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The location of organotins within the erythrocyte membrane in relation to their toxicity

Dorota Bonarska-Kujawa *, Halina Kleszczyńska, Stanisław Przestalski

Department of Physics and Biophysics, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland

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ABSTRACT

The aim of the present study on organotin compounds, which are toxic to biological systems, was to determine the relationship between the compounds' toxicity and their location in the lipid bilayer of the biological membrane. It was assumed that the degree of disturbance caused within the lipid bilayer of the membrane, which in turn depends on the depth of incorporation, was an appropriate measure of toxicity.

Previous results from our studies on the effect of organotin chlorides on membranes, made by using infrared radiation and hemolysis of erythrocytes, indicated that tributyltin (TBT) is the most active in terms of its interaction with the erythrocyte membrane. This compound causes the most severe hemolysis of erythrocytes and dehydration of membrane constituents. In order to connect the changes induced within the membrane structure with the compounds' location, we have investigated erythrocyte shape changes using both microscopic and fluorimetric methods. The microscopic results show that organotin compounds accumulate in the outer monolayer of the membrane. The fluorimetric studies indicate that all the compounds are present in the hydrophilic part of the outer lipid monolayer, and change the order parameter of the layer. However, only tributyltin, by being incorporated into the hydrophobic region of the monolayer, changes the fluidity in the alkyl chain region of the erythrocyte membrane.

Furthermore, only TBT is present in both the hydrophilic and hydrophobic regions, as evidenced by the changed order parameter of the polar groups and fluorescence anisotropy of DPH probe in the hydrophobic region, these being connected with its high toxicity.

1. Introduction

Organometallic compounds are in general more toxic to living organisms than their inorganic counterparts (Fent, 1996; Craig, 2003; White and Tobin, 2004). In particular, organotins are toxic to humans, animals and plants. Owing to their numerous applications in industry and agriculture, contamination of the environment is on the increase, as is the threat they pose to living organisms (Eng et al., 1991; Nagase et al., 1991; Krug, 1992; Musmeci et al., 1992; Huang et al., 1996; Lascourreges et al., 2000; Nielsen and Rasmussen, 2004; Marcic et al., 2006; Lespes et al., 2009). The absorption of organotins by the human organism occurs mainly through skin and the digestive system. All the organotins, in few minutes of intensive contact, cause local disturbances, irritation and damage of the skin, irritation of eyes, disturbance in eyesight and breathing. They also adversely affect the nervous and nutritional system (e.g. disturbed sense of smell, neuron necrosis, encephalopathy, amnesia, headache, disturbance in equilibrium, vomiting, nausea, loss of appetite, stomachache), disorder and damage of liver and kidney, anemia and leukopenia (Craig, 2003).

The mechanism of these compounds' action, and that of many other toxicants, at the molecular level has not yet been fully explained, and concerns the cell membrane, which is the prime target for any toxicants. As shown by Ortiz et al. (2005) and Falcioni et al. (2008), among the alkyltin compounds high toxicity is shown by butyl and phenyl derivatives, whereas Buck-Koehntop et al. (2005) emphasize the special toxicity of trimethyltin chlorides. In order to explain the mechanism of the action of organotins (and that of other compounds) on the main components of membranes, model lipid membranes are often used in investigations (Radecka et al., 1999; Zielińska et al., 2000; Sarapuk et al., 2001; Chicano et al., 2002; Petrosyan et al., 2003;

Abbreviations: TPhT, triphenyltin chloride—$(C_6H_5)_3SnCl$; TBT, tributyltin chloride—$(C_4H_9)_3SnCl$; DPhT, diphenyltin chloride—$(C_6H_5)_2SnCl_2$; DBT, dibutyltin dichloride—$(C_4H_9)_2SnCl_2$; Laurdan, fluorescent probe—6-dodecanoyl-2-dimethylaminonaphthalene; DPH, fluorescent probe—1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, fluorescent probe—1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate; GP, generalized polarization

* Corresponding author. Fax: +48 71 3205167.
E-mail address: dorota.bonarska-kujawa@up.wroc.pl (D. Bonarska-Kujawa).

