Ca\textsuperscript{2+} Interaction with Phospholipid Bilayers Studied by Multifrequency Phase Fluorometry

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Calcium interaction with phospholipid membranes containing phosphatidic acid is studied by multifrequency phase fluorometry, using DPH as fluorescent molecule. DPH decay is analysed by a continuous distribution of lifetimes. The results suggest an increase of membrane heterogeneity at low calcium concentrations, without changes in the polarity of the environment surrounding the probe.

KEY WORDS: calcium; phosphatidate; DPH; phase fluorometry; distributional analysis.

INTRODUCTION

The interaction of calcium with phospholipid membranes is of interest for the important role of this ion in many cellular processes such as fusion, neuronal transmission (Poste and Allison, 1973) and in general signal transduction; in particular the involvement of negative phospholipids such as phosphatidic acid (PA) and cardiolipin have been widely investigated as calcium binding sites in membranes. Phase separation (Kouaouci et al., 1985; Jacobson and Papahadjopoulos, 1975), bilayer-non-bilayer transition (Farren et al., 1983), changes of membrane permeability (Smaal et al., 1986) and fusion (Chernomordik et al., 1987) have been shown to result from calcium interaction. A sequence of events can be hypothesized in which phase separation, inducing discontinuities in membrane structure, could be the initiation site for fusion (Duzgunes and Papahadjopoulos, 1983); moreover the possibility that non-bilayer organizations are involved as structural intermediates to membrane fusion has been discussed (Verkleij, 1984). PA is considered to be the only phospholipid capable of acting as a calcium ionophore (Reusch, 1985) that allows the formation of a lipid-soluble complex inside the membrane structure. At present many investigations are devoted to clarifying the peculiar structural modifications induced by calcium on membranes containing PA.

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In our previous work we have shown that multifrequency phase fluorometry is a sensitive technique to study changes of membrane heterogeneity due to the coexistence of microdomains by using 1,6-diphenyl-1,3,5-hexatriene (DPH) (Fiorini et al., 1987). The excited state decay of DPH is dependent on the dielectric constant of the environment surrounding the probe and is independent of local microviscosity (Zannoni et al., 1983); therefore, changes in DPH lifetime value have been related to probe environments of different polarity.

In the present study we investigate the changes of membrane polarity and heterogeneity following calcium interactions with liposomes obtained from a mixture of neutral phospholipids and PA. The results are correlated with the data on membrane permeability obtained by other authors using different techniques.

MATERIALS AND METHODS

Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL) and egg phosphatidic acid (PA) from Lipid Products (Redhill, Surrey, UK). The lipids were used without further purification; in the experimental conditions used in this work the background fluorescence was negligible. DPH was obtained from Molecular Probes Inc. (Oregon).

Lipids suspended in chloroform or chloroform/methanol were mixed in a molar ratio 85/15, and dried under nitrogen flux in the presence of a sufficient quantity of a solution DPH in tetrahydrofuran to obtain a final probe/lipid molar ratio 1/800. Multilamellar liposomes were formed by resuspending lipids at a final concentration of 0.4 mM in 0.1 M NaC1, Tris/HCl 20 mM pH 7.4, at a temperature well above the higher transition. Water was deionized and double distilled.

Steady state fluorescence measurements were performed using a MPF66 Perkin Elmer fluorometer. Excitation and emission wavelengths were 360 nm and 430 nm respectively. Scattering contribution to fluorescence was subtracted by using liposomes without fluorescent probe. Fluorescence Polarization Analysis was performed as already described (Becceria et al., 1988).

Light scattering was measured at 400 nm with a Kontron UVIKON 810. Liposomes were incubated with calcium for 3 min, and light scattering was determined as an increase of absorbance in comparison with a calcium-free sample.

Lifetime measurements were performed with a multifrequency phase fluorometer (ISS GREG 200) Interfaced with a M24 Olivetti computer for data collection and analysis. Excitation wavelength was 325 nm (UV line of an HeCd Liconix Model 4240 NB laser). A large range of modulation frequencies was used. POPOP in absolute ethanol (1.35 ns lifetime) was used as reference (Lakowicz et al., 1981). Measurements were collected through a Corion LG 370 S filter in emission; data were accumulated until the standard deviations of phase and modulation values at a given frequency were below 0.1 and 0.002 degrees respectively. The data analysis was performed using a non-linear least-square routine for the multiexponential fit described by Lakowicz et al. (1984) and a routine based on the simplex method for lifetime distribution analysis (Caceri and
Cacheris, 1984). The distribution used in this work is characterized by a Lorentzian shape centered at a decay time $\tau$ and having a Full Width at Half Maximum (FWHM). Measurements were made immediately after calcium addition to the samples. All measurements were made at 22°C.

