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# Transmembrane Calcium Movements Mediated by Ionomycin and Phosphatidate in Liposomes with Fura 2 Entrapped†

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*Received October 14, 1987; Revised Manuscript Received March 24, 1988*

ABSTRACT: A novel liposomal method permits studies of Ca movements across the bilayers of multilamellar vesicles (MLV) which had entrapped the Ca-dependent, fluorescent indicator dye Fura 2. Ionomycin-mediated Ca translocation across MLV of phosphatidylcholine (PC)/dicetyl phosphate (DCP), 9:1, obeyed simple first-order kinetics since log–log plots of initial rates versus ionomycin or Ca concentration yielded slopes of approximately 1. Since Ca is translocated in a Ca-dependent fashion in the course of stimulus–response coupling of cells which form diacylglycerol (DAG) and phosphatidate (PA) from polyphosphoinositides, we compared effects of PA with those of DAG. PA and DAG were preincorporated in PC/DCP vesicles, in which trace amounts of ionomycin provided transmembrane potential (due to $Ca^{2+}/H^+$ exchange). Significant increases in Ca movements were observed in the presence of egg lecithin PA, dioleoyl-PA, and dipalmitoyl-PA when compared with DCP- or DAG-containing MLV. DAGs such as 1-oleoyl-2-acetoylglycerol or 1,2-dioleoylglycerol in liposomes decreased rates of Ca translocation. Ca influx into PA-containing MLV was dependent on the mole percent of the PA in bilayers; the complex kinetics of Ca influx were compatible with the formation of nonbilayer states. Incorporation of cholesterol into the liposomes inhibited initial rates of Ca uptake by MLV presumably by condensing the bilayers. Ca influx increased with increasing pH of the external medium from 6.9 to 7.9 in liposomes with an internal pH of 7.4. The results not only indicate that transmembrane pH gradients and the extent of ionization of the ionophore affect rates of Ca translocation across lipid bilayers but also demonstrate that in model systems PA but not DAG promotes Ca translocation, the rate of which is altered by lipid composition of the bilayer and the pH of the suspension.

The levels of free cytoplasmic Ca ($[Ca]_i$)[1] play a critical role in mediating cell responses to extracellular signals (Berridge & Irvine, 1984; Kikkawa & Nishizuka, 1986; Spat et al., 1986). These responses are probably initiated by ligand-induced hydrolysis of inositol phospholipids (Hokin & Hokin, 1953; Nishizuka, 1984a; Berridge, 1984) followed by a rise in the $[Ca]_i$ (Michell, 1975). In absence of phospholipid remodeling, $[Ca]_i$ may be modulated by ionophores (Serhan et al., 1983; Ransom & Cambier, 1986). The ionophore-

†Supported by Grants AR-11949, HL-19721, and AR-01431 from the National Institutes of Health to G.W.
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[1] Abbreviations: $[Ca]_i$, Ca concentration in cytoplasm or internal medium of liposomes; PA, phosphatidic acid or phosphatidate; DAG, diacylglycerol; $IP_3$, inositol 1,4,5-triphosphate; $H_{II}$, inverted hexagonal phase; MLV, multilamellar vesicle(s); PC, egg L-α-phosphatidylcholine; DOPA, dioleoyl-L-α-phosphatidic acid; DPPA, dipalmitoyl-L-α-phosphatidic acid; OAG, 1-oleoyl-2-acetoyl-*sn*-3-glycerol; DOG, 1,2-dioleoyl-*sn*-3-glycerol; DCP, dicetyl phosphate; TX-100, Triton X-100; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $[Ca]_o$, Ca concentration in the external medium.

