THE CHEMICAL TOXIC BENZO[a]PYRENE PERTURBS THE PHYSICAL ORGANIZATION OF PHOSPHATIDYLCHOLINE MEMBRANES

MARÍA JIMÉNEZ, FRANCISCO J. ARANDA, J. ANTONIO TERUEL, and ANTONIO ORTIZ*
Department of Biochemistry and Molecular Biology-A, Veterinary Faculty, University of Murcia, Campus de Espinardo, E-30100 Espinardo, Murcia, Spain

(Received 3 May 2001; Accepted 5 October 2001)

Abstract—The interaction of the chemical carcinogen benzo[a]pyrene (BaP) with phosphatidylcholine membranes has been investigated by using various physical techniques. Differential scanning calorimetry showed that BaP, at concentrations as low as 2 mol% in mixtures with dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine, abolished the pretransition and broadened and shifted to lower temperatures the main gel–to–liquid crystalline phase transition. The effect was stronger as the lipid acyl chain length decreased. Infrared spectroscopy indicated that inclusion of BaP had a strong influence on the carbonyl (C=O) stretching band. Fitting of this band resulted in the presence of three components in the case of samples containing BaP, centered at 1,742, 1,727, and 1,704 cm⁻¹ (only two components, at 1,742 and 1,727 cm⁻¹, were observed for pure DMPC). The component at 1,704 cm⁻¹ corresponded to a disolvate and strongly supported the notion that inclusion of BaP increases hydration of the bilayer C=O. Small-angle x-ray diffraction showed that incorporation of 10 mol% BaP into DMPC increased bilayer thickness from 66.7 to 71 Å in the gel state and from 58.9 to 60.9 Å in the fluid phase. It is proposed that, when BaP is incorporated into a phospholipid bilayer, it locates in the most apolar region of the phospholipid palisade, resulting in expansion and swelling of the membrane. These two effects will facilitate water penetration into the polar region of the membrane, as observed by the increased hydration of the C=O groups, and thus perturb both membrane integrity and functionality.

Keywords—Lipid membranes Benzo[a]pyrene Infrared spectroscopy X-ray diffraction Differential scanning calorimetry

INTRODUCTION

The widespread use of cyclic hydrocarbons (e.g., as fuels, as solvents) and their release into the environment make knowledge regarding their toxic action of great importance. Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants, and exposure to urban-air PAHs may result in increased risk of lung cancer as well as many other pathologies [1]. These carcinogens are generally mutagens that induce a complex pattern of mutations [2] and other toxic effects on cells [3]. Thus, taking into account their strong hydrophobic nature, the mechanism by which PAHs perturb the structure of cell membranes is of considerable importance. Benzo[a]pyrene (BaP) (Fig. 1), one of the most potent members of this family, like most PAHs, has a very low water solubility (10⁻⁸ M [4]); therefore, it is expected that, on uptake, it will readily partition into cellular membranes [5]. Toxic and mutagenic effects of BaP have been extensively reported [6–9].

Cyclic aromatic hydrocarbons interact with biological membranes [10,11]. These interactions lead to changes in structure and function of the membrane, which may impair growth and cellular activity [3]. The interaction of hydrophobic compounds with biological membranes is an important factor in their biological activity. Some PAHs have been described to accumulate in the membrane lipid bilayer, affecting the structural and functional properties of these membranes [12], although to our knowledge, a systematic study on the effects of BaP has not been carried out so far. Thus, a fundamental insight regarding these mechanisms can be achieved by examining the influence of these substances on the organization of model membrane systems, prepared from synthetic phospholipids, that have been widely used as models for biological membranes [13,14].

This study deals with the membrane-perturbing effect of BaP. For this purpose, the interactions of the aromatic compound with membrane phospholipids has been studied by means of various physical techniques to gain insight regarding its membrane toxic action leading to impaired growth and activity of cells.