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Man, 2008), and erythrocyte membranes (RBC), which are treated as an example and model of the biological membrane (Byington et al., 1974; Gray et al., 1987; Hamasaki et al., 1995; Falcioni et al., 1996; Sato et al., 1997; Kleszczyńska et al., 1997; Kleszczyńska et al., 1998; Langner et al., 1998; Suwalsky et al., 2004). The above-mentioned studies have shown that the compounds are incorporated into the biological membrane and disturb its structure as a result of their interaction with the lipid and protein phases (Przestalski et al., 2000). The toxic effect of organotins on the membrane, as documented by numerous studies, depends on the dose. At very small concentrations (μM) the compounds are deleterious to the nervous system (Buck-Koehntop et al., 2005), and at higher concentrations (mM) they do damage to the membrane, inducing erythrocyte hemolysis (Byington et al., 1974; Hamasaki et al., 1995; Kleszczyńska et al., 1997, 1998; Burda et al., 2002).

The cause of hemolysis is thought to be the incorporation of a sufficient number of biologically active molecules. It is also known that membrane active substances, especially those of lipophilic structure, concentrate in the lipid phase of the membrane, weakening the interaction between its constituents and resulting in a breakdown of the membrane structure.

The present investigations aimed at determining the toxicity of tin organic compounds towards the biological membrane and finding the relationship between their location in the lipid bilayer of the erythrocyte membrane and toxicity. For that purpose, the effect of triphenyl- and tributyltin chlorides and dichlorides of diphenyl- and dibutyltin on the shape of erythrocytes and fluidity of the erythrocyte membrane was studied. Studies performed by many authors (Bessis, 1977; Danieluk et al., 1998; Deuticke, 1968; Iglič et al., 1998; Isomaa et al., 1987; Ponder, 1948; Przybylska et al., 1998; Schwarz et al., 1999a; Schwarz et al., 1999b; Sheetz and Singer, 1974; Tachev et al., 2004; Wong, 1999; Vranić-Manduscić et al., 2004) with various biologically active compounds have made it possible to determine, on the basis of red blood cell shapes induced by incorporated substances, the location of the substances in the lipid bilayer of the erythrocyte membrane. Fluidity was investigated in different regions of a modified red blood cell membrane after incorporation of the compounds by using three fluorescence probes that emit fluorescence from various depths of the lipid phase of the membrane. The extent of changes in the packing order of a lipid layer of the erythrocyte membrane was assumed to be a measure of the concentration of the toxicants in a specific region of the membrane.

2. Materials and methods

The studies were conducted on pig erythrocytes and on isolated erythrocyte membranes, which were obtained from fresh blood using the Dodge et al. (1963) method. The choice of pig erythrocytes was dictated by the fact that this cell's percentage share of lipids is closest to that of the human erythrocyte, and the blood was readily available. Fresh blood was taken each time to physiological solution of sodium chloride to which heparin had been added.

The following tin organic compounds, all purchased from Sigma-Aldrich, were used in the investigation: triphenyltin chloride (TPhT)—$(C_6H_5)_3SnCl$; tributyltin chloride (TBT)—$(C_4H_9)_3SnCl$; diphenyltin dichloride (DPhT)—$(C_6H_5)_2SnCl_2$; and dibutyltin dichloride (DBT)—$(C_4H_9)_2SnCl_2$. The fluorescent probes 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) were purchased from Molecular Probes (Fig. 1).

2.1. Microscopic investigation

For investigation using the optical microscope the red cells, when separated from plasma, were washed four times in saline solution and suspended in the same solution but containing an appropriate amount of the compounds studied. Hematocrit of the erythrocytes in the modification solution was 2%, the modification lasting 1 h at 37 °C. After modification the erythrocytes were removed from the solution and fixed with a 0.2% solution of glutaraldehyde. Then the red cells were observed under a biological optical microscope (Nikon Eclipse E200) equipped with a digital camera. The photographs obtained made it possible to count erythrocytes of various shapes, and then the percentage share of the two basic forms (echinocytes and stomatocytes) in the population of ca. 800 cells was determined. In the investigation the concentration of tin chlorides was 0.01 mM. The individual forms of erythrocyte cells were ascribed morphological indices according to Bessis scale (Bessis, 1977), which for stomatocytes assumes negative values from −1 to −4, and for echinocytes from 1 to 4.

