL, which did not contain detectable Ca^{2+} and had less than 0.02 dienes per CL, was used in the experiments reported. Lipids (generally 288 μmol) were dried under a N_2 stream, resuspended in 1.5 ml cyclohexane, and frozen in liquid N_2. The frozen lipids were lyophilized overnight to yield a powdery white residue. (Lyophilizing from chloroform was discontinued due to the difficulty of effecting complete solvent removal from the gel remaining after evaporation under N_2.)

Large unilamellar liposomes were prepared essentially as described by Hope and co-workers [18]. Briefly, multilamellar vesicles were formed by dispersing (vortex mixing) the dried lipids in aqueous buffer (125 mM KCl, 20 mM Mops (pH 7.2), 3 mM Arsenazo III) at a final concentration of 32 or 64 mM. The vesicle preparations were frozen in liquid N_2 and thawed in a water bath (55°C) a total of five times. The preparations were then extruded ten times, under N_2 pressure, through two (stacked) 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) utilizing an apparatus supplied by Lipex Biomembranes Inc. (Vancouver, B.C., Canada). Following extrusion, the liposomes were subjected to an additional freeze/thaw cycle and extrusion (one pass) through two (stacked) 200 nm polycarbonate filters. This final freeze/thaw and extrusion sequence was repeated once. The extrusion apparatus was maintained at 50–55°C throughout. Before each use, the extruder (assembled) and all labware coming in contact with the liposome preparations were washed with EDTA and EGTA (150 mM each), then rinsed extensively (10–12 times) with distilled water. After processing, the liposome preparations were dialyzed in the dark at 4°C against standard buffer (125 mM KCl, 20 mM Mops (pH 7.2)) to remove unentrapped dye. Liposomes were stored at 4°C in the dark under N_2. Ca^{2+} uptake behavior was stable for at least three weeks.

Determination of liposome encapsulated volume. The encapsulated metallochromic indicator Arsenazo III was utilized in determinations of both internal volume and Ca^{2+} uptake. Measurements were performed with an SLM-Amino DW-2000 dual wavelength spectrophotometer in the dual wavelength mode at the wavelength pair 650–700 nm. Temperature was maintained by means of a circulating water bath. For volume determinations an aliquot (75 –150 μl) of the vesicle preparation was added to 2.5 ml of standard buffer containing 1 mM $CaCl_2$ to saturate any external Arsenazo III. Pulses (2.5 μmol) of EGTA were then added until no additional decrease in absorbance was observed. The absorbance change induced by EGTA (E) was used to calculate the concentration of dye outside the vesicles ($E/\Delta\epsilon'$). ($\Delta\epsilon'$, the molar differential absorption coefficient was determined for each batch of encapsulated dye by measuring the difference in absorbance between Ca^{2+}-saturated and Ca^{2+}-free Arsenazo III at multiple dilutions.) Triton X-100 (50 μl of a 20% solution) was added to lyse the liposomes, followed by pulses (6.25 μmol) of $CaCl_2$ until the maximum obtainable absorbance was reached. The absorbance change elicited by Ca^{2+} (T) is an indication of the total Arsenazo III concentration in the cuvette ($T/\Delta\epsilon'$). The amount of dye entrapped in the liposome aliquot is thus

$$2.5 \cdot 10^{-3} \cdot (T - E)/\Delta\epsilon'$$

which can be converted to an encapsulated volume by assuming that Arsenazo III was encapsulated at its original concentration of 3 mM. Encapsulated volumes determined with methylumbelliferyl phosphate [19] did not differ significantly from those obtained with Arsenazo III. Any Ca^{2+} or dye redistributions occurring during the relatively rapid sizing measurements were thus too small to influence the results. Encapsulated volume and phospholipid phosphorus content [20] were used to calculate liposome size according to the equation [21]

$$D = 30.7V + 10.0$$

where D is the liposome diameter (in nm) and V is the encapsulated volume (in l/mol phosphate). This formulation is based on an average phospholipid area of 65 $Å^2$ and a bilayer thickness of 50 Å. The values obtained are summarized in Table I and are in good agreement with

TABLE I

Mean volume and diameter of liposome preparations

Means ± S.D. are shown for the number of determinations indicated in parentheses. The volume and diameter of PC/CL (4:1) liposomes differ significantly ($P < 0.05$, Student's t-test) from those for other liposome preparations. Other details are outlined under Materials and Methods.