MATERIALS AND METHODS

Materials

The 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The BaP was purchased from Sigma-Aldrich (Madrid, Spain). Water was twice-distilled and deionized using a Milli-Q system from Millipore (Bedford, MA, USA). All other reagents were of the highest purity available. Stock solutions of BaP and the various phospholipids were prepared in chloroform:methanol (2:1, v/v) and stored at −20°C for as long as one week. Lipid degradation was not detected within this period as checked by analytical thin-layer chromatography.

Differential scanning calorimetry

This physical technique allows precise determination of the temperature and enthalpy change associated to thermotropic transitions (in our case, of phospholipids organized in a bilayer structure). Incorporation of a foreign molecule within this bilayer will perturb the transition characteristics, which can be used to obtain information regarding its interaction with the...
phospholipids, its location in the membrane, and the mechanism by which it is perturbing the bilayer structure.

Samples for differential scanning calorimetry (DSC) were prepared by mixing the appropriate amounts of phospholipid (usually 3 μmol) and BaP (as indicated) in chloroform:methanol (2:1, v/v). The solvent was gently evaporated under a stream of dry N₂ to obtain a thin film at the bottom of a small, thick-walled glass tube. Last traces of solvent were removed by a further 2 h of desiccation under high vacuum. To the dry samples, 1 ml of a buffer containing 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid, and 20 mM Tris (pH 7.4) was added, and vesicles were formed by vortexing the mixture, always keeping the temperature greater than the highest gel–to–liquid crystalline phase transition of the sample. Samples were pelleted down in an Eppendorff bench microfuge (Hamburg, Germany) at 12,000 rpm for 10 min, collected with a spatula, placed into small aluminum calorimetry pans, sealed, and scanned. Scans were carried out in Perkin-Elmer DSC-7 equipment (Norwalk, CT, USA) at heating and cooling rates of 4°C min⁻¹. The calorimeter was calibrated using indium as standard as well as using the transition temperature and enthalpy of the pure phospholipids.

Partial phase diagrams for the phospholipid component were constructed as previously described [15]. The solidus and fluidus lines of the diagrams were defined by the onset and completion temperatures, respectively, from heating scans. To avoid artifacts due to the thermal history of the sample, the first scan was never considered. Second and later scans were carried out until a reproducible and reversible pattern was obtained, which usually occurred with the second scan.

**Small-angle x-ray diffraction**

Normally, phosphatidylcholine water suspensions adopt a bilayer organization. Small-angle x-ray diffraction (SAXD) is a unique physical technique to determine bilayer thickness. Stacked phospholipid bilayers (as they occur in multilamellar liposomes) behave as a crystalline structure in terms of the Bragg reflections. After proper calibration, the diffraction pattern allows determination of bilayer thickness, which is a summation of the width of the phospholipid palisades plus the water layers associated to the polar headgroups. Modification of this parameter by, in our case, BaP gives information regarding how this compound is modifying the bilayer structure.

Samples for SAXD analysis were prepared essentially as described above for DSC with some minor modifications. Usually, 10 to 15 mg of the corresponding phospholipid were used, and the dry films were resuspended in 1 ml of the same buffer. The vesicle suspensions were pelleted down by centrifugation in an Eppendorff bench microfuge and placed in the x-ray diffractometer holder.

Nickel-filtered Cu Kα (λ = 1.54 Å) x-rays were obtained from a Philips model PW1830 anode (Eindhoven, The Netherlands). The x-rays were focused using a flat, gold-plated mirror and were recorded using a linear position–sensitive detector model 210 (Bio-Logic, Echirolles, France). Unoriented lipid dispersions, prepared as described above, were measured in aluminum holders with Mylar (Grafax Plastics, Cleveland, OH, USA) windows. The sample temperature was kept to the desired value (±0.5°C) using a circulating water bath. The system was allowed to equilibrate for approximately 5 min at each temperature before measurements were obtained. Typical x-ray exposure times were 10 to 15 min for each sample.