RESULTS

Steady state fluorescence of DPH shows only very small changes of polarization at increasing calcium concentrations in MLV of PC/PA (80/15, mol%) (Table 1). The polarization values obtained are similar to those observed for liquid-crystalline phases in MLV by Parkson et al. (1983), suggesting the calcium does not induce modifications of membrane microviscosity. The aggregation measured as light scattering at 400 nm, after 3 min incubation with calcium, is presented as the percentage of the increase detected at the highest concentration used (10.8 mM) (Fig. 1). At a Ca$^{2+}$ concentration below 6 mM there is a slight increase in light scattering, while above this threshold light scattering shows a remarkable increase. Similar (Smaal et al., 1987a) studies with LUV showed an analogous threshold concentration for aggregation of dioleyl phosphatidic acid (DOPA)/dioleyl phosphatidyl choline (DOPC) (20/80, mol%); the slight differences compared with our data could be due to the use of different membrane systems. The data of DPH fluorescence decay in our experimental conditions are analysed by both the exponential and the continuous lifetime distribution approaches. The goodness of the fit is judged by comparing the value of reduced chi square (Lakowicz et al., 1984). A double exponential analysis is necessary to obtain a good fit (Table 2A). Lifetime values of all samples correspond to those obtained for a liquid crystalline phase (Fiorini et al., 1987a), showing a long lifetime component of about 8.3 ns and 0.94 fractional intensity and a short-lifetime component of about 2 ns and 0.06 fractional intensity. The origin of the short lifetime component is still debated; tentatively it was attributed to a little DPH localised in a very polar environment (Karnovsky et al., 1982). Table 2A shows substantially no change in lifetime values, at increasing calcium concentration, indicating no changes in dielectric constant value in the hydrophobic part of the membrane (Zannoni et al., 1983). The same DPH fluorescence

<table>
<thead>
<tr>
<th>Calcium (mM)</th>
<th>Polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.135 ± 0.003</td>
</tr>
<tr>
<td>1</td>
<td>0.140 ± 0.003</td>
</tr>
<tr>
<td>2.07</td>
<td>0.141 ± 0.002</td>
</tr>
<tr>
<td>3.10</td>
<td>0.141 ± 0.004</td>
</tr>
<tr>
<td>8.24</td>
<td>0.143 ± 0.002</td>
</tr>
<tr>
<td>11.32</td>
<td>0.142 ± 0.002</td>
</tr>
</tbody>
</table>
Fig. 1. Aggregation of PC/PA (85/15, mol %) liposomes in media with different calcium concentrations. The light scattering is measured as an increase in absorbance at 400 nm. Further details are described in the text.

Table 2. Analysis of fluorescence emission decay of DPH in PC/PA liposomes (85/15, mol %), dependence on calcium concentration (A) exponential analysis; (B) distributional analysis.

<table>
<thead>
<tr>
<th>Calcium (mM)</th>
<th>$\tau_1$</th>
<th>$f_1$</th>
<th>$\tau_2$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.47</td>
<td>0.95</td>
<td>2.23</td>
<td>0.232</td>
</tr>
<tr>
<td>1</td>
<td>8.45</td>
<td>0.95</td>
<td>2.14</td>
<td>0.864</td>
</tr>
<tr>
<td>2</td>
<td>8.36</td>
<td>0.94</td>
<td>2.11</td>
<td>0.941</td>
</tr>
<tr>
<td>10</td>
<td>8.27</td>
<td>0.93</td>
<td>2.20</td>
<td>0.323</td>
</tr>
</tbody>
</table>

(A)