Liposome composition (mol/mol)	Volume (l/mol)	Diameter (nm)
PC/CL (4:1)	4.57 ± 1.05 (4)	150.2 ± 32.4
PC/PE/CL (2:2:1)	2.56 ± 0.33 (7)	88.6 ± 10.1
PC/PE/CL (4:4:1)	2.54 ± 0.22 (4)	88.1 ± 6.6
PC/PE/PE_{pl}/CL (4:3:1:1)	2.34 ± 0.37 (3)	81.7 ± 11.5

reports in which $^{22}Na^+$ and [^{14}C]inulin were used to determine the internal volume of liposomes prepared by this method [18].

Measurement of Ca^{2+} uptake. Ca^{2+} uptake was measured by a modification of the method of Smaal et al. [22]. Liposomes (2.5 μmol phosphate/ml) were added to standard buffer containing 0.5 to 10 mM $CaCl_2$. Samples (0.75 ml) were removed at intervals following liposome addition. EDTA (18.75 μmol) was added to chelate external Ca^{2+} followed by A23187 (7.5 μl of a 0.1 mg/ml methanol solution) to permit chelation of internal Ca^{2+}. The resultant absorbance decreases are proportional to the amounts of Ca^{2+}-Arsenazo III complex outside and inside the liposomes, respectively, at the time of sampling. The initial Ca^{2+} content of the liposomes was determined by means of a similar titration carried out in the absence of external Ca^{2+}. Ca^{2+} concentration inside the liposomes and percentage Arsenazo III efflux were calculated as previously described [23]. To permit comparison of liposomes with different entrapped volumes, Ca^{2+} uptake was normalized to phospholipid by multiplying the internal Ca^{2+} concentration (millimolar) by the entrapped volume (in l/mol phosphate), yielding uptake in units of mmol Ca^{2+} per mol phospholipid (PL). All data shown are representative of multiple ($n \geq 3$) experiments.

In earlier experiments, Triton X-100 was utilized to expose internal Ca^{2+} to chelator [23]. The large turbidity changes induced by the detergent were not, however, cancelled out by the use of dual-wavelength methodology, with the result that Ca^{2+} uptake was overestimated. The divalent cation ionophore A23187, which is not subject to these difficulties, has been used exclusively in the uptake measurements reported here.

Measurement of light scattering. For light scattering measurements, vesicles were prepared as described above but in the absence of Arsenazo III. Liposomes (2.5 μmol phosphate/ml) were added to standard buffer containing 0.5 to 10 mM $CaCl_2$ at 37°C. Samples (1.0 ml) were removed 2 h after liposome addition, and absorbance at 450 nm was measured both before and after the addition of 25 μmol EDTA. EDTA-reversible light-scattering was taken as an indicator of Ca^{2+}-dependent liposome aggregation and EDTA-irreversible scattering as a reflection of the presence of fused structures [6].

Assessment of liposome integrity. Liposomes loaded with methylumbelliferyl phosphate were used to determine the extent to which Ca^{2+} and Arsenazo III fluxes were correlated with a more general breakdown of the liposomal permeability barrier. Methylumbelliferyl phosphate release was quantitated by treating liposome samples with phosphodiesterase (0.5 units/ml), measuring the fluorescence increase due to production of methylumbelliferone [19], and normalizing the results to the amount of methylumbelliferone detected upon phosphodiesterase treatment of an equivalent Triton-permeabilized sample. Standard curves for methylumbelliferone fluorescence were determined in the presence and absence of Triton; detergent effects on fluorescence were substantial.