For the measurement of lattice spacing, crystalline cholesterol was used as standard. This compound shows a sharp reflection corresponding to a 33.6 Å spacing, which is very adequate for calibration. The relative position of the peaks in the diffractogram allows specification of the packing symmetry of the phase (principally lamellar, L or hexagonal-HII, H II ), and the distance of the peaks to the center (nondiffracted beam) allows calculation of the repeat or d-spacing of that particular phase.

**Infrared spectroscopy**

The carbonyl (C=O) groups of phospholipids present a strong absorption band in the infrared centered at approximately 1,730 cm⁻¹. In water suspensions of phospholipids, these C=O groups may be in a hydrated and a dehydrated state, so that this band is the summation of, usually, two other bands centered at 1,742 and 1,727 cm⁻¹. In our case, the displacement of these maxima induced by BaP allows determination of the proportion of hydrated and dehydrated C=O and, thus, information regarding the perturbation exerted at the polar region of the membrane.

For the infrared measurements, multilamellar vesicles were prepared in 40 μl of D₂O as described above. Samples were placed between two CaF₂ windows (25 × 2 mm) separated by 50-μm Teflon® (TTS, Nashville, TN, USA) spacers and transferred to a Symta (Madrid, Spain) cell mount. Infrared spectra were acquired in a Nicolet MX-1 Fourier-transform infrared spectrometer (Madison, WI, USA) provided with computer data collection. Each spectrum was obtained by collecting 27 interferograms. The D₂O spectrum taken at the same temperature was subtracted interactively using either GRAMS/32 or Spectra-Calc (Galactic Industries, Salem, MA, USA) as described previously [16].

**Analytical assays**

Phospholipid phosphorous was determined according to the method of Böttcher et al. [17].

**RESULTS**

In this study, the interaction of the hydrophobic aromatic toxic BaP with phospholipid membranes was examined using various physical techniques of wide application in this type of research.

The influence of BaP on the thermotropic phase transition of various phosphatidylcholines of different acyl chain length is shown in Figure 2. In the absence of BaP, DMPC exhibited a pretransition at approximately 15°C and a main gel–to–liquid crystalline phase transition at 25.5°C. Similar DSC scans were obtained for DPPC (transitions at 35 and 41°C) and DSPC.
Benzo[a]pyrene perturbs phosphatidylcholine membranes

Benzo[a]pyrene was essentially zero at 40 mol%.

The increase in the enthalpy change (ΔH) of the transition was especially abolished in mixtures containing 40 mol% B.

The concentration of benzo[a]pyrene (in mol%) is expressed on the curves.

Fig. 2. Differential scanning calorimetry heating scans for mixtures of benzo[a]pyrene with dimyristoylphosphatidylcholine (A), dipalmitylphosphatidylcholine (B), and distearoylphosphatidylcholine (C). The concentration of benzo[a]pyrene (in mol%) is expressed on the curves.

(transitions at 51 and 55°C). The presence of concentrations of BaP as low as 2 mol% (molar fraction 0.02) completely abolished the pretransition and slightly lowered and broadened the main transition, in mixtures with the three lipids. Increasing concentrations of BaP caused a progressive broadening of the main transition, concomitantly with a shift to lower temperatures. This effect was stronger as the acyl chain length of the phospholipid grew shorter, being particularly important for DMPC. For this lipid, the main gel–to–liquid crystalline phase transition was essentially abolished in mixtures containing 40 mol% BaP.

Figure 3 shows the enthalpy change for the gel–to–liquid crystalline phase transition for mixtures of DMPC, DPPC, and DSPC with various concentrations of BaP. Increasing the concentration of BaP produced a progressive and significant decrease in the enthalpy change (ΔH) of the transition. This effect was particularly important for DMPC samples, because ΔH decreased to approximately 2 Kcal mol⁻¹ at 10 mol% BaP and was essentially zero at 40 mol%.