0006-2960/88/0427-5661$01.50/0 

mediated Ca transport across lipid bilayers of liposomes has been studied in the presence of A23187 (Blau et al., 1984) and ionomycin (Liu & Herman, 1978; Serhan et al., 1981). Among naturally occurring membrane components, phosphatidic acid (PA) has been shown to enhance Ca translocation across organic phases (Tyson et al., 1976; Reusch, 1985), lipid bilayers (Serhan et al., 1981, 1982; Nayar et al., 1984; Ohki et al., 1986), and plasma membranes (Putney et al., 1980; Salmon & Honeyman, 1980). Low levels of PA are present in the membrane of cells as a result of polyphosphoinositide breakdown (Serhan et al., 1983) to form diacylglycerol (DAG) (Nishizuka, 1984b) and inositol 1,4,5-triphosphate ($IP_3$) (Berridge & Irvine, 1984). $[Ca]_i$ increases due to mobilization of Ca from intracellular sources, probably by $IP_3$ (Putney, 1986; Spat et al., 1986), followed by increased permeability of the plasma membrane (Korchak et al., 1984). The mechanism of Ca influx from external sources remains a puzzle. A number of messengers have been postulated as mediators of Ca influx such as inositol 1,3,4,5-tetrakisphosphate, which is formed upon cell stimulation (Hawkins et al., 1986; Michell, 1986; Irvine & Moor, 1986) and may regulate Ca entry from extracellular sources by controlling cation channels in the plasma membrane (Von Tscharner, 1986). On the other hand, Ca may enter via channels formed by lipid interactions in the bilayer of membranes which contain newly formed DAG and/or PA (Michell, 1975; Ohki et al., 1982). Ca may also be transported by PA by forming neutral, hydrophobic complexes (Chauhan & Brockerhoff, 1984; Reusch, 1985) or via localized disruption of the bilayer and formation of nonbilayer structures such as the inverted hexagonal phase ($H_{II}$) (Gruner et al., 1985; Cullis et al., 1985).

In this study, we have used a novel method which employs Fura 2, a highly sensitive fluorescent Ca indicator (Grynkiewitz et al., 1985), to study Ca influx into multilamellar vesicles (MLV). Fura 2 containing liposomes permitted kinetic analysis of the ionophoretic properties of PA and DAG. We have also studied the effects of cholesterol and pH gradients on Ca movements. The results indicate that PA but not DAG promotes Ca translocation across lipid bilayers in a fashion that is enhanced by a $Ca^{2+}/H^+$ gradient, i.e., Ca-dependent transport of Ca.

## Materials and Methods

*Reagents.* Egg L-$\alpha$-phosphatidylcholine (PC), egg lecithin L-$\alpha$-phosphatidic acid, dioleoyl-L-$\alpha$-phosphatidic acid, and dipalmitoyl-L-$\alpha$-phosphatidic acid (egg PA, DOPA, and DPPA, respectively), 1-oleoyl-2-acetoyl-*sn*-3-glycerol (OAG), and 1,2-dioleoyl-*sn*-3-glycerol (DOG) were purchased from Avanti Polar Lipids. Cholesterol, dicetyl phosphate (DCP), Sephadex G-50, Triton X-100 (TX-100), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. Ionomycin was purchased from Calbiochem and Fura 2 (as the pentapotassium salt) from Molecular Probes. Cholesterol was recrystallized twice from methanol; all other reagents were used without further purification.

*Preparation of Liposomes.* MLV were prepared by suspending a dry film with the appropriate lipid composition in a buffered solution of 20 $\mu$M Fura 2 containing 0.145 M potassium chloride, 10 mM HEPES, and 0.5 mM EGTA. The same buffer solution was used in all experiments unless otherwise indicated. The liposomes were prepared at room temperature except for DPPA-containing liposomes which were prepared at 55 °C. The lipids were suspended by vigorous agitation on a Vortex mixer in the presence of several glass beads for about 2 min (Bittman & Blau, 1972). The MLV contained 15 $\mu$mol of lipid/mL of suspension. The resulting liposomes were kept about 2 h at room temperature, refrigerated overnight, and afterward passed, at room temperature, through a 45 × 1 cm Sephadex G-50 medium column and eluted with the buffer in order to replace the external Fura 2. The PC/DCP liposomes were at a molar ratio of 9:1 and PC/DCP/PA at 9:0.5:0.5 unless otherwise indicated.

