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Influence of organotin compounds on phosphatidylserine membranes

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Organotin compounds are widely distributed toxicants. They are membrane-active molecules with broad biological toxicity. We have studied the interaction of tributyltin and triphenyltin with phosphatidylserine model membranes using differential scanning calorimetry, infrared spectroscopy and X-ray diffraction techniques. Organotin compounds produced a broadening of the gel to the liquid-crystalline phase transition of the phospholipid and a shifting of the phase transition temperature to lower values. Infrared spectroscopy experiments showed that tributyltin exerted a fluidizing effect on the apolar part of the bilayer, and that both tributyl- and triphenyltin interact with the interfacial region of the bilayer, making the carbonyl groups less accessible to water. As seen by X-ray diffraction experiments, organotin compounds were unable to change the bilayer macroscopic organization of the phospholipid, but they were able to reduce the long-range order of the multibilayer system and to disorder the packing of the phospholipid molecules. The observed interaction between organotin compounds and phosphatidylserine membranes promotes physical perturbations that could affect membrane function and may mediate some of their toxic effects.

KEYWORDS: organotin compounds; phosphatidylserine; model membranes; DSC; X-ray diffraction; infrared spectroscopy.

INTRODUCTION

Organotin compounds are widely distributed toxicants. These compounds are used as stabilizers or glass coatings, as catalysts for the formation of polyurethane foams, as biocides for agricultural applications and as preservatives for timber, wood textiles, paper and leather.[1,2] Several *in vivo* studies showed that organotin compounds are immunotoxic, neurotoxic, genotoxic and hepatotoxic.[3–6] Their increasing use has given rise to ubiquitous environmental contaminations. Tri-*n*-butyltin (TBT) and tri-*n*-phenyltin (TPT) are very common derivatives in antifouling paints, and they are also two of the most toxic species to mammalian cells. TBT and TPT are membrane-active molecules, and their mechanism of action appears to be strongly dependent on organotin lipophilicity.[7,8] They function as ionophores[9] and produce haemolysis,[8] release calcium from sarcoplasmic reticulum,[10] alter phosphatidylserine-induced histamine release,[11] alter mitochondrial membrane permeability,[12] perturb membrane enzymes[13,14] and induce apoptosis in lymphocytes.[15] Organotin compounds have been shown to affect cell signalling; they activate protein kinase C[16] and increase free arachidonic acid through the activation of phospholipase A_2.[17]

Hydrophobicity of organotin compounds suggests that their interaction with membranes may play an important role in their toxic mechanism. In this respect, the understanding of the interaction of organotin compounds with the lipid component of membranes is of considerable interest. Fluorescence polarization measurements[18] suggested that the effect of TBT on liposomal membranes is dependent on the anion moiety. Studies on the release of liposome-bound praseodymium[19] indicated that the lipophilicity and polarity of organotin compounds and the surface potential and environment of the lipid molecules are important factors in their interaction with membranes. From the study of the interaction of several organotin compounds (differing in their polar and hydrophobic moieties) with erythrocytes[20] it was concluded that the different effects can result from

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a different location of the organotin compound in the lipid bilayer. Differential scanning calorimetry (DSC) and infrared spectroscopy studies showed that TBT affected the thermotropic properties of dipalmitoylphosphatidylcholine, suggesting a location of the toxicant in the hydrophobic region of the membrane.[21] We have shown that the effects on the thermotropic properties of phosphatidylcholine are more pronounced in the case of TBT than in the case of TPT, being quantitatively larger as the phosphatidylcholine acyl chain length decreases, and we have also shown that organotin compounds do not affect the macroscopic bilayer organization of phosphatidylcholine but that they do affect the degree of hydration of its carbonyl moiety.[22] We have also recently described that organotin compounds laterally segregate in phosphatidylethanolamine membranes without affecting the gel to liquid-crystalline phase transition, and that they have the ability to promote the formation of hexagonal H_{II} structures in unsaturated phosphatidylethanolamine systems.[23]

In an attempt to understand further the influence of organotin compounds on the lipid component of the membrane, we extended our studies to phosphatidylserine, which is a very important phospholipid from the point of view of membrane function, and we present a study of the effect of TBT and TPT on the thermotropic and structural properties of dimyristoylphosphatidylserine, using DSC, infrared spectroscopy and X-ray diffraction.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). TBT chloride (TBTCl) and TPT chloride (TPTCl) were obtained from Sigma–Aldrich (Spain). All other reagents were of the highest purity available.