Small-angle x-ray diffraction was used to check whether BaP affects the phase behavior of phosphatidylcholines and then to determine a possible perturbation of lamellar bilayer thickness. Figure 4 shows the diffraction pattern profiles corresponding to pure DMPC and DMPC containing 10 mol% BaP. In Figure 4A, a typical raw SAXD pattern obtained for pure DMPC is presented, in which sharp Bragg reflections with a good signal-to-noise ratio are shown. The diffractograms were symmetrical, as expected for the nonoriented samples used. The center of the diffractogram had a deep trough as a normal consequence of the presence of the nondiffracted beam stopper.

For the results shown in Figure 4B, only one side of the diffractogram was considered. The detector was calibrated, and the channel number units were transformed into distance in angstroms, presenting the scale in a logarithmic way for better clarity. Figure 4B shows that DMPC gave rise to two reflections with distances that related as 1:1/2, consistent with the expected multilamellar organization. With this technique, not only the macroscopic structure itself can be defined, but also the interlamellar repeat distance. The largest first-order reflection corresponds to the interlamellar repeat distance, which is comprised of bilayer thickness plus the water layer between bilayers. The DMPC gave rise to a first-order reflection with a d-value of 66.7 Å in the gel state (10°C), which decreased to approximately 59 Å in the liquid-crystalline state (30°C). Samples containing 10 mol% BaP also displayed a lamellar pattern in the whole temperature range under study, indicating that BaP did not modify the organization of the lamellar structure of DMPC. A similar behavior was observed for DPPC and DSPC (data not shown). However, both below and above the gel–to–liquid crystalline phase transition, some effect was observed on the d-value. At 10°C, the interlamellar repeat distance was increased from 66.7 Å for pure DMPC to 69.3 Å for mixtures containing 10 mol% BaP, with essentially no effect on the intensity of the reflections (Fig. 4B). The increase in d-spacing was approximately 2.6 Å. At 30°C, the effect was of the same order of magnitude, increasing the d-spacing by approximately 2 Å.

Using the data from the DSC scans shown in Figure 2, partial phase diagrams for the phospholipid component were constructed for the mixtures of BaP with the three phosphatidylcholines under study (Fig. 5). Despite quantitative differences, the behavior found was the same for DMPC, DPPC, and DSPC. The BaP did not undergo any phase transition in the range of temperature studied; therefore, all the thermotropic transitions observed arose from the phospholipid component of the mixture. The onset and completion temperatures of the main transition provided the points to define the solidus and fluidus lines (boundary lines of the gel–to–liquid crystalline phase transition), respectively. Increasing the concentration of BaP from to 0 to 50 mol% produced a decrease in the temperature of both lines in a near ideal manner, so that no immiscibilities or critical points were observed. At a given BaP molar ratio, increasing temperature made the systems evolve from a gel phase (G) to a lamellar liquid-crystalline phase (F) through a region of coexistence of both phases (G...).
Fig. 4. A. Raw, representative x-ray diffraction pattern obtained for pure dimyristoylphosphatidylcholine at 10°C. B. x-ray diffraction profiles for pure dimyristoylphosphatidylcholine (bottom) and dimyristoylphosphatidylcholine/benzo[a]pyrene (10 mol%; top) at 10°C.

+ F), which became wider as the concentration of BaP increased. In relative terms, the effect was stronger with DMPC than with DPPC or DSPC (i.e., the effect was stronger as the lipid acyl chain was shorter).

The perturbation exerted by BaP on different parts of the phospholipid molecule was investigated by Fourier transform infrared spectroscopy. Figure 6 shows the C=O stretching band for pure DMPC and a mixture with 10 mol% BaP in the gel state. Pure DMPC showed an absorption maximum in the gel state centered at 1,733 cm⁻¹ (Fig. 6A). The presence of BaP shifted this absorption maximum to lower values, being 1,726 cm⁻¹ for the mixture containing 10 mol% BaP. Similar displacements were obtained in the fluid state (data not shown).