*Characterization of Liposomes.* The total lipid concentration of the liposomes was estimated by phosphate analysis (Chen et al., 1956). The trapped volume was calculated by measuring the excitation fluorescence intensity at 340 nm of Fura 2 in the presence of excess Ca on a Perkin-Elmer 650-10S fluorescence spectrophotometer. In all experiments, the excitation slit width was 2 nm, and the emission wavelength was set to 510 nm with a slit width of 5 nm. The internal volume of MLV was 6.7 ± 0.5 L of $H_2O$/mol of lipid; this value was used in all calculations. Excitation fluorescence spectra were taken for each preparation in the presence of external Ca ($[Ca]_o$) without and with ionomycin. The absence of Fura 2 efflux was shown by addition of EDTA to a final concentration of 9 mM to the external medium of the liposomes, trapping the Fura 2/Ca complex.

*Calcium Influx Assay.* In preliminary experiments, liposomes containing arsenazo III (Weissmann et al., 1976) were compared with liposomes that had entrapped Fura 2, and the dyes were examined with respect to their sensitivity as Ca indicators. Fura 2 proved to be 2–3-fold more sensitive on a molar basis than arsenazo III. Liposomes with Fura 2 entrapped, at 20 $\mu$M, were used in all the Ca influx measurements. Fura 2 was chosen because of its high sensitivity and since it was demonstrated to be well-suited to measure rapid changes in $[Ca]_i$ (Jackson et al., 1987). Calcium translocation across the lipid bilayer was measured within 40–48 h after liposomes had been prepared, or at 37 °C in a 2.0-mL volume containing approximately 0.62 or 0.8 $\mu$mol of lipid. Lower rates were observed at higher lipid concentrations. To aliquots of buffered MLV (1.8 mL), various concentrations of ionomycin (5 $\mu$L in ethanol) were added and permitted to equilibrate for 5 min. Ca influx was initiated by addition of buffered $CaCl_2$ solution (0.2 mL). The rate of Ca influx was monitored by following the fluorescence change of Fura 2 at excitation wavelengths of 340 and 380 nm as a function of time. The Ca concentration in the internal medium, $[Ca]_i$, was calculated from the fluorescence intensity ratio at 340 and 380 nm (Grynkiewitz et al., 1985) assuming zero $[Ca]_i$ at time zero. Initial rates of Ca influx were estimated from the linear portion of plots of $[Ca]_i$ versus time or from the $[Ca]_i$ changes within the first 10–20 s after Ca addition and equilibration. The results are reported either as nanomoles of Ca per minute considering an internal volume of 6.7 L of $H_2O$/mol of lipid or as nanomolar Ca per minute calculated directly from the fluorescence measurements. No corrections were required for fluorescence changes due to addition of Ca to liposomes since these were minimal when empty liposomes (no Fura 2) were used.

## Results

*Encapsulation of Fura 2 in Liposomes.* The excitation spectra of Fura 2 entrapped in PC/DCP and PC/DCP/PA MLV, measured within about 3 min after the adjustment of the $[Ca]_o$ to 6.0 mM, are given in Figure 1. Similar spectra were obtained with a $[Ca]_o$ of 0–6 mM. Incubation of the liposomes with 2–10 nM ionomycin, in the presence of $[Ca]_o$, induced a drastic increase in the fluorescence intensity at 340

Table I: Effect of Lipid Composition on Initial Rates of Ca Influx into Liposomes[a]

| | $[Ca]_i$ ($\Delta$nM/min)[b] | | | |
|---|---|---|---|---|
| | PC/DCP | | PC/cholesterol/DCP | |
| added lipid | -ionomycin | +ionomycin | -ionomycin | +ionomycin |
| | 1.1 ± 0.2[c] | 93.2 ± 3.4 (12) | 0.9 ± 0.1 | 55.8 ± 3.4 (7) |
| OAG | 2.1 ± 0.1[c] | 63.2 ± 2.9 (6) | ND[d] | ND[d] |
| DOG | 1.0 ± 0.3[c] | 55.2 ± 3.5 (6) | ND[d] | ND[d] |
| egg PA | 5.8 ± 0.3[c] | 155.8 ± 8.7 (4) | 3.0 ± 0.3 | 118.0 ± 9.9 (3) |
| DPPA | 5.1 ± 2.2 | 174.1 ± 8.5 (8) | 2.2 ± 0.2 | 90.4 ± 3.5 (10) |
| DOPA | 5.0 ± 2.1 | 157.1 ± 7.3 (9) | ND[d] | ND[d] |