Differential scanning calorimetry

The lipid mixtures for DSC measurements were prepared by combining chloroform/methanol (1 : 1) solutions containing 4 μmol phospholipid and the appropriate amount of organotin compounds as indicated. The organic solvents were evaporated under a stream of dry nitrogen, and the last traces of solvents were removed by a further 3 h of evaporation under high vacuum. Multilamellar liposomes were prepared in 0.1 mM EDTA, 100 mM NaCl, and 10 mM Hepes (pH 7.4) buffer by mixing, with a bench mixer, always keeping the samples at a temperature above the gel to liquid-crystalline phase transition temperature of the lipid. The suspensions were centrifuged at 13 000 rpm in a bench microfuge and the pellets were collected into small aluminium pans. The pans were sealed and scanned in a Perkin–Elmer DSC-7 calorimeter, using a reference pan containing buffer. The instrument was calibrated using indium as standard. The heating rate was 4 °C min^{-1} in all the experiments. The construction of partial phase diagrams was based on the heating thermograms for a given mixture of phospholipid and organotin compounds at various organotin compound concentrations. The onset and completion temperatures for each transition peak were plotted as a function of the molar fraction of organotin compounds. These onset and completion temperature points formed the basis for defining the boundary lines of the partial temperature–composition phase diagram.

Infrared spectroscopy

For the infrared measurements, multilamellar vesicles were prepared in 40 μl of D_2O as described above. Samples were placed between two CaF_2 windows (25 mm × 2 mm) separated by 50 μm Teflon spacers and transferred to a Symta cell mount. Infrared spectra were obtained in a Nicolet MX-1 FT-IR spectrometer. Each spectrum was obtained by collecting 27 interferograms. The D_2O spectra taken at the same temperature were subtracted interactively using GRAMS/32 (Galactic Industries, Salem, MA), as described previously.[24]

X-ray diffraction

Simultaneous small- and wide-angle X-ray diffraction measurements were carried out using a modified Kratky compact camera (MBraum-Graz-Optical Systems, Graz, Austria), which employs two coupled linear position-sensitive detectors (PSDS, MBraum, Garching, Germany), monitoring the *s*-ranges ($s = 2 \sin\theta/\lambda$, 2θ is the scattering angle, $\lambda = 1.54$ Å) between 0.0075–0.07 $Å^{-1}$ and 0.20–0.29 $Å^{-1}$ respectively. Nickel-filtered Cu K_α X-rays were generated by a Philips PW3830 X-ray generator operating at 50 kV and 30 mA. The position calibration of the detectors was performed by using silver stearate (small-angle region, *d*-spacing at 48.8 Å) and lupolen (wide-angle region, *d*-spacing at 4.12 Å) as reference materials. Samples for X-ray diffraction were prepared by mixing 15 mg of phospholipids and the appropriate amount of organotin compounds in chloroform/methanol (1 : 1). Multilamellar vesicles were formed as described above. After centrifugation at 13 000 rpm, the pellets were resuspended in 50 μl of buffer and measured in a thin-walled Mark capillary held in a steel cuvette, which provides good thermal contact to the Peltier heating unit. X-ray diffraction profiles were obtained for 10 min exposure times after 5 min of temperature equilibration.

RESULTS

The influence of TBTCl and TPTCl on the thermotropic gel to liquid-crystalline phase transition of DMPS is depicted in Fig. 1. In the absence of organotin compounds, DMPS exhibited only one endotherm upon heating, located at 36.2 °C, in agreement with previous reports.[25] The presence of

increasing concentrations of organotin compounds produced a progressive broadening of the transition and a shift to lower temperatures, these effects being more pronounced in the case of TBTCl (Fig. 1A) than in the case of TPTCl (Fig. 1B).