The C=O groups of diacylphospholipids in lipid vesicles may be in a hydrated and a dehydrated state, with their proportion depending on the physical state of the phospholipid bilayer [18,19]. The spectra of pure DMPC represent a summation of the component bands centered at 1,742 and 1,727 cm⁻¹ (and attributed to dehydrated and hydrated C=O groups, respectively) [20]. The spectra shown in Figure 6 (solid lines) were subjected to curve fitting to two bands centered at 1,742 and 1,727 cm⁻¹, simulated by a Gaussian-Lorentzian function. However, whereas the band corresponding to pure DMPC could be perfectly fitted (Fig. 6A), no good fit could be achieved for the spectra of samples containing BaP. Under certain circumstances, a third component band, centered at 1,704 cm⁻¹ and corresponding to doubly hydrated C=O, might occur [17]. Thus, a best-fit estimate could then be obtained for the samples containing BaP by including this third, low-frequency component (Fig. 6B). The relative areas of these simulated bands were calculated for pure DMPC and a mixture with 10 mol% BaP and are shown in Figure 7. It can be seen that the presence of BaP substantially increased the overall contribution of hydrated components (1,727 and 1,704 cm⁻¹) at the expense of the nonhydrated one (1,742 cm⁻¹). Further-

Fig. 5. Partial phase diagrams for dimyristoylphosphatidylcholine (A), dipalmitoylphosphatidylcholine (B), and distearoylphosphatidylcholine (C) in mixtures with benzo[a]pyrene. Open and closed circles were obtained from the onset and completion temperatures of the main gel-to-liquid crystalline phase transitions shown in Figure 2 and represent the solidus and fluidus lines, respectively. G = gel phase; F = liquid-crystalline phase.
Benzo[a]pyrene perturbs phosphatidylcholine membranes

Environ. Toxicol. Chem. 21, 2002 791

Fig. 6. Infrared spectra of the carbonyl stretching band of dimyristoylphosphatidylcholine (A) and dimyristoylphosphatidylcholine/benzo[a]pyrene (10 mol%; B) at 10°C. The dashed lines represent the carbonyl band components obtained by band fitting.

Fig. 7. Relative area of the dehydrated (black), monohydrated (light gray), and dihydrated (dark gray) components of the carbonyl stretching band for dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylcholine/benzo[a]pyrene (DMPC + BaP; 10 mol%) in the gel (10°C) and fluid (30°C) state.

more, BaP induced the appearance of another component band at 1,704 cm⁻¹, which corresponded to a disolvate. These effects were observed both below and above the phase transition.

When the CH₂ stretching bands of DMPC were studied, no change in the frequency of these absorption bands was found by the presence of BaP, neither below nor above the phase transition (data not shown).

Computer simulation of the interaction of BaP with DMPC adopting a bilayer configuration was performed with HyperChem (Hypercube, Gainesville, FL, USA) molecular modeling software using a Steepest Descent algorithm with a termination condition of 0.0001 Kcal Å⁻¹ mol⁻¹. Thus, in the minimum-energy configuration obtained, BaP was placed in the most apolar region of the bilayer, that is, near the terminal CH₃ groups of the acyl chains and laying parallel to them (Fig. 8), so that hydrophobic interactions were maximized.

DISCUSSION

The present study was carried out to investigate the nature of the interaction between a well-known carcinogen, BaP, and lipid membranes using lipid vesicles composed of phosphatidylcholines of various acyl chain length, the most important class of phospholipid. The aim of this work was to explain some other noncarcinogenic, cytotoxic actions of BaP that might take place at the level of the cellular membrane. Actually, the carcinogenic action of BaP is a long-term event in comparison with other alterations of normal cell functioning that begin as soon as BaP is taken up by cells. The DSC was used to characterize the influence of BaP on the thermotropic properties of phosphatidylcholines. The effect of BaP on the macroscopic organization of DMPC was evaluated by mean of SAXD, whereas infrared spectroscopy was used to analyze the interaction of BaP with defined parts of the phospholipid moiety.