[a] Total lipid in 2.0 mL was 0.62 $\mu$mol, PC/DCP was 9:1, and PC/cholesterol/DCP was 5.7:3.3:1.0. The added lipids were preincorporated at 5 mol %, replacing 5 mol % of DCP. $[Ca]_o$ = 2 mM. Ionomycin, when present, was added to the preformed MLV to a final concentration of 2 nM. The data are expressed as the increase in $[Ca]_i$, mean ± SE ($n$). Measurements were made at 37 °C. [b] Apparent change in $[Ca]_i$ of liposomes as determined by Fura 2. [c] $[Ca]_o$ = 6.0 mM. [d] Not determined.

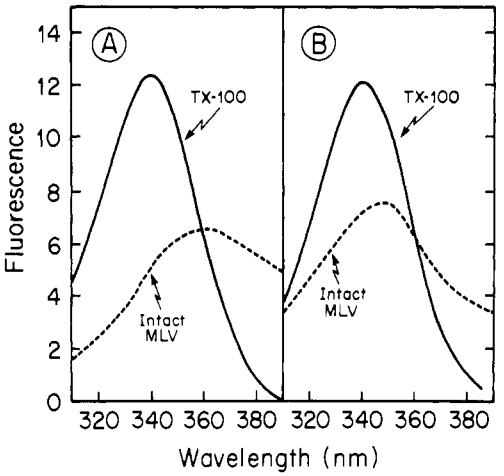


FIGURE 1: Excitation spectra of MLV entrapping Fura 2 (---) and disrupted with 0.2% TX-100 (—). The total lipid concentration was 0.31 $\mu$mol/mL with 20 $\mu$M Fura 2 entrapped. $[Ca]_o$ was 6.0 mM. The lipid composition was (A) PC/DCP 9:1 and (B) PC/DCP/PA 9:0.5:0.5.

nm and decreased it above 360 nm (not shown). Disruption of the liposomes with Triton yielded a fluorescence spectrum typical of the Fura 2/Ca complex (Grynkiewitz et al., 1985) (Figure 1). Comparison of the fluorescence spectra of PC/DCP/PA (9.0:0.6:0.4) liposomes incubated 1 h with ionomycin (3 nM) and $[Ca]_o$ (2 mM) was similar in the presence or absence of EDTA (9 mM) in the external medium (Figure 2), indicating that the Fura 2 complex was present within the lipid vesicles which excluded EDTA (Serhan et al., 1981). Upon Triton treatment, the fluorescence spectrum of the EDTA-containing sample showed free Fura 2 whereas the sample without EDTA continued to show the Fura 2/Ca complex (Figure 2).

*Ionomycin-Mediated Ca Influx into MLV.* Changes in the fluorescence intensity evoked by ionomycin at 340 and 380 nm as a function of time in PC/DCP (9:1) liposomes entrapping 20 $\mu$M Fura 2 were recorded. From these values, $[Ca]_i$ was calculated. In Figure 3, the initial rates are given of ionomycin-induced Ca uptake by liposomes in nanomoles of Ca taken up by liposomes (2.0 mL containing 0.8 $\mu$mol of lipids) trapping a total volume of 5.36 $\mu$L of aqueous solution of Fura 2. The initial rates increased with increasing ionomycin concentration at constant $[Ca]_o$ (1.5 mM) as well as with increasing $[Ca]_o$ in the presence of ionomycin (10 nM). The logs of initial rates versus the logs of ionomycin concentration and $[Ca]_o$ were plotted and yielded slopes of 0.99 and 1.03 (panels A and B, respectively, of Figure 3). These results support a first-order reaction with respect to both ionomycin and Ca. The apparent second-order rate constant for this process was found to be 6.0 ± 0.4 $mM^{-1}$ $min^{-1}$.