DSC data were used to construct partial phase diagrams for the DMPS component in mixtures of the phospholipid and organotin compounds. The onset and completion temperatures of the heating thermograms shown in Fig. 1 provided the data necessary for obtaining the solid and fluid lines of the phase diagrams respectively. In both systems (Fig. 2), the solid and fluid lines displayed near ideal behaviour, the temperature decreasing as the TBTCl (Fig. 2A) or TPTCl (Fig. 2B) concentration increased. The system evolved from a lamellar gel phase (G phase) to a lamellar liquid-crystalline phase (F phase) through a coexistence region (G + F), which became wider as more organotin compound was added to the system. The effect of low concentrations of organotin compounds on the solid line and the width of the coexistence region was more pronounced for TBTCl than for TPTCl.

To investigate the effect of organotin compounds on different parts of the DMPS molecule, infrared spectroscopy was used. Figure 3 shows the infrared spectra corresponding to the antisymmetric and symmetric absorption bands of the methylene groups of the phospholipid acyl chains, in the gel phase. Pure DMPS shows absorption maxima near 2849.5 cm^{-1} and 2917 cm^{-1} for the symmetric and antisymmetric bands respectively. The presence of TBTCl shifted both the symmetric and antisymmetric stretching band maxima to higher values (2851 cm^{-1} and 2919.8 cm^{-1} respectively). The presence of TPTCl did not change the frequency of these bands in the gel phase, and neither TBTCl nor TPTCl changed the frequency of these bands in the liquid-crystalline phase (data not shown).

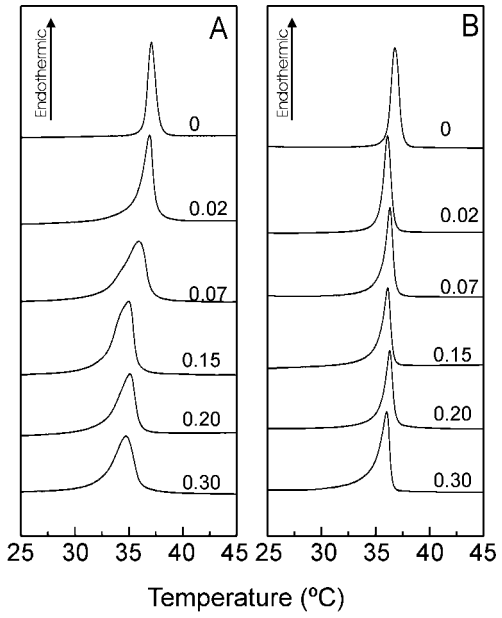

Figure 1. DSC thermograms for DMPS and mixtures of DMPS–TBTCl (A) and DMPS–TBTCl (B). The concentration of organotin compound in the membrane (molar fraction) is expressed on the curves.

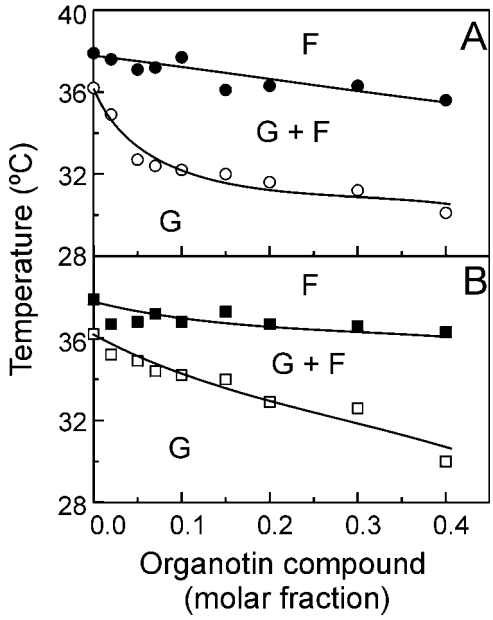

Figure 2. Partial phase diagrams for DMPS in DMPS–TBTCl mixtures (A) and DMPS–TPTCl mixtures (B). Open and closed symbols were obtained from the onset and completion temperatures of the main gel to liquid-crystalline phase transition. The phase designations are as follows: G, gel phase; F, liquid-crystalline phase.