The effect of incorporation of increasing concentrations of BaP on DMPC membranes (and, by extension, on the other phosphatidylcholines under study) as seen by DSC can be summarized as follows: First, the disappearing of the pretransition at very low BaP concentrations; second, the broadening and shifting to lower temperatures of the main transition; and third, the decrease in the transition enthalpy. These effects were considerably more pronounced in the case of DMPC than in the case the longer-acyl-chain species like DPPC and DSPC, in which the stronger van der Waals forces characteristic of saturated and longer-chain phospholipids increase the tightness of the packing of these molecules. We can then conclude that the miscibility of BaP with any particular phospholipid is inversely related to the degree of order of the tightness of packing that is characteristic of that phospholipid at a given temperature and phase state. Although other, more specific interactions may occur in any particular phospholipid–BaP system, it seems that, in general, BaP has the ability to maximize BaP–phospholipid interactions and to minimize phospholipid–phospholipid interactions, and that this will depend on the strength of phospholipid–phospholipid interactions, which, in our case, are less important in DMPC. A concomitant decrease in the transition enthalpy also will occur as a consequence of the diminished hydrophobic interactions between the acyl chains themselves due to intercalation of BaP. No immiscibilities were observed in these mixtures whatsoever, which indicates a good miscibility as well as a homogeneous distribution of BaP in
the membrane, due to the more favorable interaction of the BaP molecules with the phospholipid molecules compared with the interaction between themselves, as discussed above.

As we have determined by SAXD, BaP does not affect the lamellar organization of phosphatidylcholine, at least in the species and concentration ranges studied here. However, it does increase bilayer thickness to a considerable extent. Bilayer thickness is a parameter comprised of two others: The net length of the phospholipid palisade, and the hydration layer thickness discussed above.

The interaction of BaP into the hydrophobic region of the membrane may be a cause of bilayer swelling, as has been described for other PAHs of smaller size, like anthracene or phenanthrene [12].

To investigate the effect of BaP on the polar region of the membrane, infrared spectroscopy was used. Our results clearly show that incorporation of BaP into DMPC membranes strongly increases hydration of the C=O groups in such a way that a disolvate of the C=O group (absorption maximum = 1,704 cm⁻¹) is obtained. This will be the result of the spreading of the phospholipid molecules within the plane of the bilayer due to intercalation of BaP, which will allow a deeper water penetration into the region of the glycerol moiety (increasing hydration) and could also account for the augmented bilayer thickness discussed above.

Considering our results together, the following picture can be formed regarding the interaction of BaP with phosphatidylcholine membranes: Given its planar aromatic character, BaP will penetrate the hydrocarbon region of the bilayer, establishing strong hydrophobic interactions with the phospholipid acyl chains. This will cause expansion and swelling of the bilayer, resulting in increased water penetration into the region of the glycerol moiety, where the C=O groups are located.

The interaction of BaP with a DMPC bilayer was simulated by means of computer-aided molecular modeling. Using different approaches, a minimum-energy configuration was achieved in which BaP was located at the most apolar region of the membrane, that is, near the acyl chain terminal CH₃ groups (Fig. 8). This most favorable geometrical disposition is in total agreement with the experimental results described in this work, explaining the strong perturbation of phospholipid membranes exerted by BaP.

Despite BaP mainly being known for its strong mutagenic character [7], this is not necessarily the only toxic action of this aromatic compound. It should not be forgotten that, on exposure to cells, BaP will readily partition into cellular membranes [5], where its toxic action will begin. Thus, in a biological membrane, as a result of the accumulation of BaP molecules in the membrane lipid bilayer, the structural and functional properties of these membranes will be altered in the way described here, which will produce a loss of membrane integrity and increased permeability to protons and ions [12,21], resulting in impaired normal functioning.

Acknowledgement—This work was supported by Fundación Séneca, Comunidad Autónoma Región de Murcia, Spain.

REFERENCES