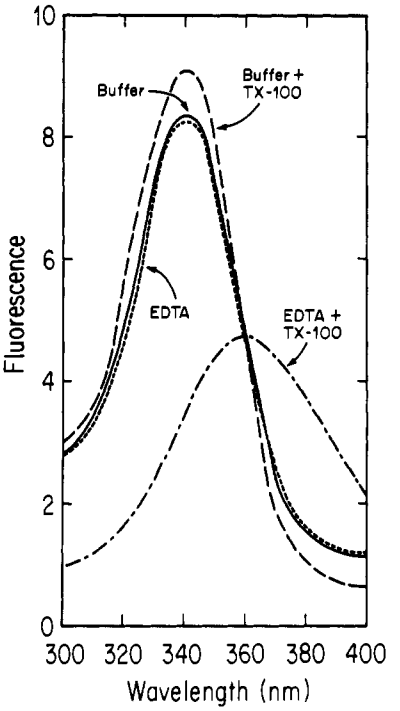


FIGURE 2: Excitation spectra of PC/DCP/PA (9:0.6:0.4) liposomes entrapping Fura 2/Ca complex incubated 1 h with 3 nM ionomycin in 2.0 mM $[Ca]_o$ with no EDTA in the external medium (—) and with 9 mM EDTA outside (---) and after addition of 0.2% TX-100 to the EDTA-free sample (– –) and the one containing EDTA (–·–). The total lipid concentration was 0.28 $\mu$mol/mL with 20 $\mu$M Fura 2 entrapped.

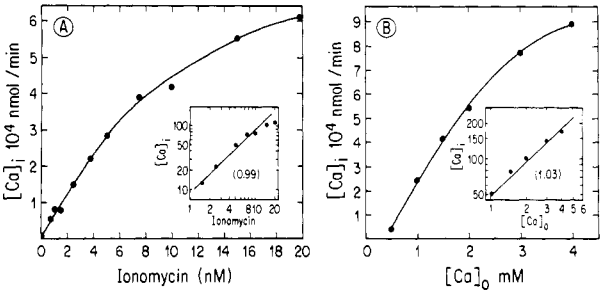


FIGURE 3: Initial rates of calcium influx into PC/DCP 9:1 liposomes as a function of (A) ionomycin concentration and (B) calcium concentration. The total lipid concentration was 0.4 $\mu$mol/mL with 20 $\mu$M Fura 2 entrapped in a total volume of 5.36 $\mu$L. Insets: Plot of the log of the initial rate of Ca entrance in liposomes in nanomolar per minute as a function of (A) log of ionomycin concentration in nanomolar and (B) log of calcium concentration in millimolar.

*Effect of Lipid Composition on Ca Uptake by MLV.* In Table I are summarized the initial rates of influx in PC/DCP (9:0.5) liposomes containing 5 mol % DAG or PA. Inhibition of 30–40% was observed in the presence of OAG and DOG

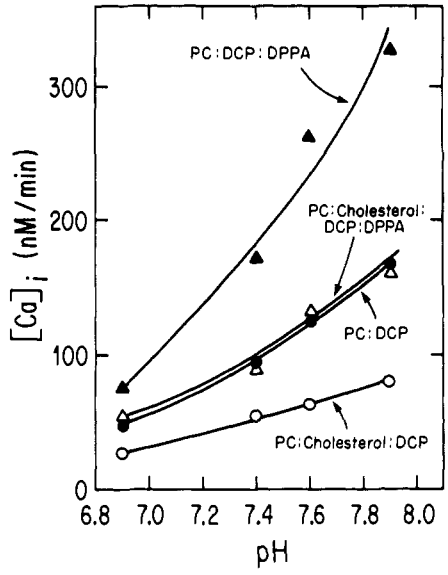


FIGURE 4: Effect of pH in the external medium of the liposome suspension on the initial rates of Ca uptake by MLV. The total lipid concentration was 0.31 μmol/mL; the lipid composition was PC/DCP 9:1 (●), PC/cholesterol/DCP 5.7:3.3:1 (○), PC/DCP/DPPA 9:0.5:0.5 (▲), and PC/cholesterol/DCP/DPPA 5.7; 3.3:0.5:0.5 (△). Ionomycin was added to the preformed MLV to a final concentration of 2 nM. $[Ca]_o$ was 2.0 mM. Rates were calculated from the increase in $[Ca]_i$ within the first 10–20 s after addition of Ca to the external medium.