Results

Smaal and co-workers [6] have reported uptake of Ca^{2+} into CL-containing liposomes from solutions containing 0.5 to 8 mM Ca^{2+}. All data reported were collected after 60 min incubation at 20°C. Under these conditions, Ca^{2+} uptake by PC/CL (4:1) liposomes (expressed as percent of maximal absorbance of the entrapped Ca^{2+}-indicator Arsenazo III) saturated at 4 mM Ca^{2+} and was accompanied by efflux of a Ca^{2+}-Arsenazo complex. Both fluxes were demonstrated to reflect a selective increase in bilayer permeability, occurring in the absence of appreciable K^+ leakage.

The aim of this investigation was to determine the properties of CL-mediated Ca^{2+} uptake in a more nearly physiological lipid environment. As an initial step, however, Ca^{2+} uptake by PC/CL (4:1) liposomes was characterized in some additional detail. A measuring temperature of 37°C was utilized to maximize Ca^{2+} uptake (see below). The time course of Ca^{2+} uptake by PC/CL (4:1) liposomes at 37°C is shown in Fig. 1A for two external Ca^{2+} concentrations. Uptake was biphasic, with a very rapid initial phase being followed by a period of slower Ca^{2+} entry. In contrast to the earlier report [6], virtually no Arsenazo III efflux could be detected (data not shown). Ca^{2+} uptake was completely dependent on the presence of CL. Uptake was undetectable with liposomes consisting of PC only and with PC/PE (6:4) liposomes (not shown).

Ca^{2+} uptake by liposomes containing the same amount of CL, but in which half of the PC was replaced by PE (PC/PE/CL, 2:2:1) differed dramatically in terms both of Ca^{2+} uptake (Fig. 1B) and dye efflux (Fig. 1C). Most notably, (1) Ca^{2+} uptake into these liposomes was dominated by a slow, sustained process.

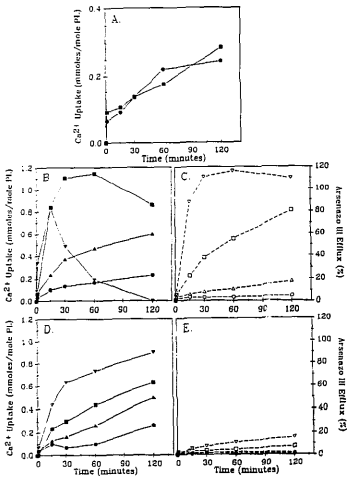

Fig. 1. Time course of Ca^{2+} uptake (filled symbols) by PC/CL (4:1) liposomes (A), PC/PE/CL (2:2:1) liposomes (B), and PC/PE/CL (4:4:1) liposomes (D) and of Arsenazo III efflux (open symbols) from PC/PE/CL (2:2:1; C) and PC/PE/CL (4:4:1; E) liposomes. Measurements were carried out at 37°C in standard buffer containing 2 mM (●, ○), 4 mM (▲, △), 6 mM (■, □), or 8 mM (▼ ▽) $CaCl_2$.

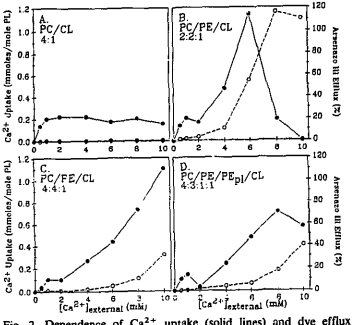

Fig. 2. Dependence of Ca^{2+} uptake (solid lines) and dye efflux (broken lines) on external Ca^{2+} concentration. Measurements were made 60 min after the addition of liposomes to standard buffer at 37°C supplemented with the indicated concentrations of $CaCl_2$. (A) PC/CL (4:1); (B) PC/PE/CL (2:2:1); (C) PC/PE/CL (4:4:1); and (D) PC/PE/PE_{pl}/CL (4:3:1:1) liposomes. The apparent lack of Ca^{2+} uptake at 2 mM Ca^{2+} shown in panel D is an artifact; it was not observed in measurements made at 120 min in the same experiment or when the experiment was repeated.