The interfacial region of DMPS was studied by means of the carbonyl stretching band. It is known that the carbonyl groups of diacylphospholipids may be found in lipid vesicles in hydrated and dehydrated states, their proportion depending

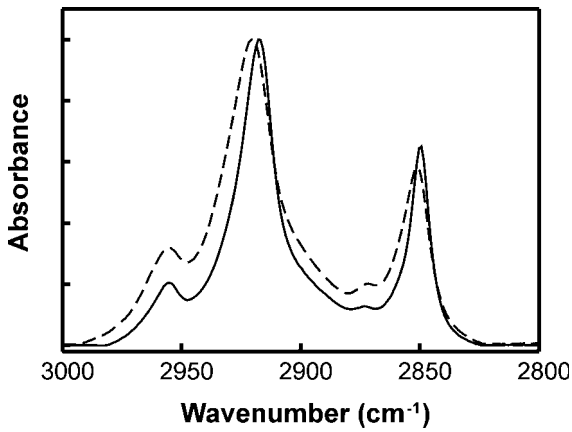

Figure 3. Infrared spectra of the methylene stretching region of DMPS for pure DMPS (solid line) and DMPS containing a 0.2 molar fraction of TBT (dashed line) at 25 °C.

on the physical state of the phospholipid bilayer.[26] Pure DMPS spectra represent a summation of two component bands centred near 1743 cm^{-1} and 1728 cm^{-1} (attributed to dehydrated and hydrated C═O groups respectively).[27] The spectra corresponding to DMPS and DMPS systems containing organotin compounds were subjected to curve fitting to two bands centred at 1743 and 1728 cm^{-1}. These bands were simulated by a Gaussian–Lorentzian function, and the relative areas of these simulated bands were calculated. It can be seen (Fig. 4) that the presence of organotin compounds increased the contribution of the dehydrated component compared with the pure phospholipid, above the phase transition in the case of TBTCl and both below and above the phase transition in the case of TPTCl.

Information on the structural characteristics of DMPS–organotin compound systems was obtained by X-ray diffraction measurements. Small-angle X-ray scattering (SAXS) was used to check whether organotin compounds affected the phase behaviour of DMPS. This technique not only defines the macroscopic structure itself, but also provides the interlamellar repeat distance in the lamellar phase. The largest first-order reflection component corresponds to the interlamellar repeat distance, which is comprised of the bilayer thickness and the thickness of the water layer between bilayers. Figure 5 shows the SAXS diffraction pattern profiles corresponding to pure DMPS and DMPS containing organotin compounds at two different temperatures. DMPS systems revealed a diffraction peak with an interlamellar repeat distance of *ca* 67 Å in the gel state (25 °C, Fig. 5A). This value decreased above the chain melting temperature to *ca* 58 Å (Fig. 5B), which is within the range of previous reported data.[28] In the gel phase, in addition to the lamellar diffraction peak, a broad shoulder is observed at *ca* 52 Å (Fig. 5A), indicative of a poorly ordered sample. A shoulder at *ca* 45 Å is also present in the diffraction pattern of DMPS in the liquid-crystalline phase (Fig. 5B). The presence of TPTCl led to the dissappearance of the main diffraction peak and the appearance of a broad peak centred at the same distance

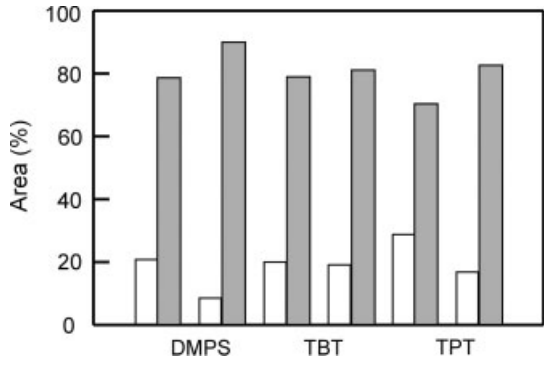

Figure 4. Relative area of the dehydrated (filled) and hydrated (empty) components of the carbonyl stretching band for DMPS and DMPS containing 0.3 molar fractions of TBTCl or TPTCl, at 25 °C (bars at the left) and 50 °C (bars at the right).