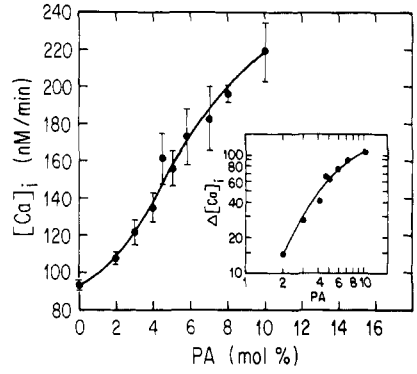


FIGURE 5: Initial rates of Ca influx as a function of the mole percent of PA in PC/DCP liposomes. The total lipid concentration was 0.31 μmol/mL; the lipid composition was PC/(DCP + egg PA) 9:1; the liposomes were incubated with 2 nM ionomycin. $[Ca]_o$ was 2.0 mM. For calculation of rates, see legend to Figure 4. Inset: Plot of log of PA-dependent rate increase as a function of log of mole percent of PA. The initial rate of Ca influx into PC/DCP (9:1) vesicles was subtracted from subsequent rates in PA-containing liposomes.

whereas a 67–69% increase in rates was observed in the presence of egg lecithin PA or DOPA and the rate increased approximately 87% in the presence of DPPA. In the absence of ionomycin, the Ca influx was 1–2 orders of magnitudes lower with slightly higher rates in PA-containing liposomes (Table I). In all the liposome preparations, replacement of PC by cholesterol to yield 33 mol % of the total lipid had an inhibitory effect on the rate of Ca influx into the vesicles (Table I and Figure 4). The presence of Mg did not affect the rate of Ca influx.

*Effect of Transmembrane Proton Gradient on Ca Movement.* Results of experiments with ionomycin-induced Ca movements are summarized in Figure 4. They show that changes in the external pH affect the initial rates of Ca translocation across the bilayer. When the pH of the external Ca-containing medium was raised above that of the internal aqueous compartment, an increase in the initial rates was observed, whereas at pH 6.9 Ca translocation was diminished. Moreover, the slopes of curves relating Ca uptake to pH indicate that cholesterol buffered the effects of pH on the rates of Ca translocation, presumably by virtue of cholesterol effects on bilayer structure (Bittman & Blau, 1972).

*Effect of PA on Ca Translocation Across Lipid Bilayers.* It has been established that the presence of PA increases the rate of Ca influx into liposomes (Serhan et al., 1982; Ohki et al., 1986). In Figure 5 we show that an increase of the concentration of PA in the bilayer (from 2 to 10 mol %) causes an increase in the initial rate of Ca movement into MLV. A log–log plot of the increase in the initial rates due to the presence of PA as compared with rates with no PA in the bilayer as a function of PA concentration yielded a curve with a slope of about 1.8 at low PA content and a slope of 1 at 7–10 mol % PA, indicating that the rate dependence on PA is complex in nature.

## DISCUSSION

Ca fluxes in liposomes have been measured by a variety of indicator dyes such as arsenazo III (Weissmann et al., 1980; Blau et al., 1984; Small et al., 1985) and quin 2 (Utsumi et al., 1985; Ohki et al., 1986). Recently, measurements of $[Ca]_i$ have been undertaken in a variety of cells by means of the permeant acetoxy methyl ester of Fura 2 (Tsien et al., 1985; Scanlon et al., 1987) which is trapped as a result of its intracellular hydrolysis.

In this study, Fura 2 containing liposomes were employed to investigate ionomycin-mediated Ca translocation across the bilayer. The order of reaction with respect to ionophore and Ca was determined by plotting the log of the initial rates of Ca influx as a function of the log of ionomycin or Ca concentration (insets to panels A and B, respectively, of Figure 3). The slopes of the log–log plots were 0.99 for the plot versus ionomycin concentration and 1.03 for the plot versus Ca concentration. It can therefore be assumed that in the concentration range of 1.5–10 nM ionomycin and 1–4 mM Ca the species that traverses the membrane is one Ca ion enveloped by one ionomycin molecule. These results are in agreement with the findings that ionomycin behaves as a dibasic acid (Toeplitz et al., 1979) binding one ion per molecule to form the Ca salt (Liu & Herman, 1978).