(2) Ca^{2+} uptake was substantially greater and was followed, at Ca^{2+} concentrations in excess of 4 mM, by apparent Ca^{2+} efflux. (3) Arsenazo III efflux from PC/PE/CL (2:2:1) liposomes was substantial.

When the cardiolipin content of the liposomes was reduced to more nearly physiological levels (PC/PE/CL, 4:4:1), the prolonged Ca^{2+} uptake process observed with PC/PE/CL (2:2:1) liposomes was preserved although somewhat slowed (Fig. 1D). Arsenazo III efflux, on the other hand, was almost completely suppressed (Fig. 1E). Replacement of 25% of the PE in PC/PE/CL (4:4:1) liposomes with PE_{pl} had essentially no effect on the time course of Ca^{2+} uptake or Arsenazo III efflux (data not shown).

The dependence of Ca^{2+} uptake on external Ca^{2+} concentration is summarized in Fig. 2 for the four vesicle types. Ca^{2+} uptake increased markedly at external Ca^{2+} concentrations exceeding 2 mM for liposomes containing PE (Fig. 2, B–D). In all cases, Ca^{2+} uptake occurred at lower external Ca^{2+} concentrations than did Arsenazo efflux. This difference was accentuated by decreasing liposome CL content. Incorporation of 25% PE_{pl} into the liposomes (Fig. 2D) may have marginally enhanced Arsenazo III efflux at the expense of Ca^{2+} uptake. In contrast to the results obtained with PC/CL liposomes (Fig. 2A and Ref. 6) for which Ca^{2+} uptake was maximal at 2 mM external Ca^{2+}, no evidence of

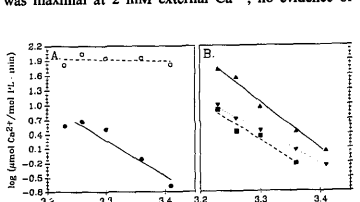

Fig. 3. Representative Arrhenius plots for the rate of Ca^{2+} uptake over the temperature range 20 to 37°C. (A) PC/CL (4:1) liposomes: initial rapid phase (○; slope = −0.32, r = 0.232) and slower phase (●; slope = −7.47, r = 0.950). (B) Ca^{2+} uptake rates for PC/PE/CL (2:2:1, ▲; slope = −9.68, r = 0.994); PC/PE/CL (4:4:1, ■; slope −8.03, r = 0.975); and PC/PE/PE_{pl}/CL (4:3:1:1, ▼; slope = −6.90, r = 0.999) liposomes. External Ca^{2+} concentration was 6 mM. The rate of the rapid uptake phase, observed only with PC/CL liposomes, was estimated from the 1 min time point. Rates for the slower uptake process were estimated from the linear portion of the time course beginning at 1 min. Correlation coefficients for the linear least-squares lines are indicated as r values.

saturation of the Ca^{2+} uptake process was seen in preparations containing PE.

The effect of temperature on Ca^{2+} uptake by all four types of vesicle preparation was also determined. An Arrhenius plot for the slow phase of Ca^{2+} uptake by PC/CL (4:1) liposomes from 6 mM external $CaCl_2$ yielded a slope of −7.47, corresponding to an apparent activation energy of 34.0 kcal/mol. The initial rapid component was essentially temperature independent (Fig. 3A). Average values of −8.62, −7.11, and −7.10 were obtained for the slopes of Arrhenius plots for Ca^{2+} entry under the same conditions into PC/PE/CL (2:1:1), PC/PE/CL (4:4:1), and PC/PE/PE_{pl}/CL (4:3:1:1) liposome preparations, respectively (Fig. 3B). These values, which correspond to apparent activation energies in the range of 30 to 45 kcal/mol, did not differ significantly from each other or from the value determined for slow uptake by PC/CL liposomes.