<table>
<thead>
<tr>
<th>Calcium (mM)</th>
<th>$c_1$</th>
<th>$w_1$</th>
<th>$f_1$</th>
<th>$c_2$</th>
<th>$w_2$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.44</td>
<td>0.100</td>
<td>0.95</td>
<td>2.22</td>
<td>0.05</td>
<td>0.269</td>
</tr>
<tr>
<td>1</td>
<td>8.25</td>
<td>0.630</td>
<td>0.97</td>
<td>1.84</td>
<td>0.10</td>
<td>0.481</td>
</tr>
<tr>
<td>2</td>
<td>8.20</td>
<td>0.496</td>
<td>0.96</td>
<td>1.93</td>
<td>0.10</td>
<td>0.766</td>
</tr>
<tr>
<td>10</td>
<td>8.24</td>
<td>0.139</td>
<td>0.94</td>
<td>2.20</td>
<td>0.10</td>
<td>0.321</td>
</tr>
</tbody>
</table>

(B)

Lifetime, $\tau$; fractional intensity, $f$; center of the distribution, $c$; full width at half maximum of the distribution, $w$; reduced $\chi^2$, $\chi^2$. 
decay data are analysed (Table 2B) by a continuous lifetime distribution approach, which assumes that heterogeneity of DPH fluorescence decay reflects the multiplicity of DPH microenvironments, and therefore membrane heterogeneity (Fiorini et al., 1987b). In all samples the center of distribution of the main component has almost the same value, which was obtained by the double exponential analysis. Moreover at low (1-2 mM) calcium concentrations the FWHM value is larger (0.6-0.5 ns) than that obtained in liposomes without calcium (0.1 ns) or with 10 mM calcium (0.139 ns). The large value of FWHM indicates a higher heterogeneity for liposomes at calcium concentrations corresponding to those showing a selective influx of this ion in DOPA-containing liposomes (Small et al., 1986). The chi square obtained by distributional analysis at the lower calcium concentrations is reduced with respect to exponential analysis (40-20% respectively) suggesting a better description of the decay behaviour of DPH.

**DISCUSSION**

Polarization data show very small changes of microviscosity of the environment in the hydrophobic core of membranes probed by DPH, and indicate a liquid-crystalline phase in agreement with NMR data of Smaal et al. (1987b), obtained with vesicles of DOPC/DOPA. These authors have demonstrated a motional restriction due to calcium in the headgroup region, but the oleate chains remain in the liquid crystalline phase; moreover in our experiments, at all calcium concentrations tested, there is no change in DPH lifetime values, indicating essentially no change in polarity of probe microenvironment. Due to the presence of double bonds in our membrane system, DPH could be preferentially located in the middle of the membrane hydrophobic core, as in the quenching experiments of Davenport et al. (1985). Light scattering measurements support the observations of Smaal et al. (1987a) who demonstrated the presence of fusion at high calcium concentration, while at low calcium the increase of light scattering can be attributed to other aspects of calcium-PA interactions, such as the formation of structures related to calcium translocation (Smaal et al., 1986). On the other hand Chauhan et al. (1986), using egg PC/PA (90/10 mol %) for studies of “transversal rate” of calcium through the membrane, found an increase of this rate up to about 7 mM Ca, and then a very slow decrease; they suggested that calcium could be involved in Ca(PA) formation and in Ca(PA·PC) complexes. Our studies with distributional analysis of DPH fluorescence decay at low calcium concentrations, which show an increase in heterogeneity in DPH microenvironments, indicate the formation of phospholipid domains, in agreement with the calcium-induced lateral phase separation observed by other techniques (Leventis et al., 1986). The possible formation of defects in the membrane, through which water and ions can cross the bilayer, is shown by the large value of distribution of lifetimes, which indicate DPH microenvironments of different polarity, although the mean value is not modified in either exponential or distributional analysis. This behaviour could be explained by the presence of structural defects as well as
of more compact domains where water and ion penetration is restricted. At high calcium concentration there is a decrease in distribution width of lifetimes, that could be related to extensive fusion, according to light scattering data. The formation of an homogeneous environment with no detectable changes in polarity, for DPH molecules, is suggested; the massive formation of Ca(PC·PC) complexes can be excluded for the low percentage of PA in the membrane.

Altogether our results show that by using multifrequency phase fluorometry it is possible to detect physico-chemical changes related to membrane structural heterogeneity which are well correlated with other aspects of membrane organization analysed by other spectroscopic and morphologic techniques.

REFERENCES