We also studied the transmembrane movement of Ca in the presence of DAG or PA. Arsenazo III studies indicate that Ca but not Mg translocation is enhanced in the presence of PA (Serhan et al., 1981; Chauhan & Brockerhoff, 1984). The initial Ca influx mediated by ionomycin established a transmembrane potential due to $Ca^{2+}/H^+$ exchange. Ohki et al. (1986) have reported that at low concentrations DAG from natural sources has no effect on Ca ionophoresis. We observed that 5 mol % DOG or OAG inhibited Ca influx into lipid vesicles (Table I). On the other hand, incorporation of 5 mol % of egg lecithin PA, DOPA, or DPPA into PC/DCP vesicles increased the rate of Ca influx significantly (Table I). These results are in agreement with data previously reported (Serhan et al., 1981; Nayar et al., 1984; Chauhan et al., 1986; Ohki et al., 1986). We have found that DPPA at 5 mol % has similar Ca ionophoretic activity to DOPA (Table I), in agreement with previous reports (Ohki et al., 1986). These results serve as further proof that PA—with saturated or unsaturated fatty acids in the $R_1$ or $R_2$ position—is a Ca ionophore in model membranes and does *not* require oxidation of its double bonds for this action (Holmes & Yoss, 1983).

To determine whether bilayer stability influences Ca movement in PA liposomes, we prepared these with and without cholesterol. Cholesterol reduces the permeability of

fluid lipid bilayers to a variety of solutes (DeGier et al., 1968; Bittman & Blau, 1972) including ionophore-mediated Ca translocation across membranes (Blau et al., 1984). We have found (Table I) that cholesterol inhibited the transmembrane movement of Ca in liposomes with or without PA. Therefore, the effect of cholesterol is most likely due to changes in overall bilayer packing rather than to the effects of cholesterol on the liganding properties of PA for Ca (Chauhan et al., 1986). In fact, the "buffering" effect of cholesterol in PC and PC/PA liposomes on the slope of $[Ca]_i$ (Figure 4) confirms the hypothesis that bilayer structures support Ca movements less well than nonbilayer states.

Imposition of a proton gradient across the liposomal membrane enhanced the initial rates of Ca influx in MLV. The more negative the external Ca-containing medium became as compared to the internal medium (pH 7.4), the greater the rate of Ca uptake by the MLV (Figure 4). This effect may be due to an increased binding of the Ca to the ionophoretic species which is more ionized at higher pHs. Ionomycin-mediated Ca movement is slow at pH 7.0 and increases with increasing pH to a maximum at about 9.5 (Liu & Herman, 1978). The enhancement of Ca movement is higher in the presence of DPPA than in MLV without PA. At pH 7.4, the bulk of PA carries one negative charge on the PA. Increased charge density and Ca-induced phase separation (Trauble & Eible, 1974; Koter et al., 1978) are likely to create sites that may permit increased Ca movement across the bilayer. The rate increase might also be attributed to a higher effective $[Ca]_o$ due to electrostatic attraction of Ca to the negatively charged membrane surface (McLaughlin et al., 1971; Haynes, 1974). However, major changes are not expected to result from variation in the effective $[Ca]_o$ since the net charge of the membrane surface was not altered when PA was replaced by equimolar DCP. Since all bilayers of MLV have identical $\zeta$ potentials in the presence of ionomycin, they would all undergo equal charge shielding by Ca, and the internal aqueous compartment would shrink (Bangham, 1968). Shrinkage would be accompanied by changes in light scattering on addition of Ca, but we obtained no such evidence. However, the inhibition of Ca influx in the presence of OAG and DAG (Table I) might be the result of a decrease in the negative charge of the membrane surface.