Fig. 4 shows Ca^{2+}-induced light scattering, measured at 450 nm, for liposomes prepared in the absence of Arsenazo III. No evidence of Ca^{2+}-induced aggregation or fusion was observed in PC/CL (4:1) liposomes (Panel A). This is in agreement with Smaal et al. [6] who failed to detect aggregation or fusion at external Ca^{2+} concentrations below 12 mM. In contrast, liposomes composed of PC/PE/CL (2:2:1) showed a large increase in light scattering, which was only partially reversed upon addition of EDTA, at external Ca^{2+} concentrations exceeding 4 mM. For liposomes composed of PC/PE/CL (4:4:1) and PC/PE/PE_{pl}/CL (4:3:1:1) increased light scattering was only seen at external Ca^{2+} concentrations exceeding 8 mM. Again, reversibility upon EDTA addition was limited.

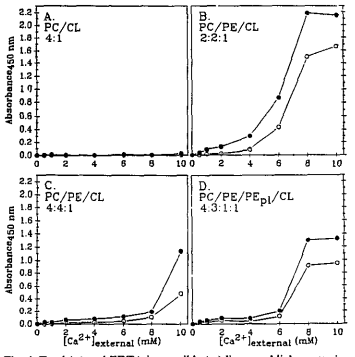

Fig. 4. Total (●) and EDTA-irreversible (○) liposomal light scattering as a function of external Ca^{2+} concentration. (A) PC/CL (4:1); (B) PC/PE/CL (2:2:1); (C) PC/PE/CL (4:4:1); (D) PC/PE/PE_{pl}/CL (4:3:1:1).

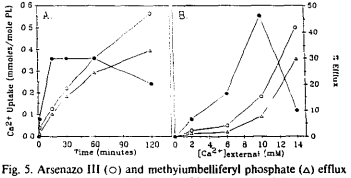

Fig. 5. Arsenazo III (○) and methylumbelliferyl phosphate (△) efflux from PC/PE/CL (4:4:1) liposomes. Liposomes containing Arsenazo III and methylumbelliferyl phosphate were prepared and assayed in parallel. (Ca^{2+} uptake data (●) are included for comparison.) All measurements were at 37°C. (A) Time course of marker release. Liposomes were added to standard buffer containing 14 mM $CaCl_2$ at time zero. In the presence of 6 mM $CaCl_2$ Ca^{2+} uptake over a two hour time course was substantial; marker release was negligible (not shown). (B) Dependence of marker release on external Ca^{2+} concentration. Measurements were made 120 min after the addition of liposomes to buffer containing the indicated Ca^{2+} concentrations.

The extent to which Ca^{2+} and Arsenazo III fluxes reflect a generalized increase in liposome permeability was assessed for PC/PE/CL (4:4:1) liposomes via parallel measurements of Arsenazo III and methylumbelliferyl phosphate release. When Ca^{2+} concentrations sufficiently high to elicit marker release were used, methylumbelliferyl phosphate release was correlated, as a function of time (Fig. 5A) and Ca^{2+} concentration (Fig. 5B), with Arsenazo III efflux. In contrast, Ca^{2+} uptake occurred, over a wide range of Ca^{2+} concentrations, in the absence of fluxes of the other markers.

Discussion

Several conclusions regarding the role of individual phospholipids in Ca^{2+} uptake by model liposome systems can be drawn based on the results summarized above. First, Ca^{2+} uptake is dependent on CL content. No uptake occurred in the absence of CL. Ca^{2+} uptake was enhanced, and occurred at lower external Ca^{2+} concentrations, when the CL content of PC/PE/CL liposomes was increased from 11% to 20% (Fig. 1, panels B and D). CL appears also to be involved in release of the Arsenazo-Ca^{2+} complex from liposomes. This phenomenon was dramatically stimulated at 20% CL as compared to 11% CL (Fig. 1, panels C and E) in PC/PE/CL liposomes. These two functions of CL are probably not identical. A comparison of Figs. 2, 4, and 5 suggests that release of the Ca^{2+}-Arsenazo complex was associated with Ca^{2+}-induced increases in light scattering (CL-dependent liposome aggregation [6]) and generalized marker leakage. It is clear from PC/PE/CL

(4:4:1) liposomes that Ca^{2+} uptake can occur, over a wide range of conditions, in the absence of Arsenazo efflux (Fig. 1), vesicle aggregation (Fig. 4), or an overall increase in bilayer permeability (Fig. 5).