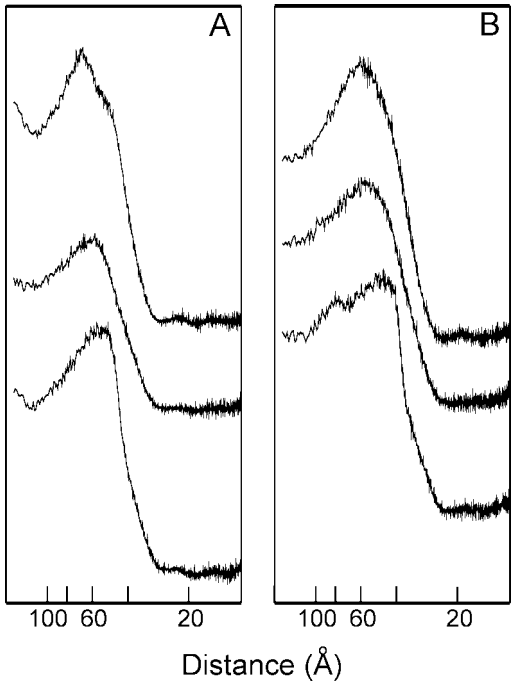

Figure 5. Small-angle X-ray diffraction profiles at 25 °C (A) and 50 °C (B), obtained from (top to bottom) pure DMPS, DMPS containing a 0.2 molar fraction of TBTCl, and DMPS containing a 0.2 molar fraction of TPTCl.

as the shoulder in the pure phospholipid, i.e. 52 Å in the gel phase and 45 Å in the liquid-crystalline phase. The presence of TBTCl decreased the interlamellar repeat distance to 58 Å and 56 Å in the gel phase and liquid-crystalline phase respectively.

Measurements in the Wide-angle X-ray scattering (WAXS) region provide information about the packing of the phospholipid acyl chains. Figure 6 shows the WAXS pattern corresponding to pure DMPS and DMPS containing organotin compounds at 25 °C (gel phase). The spacing of the wide-angle reflection for DMPS displays a value of 4.14 Å, indicating a conventional L_β phase in which the acyl chains are packed parallel to the bilayer normal on a regular hexagonal lattice, in agreement with a previous report.[29] The presence of TBTCl and TPTCl increased the spacing to 4.19 Å and 4.16 Å respectively, indicating a lower phospholipid chain packing in the gel phase. In agreement with previous results,[29] above the chain melting transition temperature, DMPS showed a very broad component centred at 4.4 Å typical of the disordered liquid-crystalline phase, which was not suitable for examination (results not shown).

DISCUSSION

The thermotropic and structural properties of mixtures of DMPS and organotin compounds have been examined to establish the extent of intermolecular interaction between the

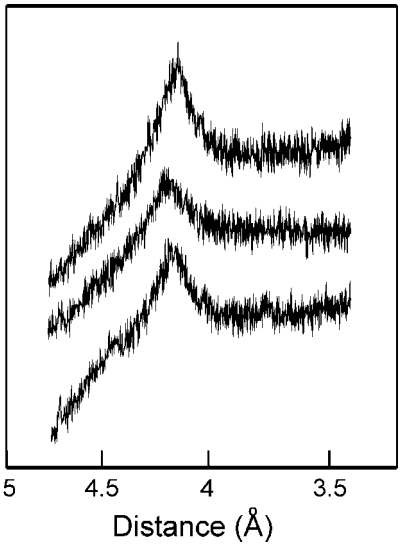

Figure 6. Wide angle X-ray diffraction profiles at 25 °C, obtained from (top to bottom) pure DMPS, DMPS containing a 0.2 molar fraction of TBTCl, and DMPS containing a 0.2 molar fraction of TPTCl.

two types of molecule. The interaction between molecules can be evidenced by the change of the thermotropic properties of the pure component of a mixture. The presence of increasing amounts of TBTCl and TPTCl produced a broadening of the gel to liquid-crystalline phase transition peak and a shift of the transition temperature to lower values. These effects were more pronounced in the case of TBTCl than in the case of TPTCl. The effect on DMPS systems described here is qualitatively similar to that described previously for phosphatidylcholine systems[22] and different from the weak interaction observed for organotin compounds and phosphatidylethanolamine systems.[23] The interaction with DMPS is less marked than in the case of phosphatidylcholine because the second thermotropic peak characteristic of the phosphatidylcholine–organotin compound thermograms[22] was absent. These observations are compatible with the hydrophobic butyl and phenyl moieties aligning themselves with the prevailing directions of the phosphatidylserine acyl chains, where they can disrupt their packing, reduce the cooperativity of the transitions and shift the phase transition temperature to lower values.