In order to further investigate the mechanism by which PA may translocate Ca, Ca influx was monitored at increasing concentrations of egg lecithin PA. It was found that the initial rates of Ca influx in liposomes increased with increasing PA concentration in the bilayer up to a total of 10 mol %. In contrast to studies which employ over 10 mol % anionic lipids in the bilayer (Lansman & Haynes, 1975; Wilschut et al., 1980), we found little change in the light scattering of the MLV preparations upon addition of Ca. We have examined membrane fusion (Dunham et al., 1977) with preparations similar to these studied, using the arsenazo III method. Unless high concentrations of fusogens such as lysolecithin were added, no fusion was observed. We attempted to establish the order of PA-mediated Ca movement from log–log plots of the initial rate of Ca influx in excess of influx in the absence of PA versus the mole percent of PA. At low PA concentration, up to 4.5 mol %, the slopes of these plots were about 2, which is in agreement with the suggestion of the formation of a hydrophobic $Ca(PA)_2$ complex (Chauhan et al., 1986) and with the stoichiometry of about 0.6 nmol of Ca/nmol of PA found in dioleoyl-PC/DOPA, 8:2, liposomes (Small et al., 1987). However, the log–log plots yielded curves, with changing slope over the range investigated, indicating that the PA-mediated Ca movement across lipid bilayers may be more complex.

PA at low pHs or in the presence of Ca tends to assume nonbilayer phases—amorphous or $H_{II}$ phase (Cullis et al., 1985). In PA-containing MLV, it is likely that Ca-induced phase separation resulted in nonbilayer structures such as inverted micelles (Siegel, 1986) or other intramembrane structures (Gruner et al., 1985), the formation of which permitted acceleration of Ca influx. Moreover, this process required the presence of modest amounts of $[Ca]_i$ translocated across the bilayers by ionomycin. These experiments suggest that model membranes can mimic $[Ca]_i$-dependent Ca translocation of natural membranes (Von Tscharner, 1986).

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# Asymmetric Distribution of Phospholipids in Spectrin-Poor Erythrocyte Vesicles†

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*Received February 12, 1988; Revised Manuscript Received March 31, 1988*

ABSTRACT: We have investigated by electron spin resonance, at 37 °C, the outside–inside passage and the equilibrium distribution of spin-labeled phospholipids, respectively, in ATP-containing ghosts, in heat-treated erythrocytes, and in heat-induced vesicles. The heat-treated vesicles were spectrin depleted to ~25% of the original content and had lost almost 100% of the other cytoskeletal proteins. Yet the vesicles, as long as they contained ATP, were capable of translocating the aminophospholipids with the same efficiency as the heat-treated erythrocytes, and almost with the same efficiency as ATP-containing ghosts. In the vesicles, sphingomyelin and phosphatidylcholine analogues underwent a very slow transverse diffusion as in native cells. We conclude that spectrin and other cytoskeleton proteins are not major factors for the establishment and maintenance of phospholipid asymmetry in human erythrocytes, which may be chiefly due to the aminophospholipid translocase activity.

In the human erythrocyte membrane, phospholipids are asymmetrically distributed between the two leaflets: phosphatidylcholine (PC) and sphingomyelin (SM) are preferentially located on the outer layer, while the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are the main components of the cytoplasmic layer (Bretscher, 1972; Verkleij et al., 1973). Several years ago it was suggested that spectrin, the main cytoskeleton component, serves as the stabilizer of the PS and PE localization at the membrane cytoplasmic face (Haest et al., 1978, 1980; Williamson et al., 1982). More recently, an ATP-dependent outside–inside transport of spin-labeled aminophospholipids was demonstrated in our laboratory (Seigneuret & Devaux, 1984; Zachowski et al., 1986; Bitbol et al., 1987). We postulated the existence of an "aminophospholipid translocase" which would be responsible for the phospholipid asymmetry in erythrocytes. Several other investigators have now shown that when exogenous PS or PE is introduced in the erythrocyte membrane outer leaflet, they are rapidly translocated into the inner leaflet provided the red cell contains ATP (Connor & Schroit, 1987;

† This work was supported by grants from the Centre National de la Recherche Scientifique (UA 526), the Université Paris 7, and the Ministère de la Recherche et de l'Enseignement Supérieur.
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0006-2960/88/0427-5666$01.50/0 