The effects of PE on Ca^{2+} uptake can be summarized as follows. (1) PE dramatically enhanced CL-mediated Ca^{2+} uptake. PE-containing liposomes (PC/PE/CL, 2:2:1) accumulated up to 1.2 mmol Ca^{2+} per mol phospholipid (Fig. 1B), whereas Ca^{2+} uptake by PC/CL liposomes with the same CL content did not exceed 0.3 mmol/mol PL. (2) Ca^{2+} uptake by PC/CL liposomes was biphasic; in the presence of PE, Ca^{2+} uptake was dominated by a single, slow process. (3) Ca^{2+} uptake by PC/CL liposomes was saturated at ≤ 2 mM external Ca^{2+} (Fig. 2A), while uptake into PE-containing liposomes increased with increasing external Ca^{2+} concentration up to 10 mM Ca^{2+} or until it was limited by released of the Arsenazo-Ca^{2+} complex (Fig. 2, B–F). (4) Ca^{2+} uptake into PE-containing liposomes was dramatically stimulated by increased temperature in the physiological range, as was the slow phase of Ca^{2+} uptake into PC/CL (4:1) liposomes. However, over the first minute, uptake in the absence of PE was largely temperature independent (Fig. 3A). Taken together, these observations suggest that PE stimulates a slow Ca^{2+} uptake process which occurs to only a limited extent in PC/CL liposomes. As this process is enhanced, its requirement for higher external Ca^{2+} concentrations can be discerned.

Smaal and his collaborators [6] reported that, for PC/CL liposomes, both Ca^{2+} uptake and Arsenazo III efflux occurred without alteration of the overall permeability properties of the liposomes. At higher external Ca^{2+} concentrations, Ca^{2+}-induced vesicle aggregation and a generalized increase in liposome permeability were observed, as had been previously reported [24]. As noted above, in this study, Ca^{2+} entry into PC/CL liposomes was not accompanied by Arsenazo III efflux. This may have been due to lower contamination of the liposomes with organic solvents. For the experiments reported here, lipids were lyophilized from cyclohexane rather than being dried from chloroform or ethanol.

For PE-containing liposomes of all three compositions, Arsenazo efflux and Ca^{2+}-induced light scattering changes were correlated in terms of dependence on external Ca^{2+} concentration (Figs. 2 and 4). For PC/PE/CL (4:4:1) liposomes Arsenazo III efflux was additionally found to be associated with methylumbelliferyl phosphate release (Fig. 5). Arsenazo efflux from PE-containing liposomes may thus occur, not as an integral portion of the Ca^{2+} translocation process, but in response to vesicle aggregation. Conversely, Ca^{2+} uptake can occur in the absence of dye loss, particularly with PC/PE/CL (4:4:1) and PC/PE/PE_{pl}/CL (4:3:1:1) liposomes. Figure 4 also suggests that aggregation and fusion events are tightly associated in PE-containing liposomes, possibly as a result of the fusogenic properties of PE [25]; very little EDTA-reversible scattering was detected.

In isolation, under physiological conditions of temperature and pressure, PE adopts inverted configurations in preference to forming a bilayer [26]. It can be proposed that such structures are involved in Ca^{2+} uptake by PE-containing liposomes. The apparent activation energies measured for the process (30–45 kcal/mol) are substantially larger, however, than those reported for the lamellar to hexagonal transition of purified PE [27]. It must therefore also be suggested that, if inverted structures participate in Ca^{2+} translocation by PE-containing model systems, then either some step other than formation of the inverted structures is rate-limiting or ease of inverted phase formation is strongly influenced by lipid composition and/or liposome curvature.