When organotin compounds are incorporated into phospholipid systems, they will change the transition temperature of the phospholipid if both types of molecule are miscible. The phase diagram shows that the temperature of both the solid and fluid lines decreases as more organotin compounds are added to the system. This indicated that DMPS and organotin compounds are miscible in the gel and in the liquid-crystalline phase, and that the intercalation of organotin compounds molecules in the phospholipid palisade perturbs its thermotropic properties. Similar to what we have previously described for phosphatidylcholine and phosphatidylethanolamine systems, we found that in the presence of TPTCl the region of phase coexistence is smaller than in the case of TBT, reflecting the tendency of TPT to aggregate in the membrane. This aggregative behaviour of TPT would help to explain the observation that TPTCl is less toxic[30] and induces less drastic lesions[31] than TBTCl.

The influence of TBTCl on the methylene stretching band of DMPS indicates an effective interaction with the phospholipid acyl chains in the gel phase, resulting in a fluidizing effect of the apolar part of the bilayer. TPTCl lacks this fluidizing effect, suggesting that this toxicant is located closer to the lipid–water interface. The effect of organotin compounds on the carbonyl stretching band of DMPS suggests that these compounds interact with the interfacial region of the phospholipid and make the carbonyl groups less accessible to water. The dehydrating effect is stronger for TPTCl than for TBTCl, supporting the idea of a more polar location for TPTCl. The effect on the phosphatidylserine interfacial region is much weaker than the reported effect on phosphatidylcholine systems,[22] but it is clearly stronger than that exerted on phosphatidylethanolamines,[23] emphasizing the importance of the polar head group of the phospholipids in their interaction with these toxicants.

SAXS measurements on DMPS systems showed diffraction peaks with interlamellar repeat distances in agreement with the literature.[32] In addition, we found a shoulder at shorter distances. The presence of such a shoulder has previously been reported for DMPS systems,[33] and the absence of sharp Bragg diffraction peaks and the presence of broad scattering peaks have been described for dimyristoylphosphatidylglycerol systems.[34] In both cases, broad peaks have been related to the presence of non-organized multibilayers. In the presence of TPTCl, both in the gel and the liquid-crystalline phases, only the shorter distance broad peak was found, which indicates that this toxicant reduced the long-range order in the multilamellar DMPS system. The decrease in the interlamellar repeat distance found for TBTCl in the gel phase can be related to the fluidizing effect on the apolar part of the bilayer, as seen through the methylene stretching absorption band of the phospholipid.

From WAXS analysis, the lateral distance between neighbouring acyl chains is measured directly. Our measurements showed that both the organotin compounds increased the distance between DMPS molecules and disordered their packing, this effect being more evident in the case of TBTCl.

In summary, this study has shown that organotin compounds are incorporated into a very important phospholipid of eukaryotic membranes, i.e. phosphatidylserine, where they perturb its thermotropic and structural properties. Organotin compounds interact in a quantitative different way with phosphatidylserine than with phosphatidylcholine[22] and phosphatidylethanolamine.[23] The evidence supports the hypothesis that organotin compounds are located in the upper part of the phosphatidylserine palisade. The butyl and phenyl groups intercalate between the initial methylene segments,

perturbing their packing and affecting the hydration of the interfacial region. According to the different effects of TBTCl and TPTCl on the fluidity of the acyl chains and the hydration of the interfacial region of phosphatidylserine, it seems that TBTCl is located more deeply in the phospholipid palisade that TPTCl, which is closer to the lipid–water interface. The observed interaction between organotin compounds and phosphatidylserine promotes physical perturbations, which could affect membrane function and may mediate some of their toxic effects.

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