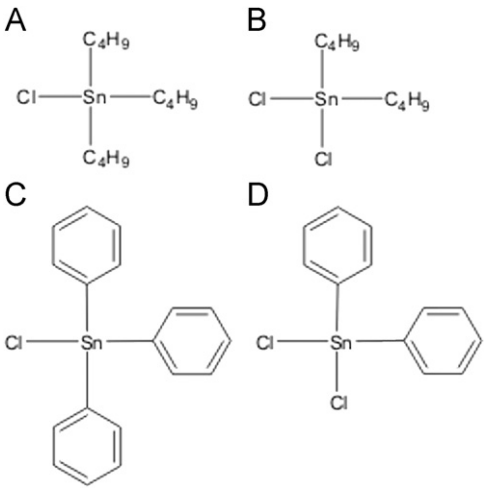

Fig. 1. Chemical structures of organotins compounds: (A) TBT, (B) DBT, (C) TPhT, (D) DPhT.

For investigation using the electron microscope the red cells were fixed for 12 h in 2.5% solution of glutaraldehyde buffered with PBS of pH 7.4 at 18 °C. After fixation the material was fixed again for 1 h in 1% solution of osmium tetroxide in the same buffer at 4 °C. Mica slates covered with the red cells were dehydrated with solutions of alcohol and acetone of increasing concentrations. The preparations were then dried with a method based on the critical point of CO_2 in a Balzers instrument CP-010. Afterwards, mica fragments covered with cells were placed on metal plates and sprayed with carbon and silver in a sputter coater (VEB Hochvakuum-Dresden B30.1). The preparations were viewed and photographed in the (Tesla BS 300) scanning electron microscope at 20 kV.

2.2. Fluorimetric method

The effect of organotins on the degree of order of lipids in the erythrocyte membrane (ghosts) was investigated using the fluorimetric method. The ghosts were suspended in an isotonic phosphate solution of pH 7.4, of such quantity that the protein concentration in the samples amounted to approximately 100 mg/ml. Control samples contained an erythrocyte ghost suspension and a fluorescent probe, while the investigated samples in addition contained appropriate concentrations of the compounds studied. Fluorescence intensity was measured by using three fluorescent probes – Laurdan, DPH and TMA-DPH – whose concentration in the samples was 10 μM, while concentrations of the compounds were within the range 5–25 μM. Measurements were conducted with a fluorimeter (CARRY Eclipse of VARIAN) at temperature 37 °C. The excitation and emission wavelengths were as follows: for the DPH probe $\lambda_{ex}=360$ nm, $\lambda_{em}=425$ nm, and for the TMA-DPH probe $\lambda_{ex}=358$ nm, $\lambda_{em}=428$ nm. The excitation wavelength for Laurdan was 360 nm, and the emitted fluorescence was recorded at two wavelengths, 440 and 490 nm.

Fluorescence anisotropy (A) for probes DPH and TMA-DPH was calculated using the formula (Lakowicz, 2006):

$$A=\frac{(I_{II}-GI_{\perp})}{(I_{II}+2GI_{\perp})} \quad (1)$$

where I_{II} and $I_{\perp}$ are fluorescence intensities observed in directions parallel and perpendicular, respectively, with respect to the polarization direction of the exciting wave. G is an apparatus constant dependent on the emission wavelength.

Changes in the packing order of the hydrophilic part of the membrane were investigated using the Laurdan probe, on the basis of generalized polarization (GP), and were calculated with the formula (Parasassi et al., 1998):

$$GP=\frac{(I_b-I_r)}{(I_b+I_r)} \quad (2)$$

where I_b is fluorescence intensity at $\lambda=440$ nm, while I_r is fluorescence intensity at $\lambda=490$ nm.