Incorporation of PE-plasmalogen into liposomes composed of PC/PE/CL did not appreciably enhance Ca^{2+} uptake or Arsenazo III efflux. This is in contrast to results obtained by Chauhan et al. [15] who found that substitution of diether-PC for diacyl-PC enhanced Ca^{2+} transport through membranes containing phosphatidic acid and that PC inhibited P_i-mediated Ca^{2+} uptake but PC-plasmalogen did not [16]. The lack of effect of the vinyl ether-linked fatty acids in PE_{pl} on Ca^{2+} uptake may be due to their presence in PE rather than PC. Alternatively, Ca^{2+} translocation mediated by doubly charged CL may be insensitive to the nature of the fatty acid linkages in adjacent lipid molecules.

The mitochondrial inner membrane supports several Ca^{2+} translocation processes: Ca^{2+} uptake via an electrophoretic uniporter and Ca^{2+} efflux by both Na^+-dependent and Na^+-independent routes (for a review see Ref. 28). Attempts to isolate mitochondrial transport proteins have not, however, met with success (Refs. 29, 30, but see also Refs. 31, 32). Involvement of CL in one or more of the mitochondrial Ca^{2+} transport processes can be postulated. The data presented here permit a preliminary evaluation of this hypothesis.

Liposomal Ca^{2+} uptake was most rapid in the experiments summarized in Fig. 1B. Over 30 min, uptake amounting to 1 mmol Ca^{2+}/mol phospholipid (PL) occurred from a 6 mM Ca^{2+} solution. A maximal uptake rate of 0.35 mmol/mol PL was observed after 1 min at 8 mM Ca^{2+}. These values correspond to uptake rates of $5.6 \cdot 10^{-7}$ and $5.8 \cdot 10^{-6}$ mol Ca^{2+}/mol PL per s, and, assuming a PL headgroup area of 65 $Å^2$ [33], Ca^{2+} fluxes of $2.8 \cdot 10^{-16}$ and $3.0 \cdot 10^{-15}$ mol/cm^2 per s. When these fluxes are divided by the initial concentration gradients across the liposome bilayer ($6 \cdot 10^{-6}$ and $8 \cdot 10^{-6}$ mol/cm^3), permeability coefficients of 4.7 $\cdot 10^{-11}$ and $3.7 \cdot 10^{-10}$ cm/s can be calculated. Permeability coefficients on the order of 10^{-12} cm/s have been reported for K^+ and Na^+ in liposome systems. It would

thus appear that Ca^{2+} uptake into PC/PE/CL liposomes is in some way facilitated.

Maximal Ca^{2+} uptake rates of > 350 and 15 nmol/min per mg protein have been reported for the electrophoretic uniporter and Na^+/Ca^{2+} exchanger of mitochondria, respectively [28]. The surface area of the inner membrane is estimated at 1060 cm^2/mg [34]. Maximal Ca^{2+} fluxes of $5.5 \cdot 10^{-12}$ and $2.4 \cdot 10^{-13}$ mol/cm^2 per s can therefore be calculated for the uptake and efflux systems of mitochondria. The largest Ca^{2+} flux measured in the PC/PE/CL liposomes examined here is thus two orders of magnitude smaller than the Ca^{2+} fluxes reported for even the slowest of the mitochondrial transport systems. The Ca^{2+} concentrations utilized (0.5 and 10 mM) also greatly exceeded cytosolic levels.

Data from this simple model appear inadequate to support the hypothesis that mitochondrial lipids mediate mitochondrial Ca^{2+} transport processes. They demonstrate convincingly, however, the ability of CL-containing liposomes to accumulate Ca^{2+} and the stimulatory effect of PE on that process.

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