Different Effects of Di- and Triphenyltin Compounds on Lipid Bilayer Dithionite Permeabilization

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Phenyltins, Lipid Bilayer, Membrane Permeability

Phenyltins are chemicals widely used in industry, hence their occurrence in the human environment is frequent and widespread. Such compounds include hydrophobic phenyl rings bonded to positively charged tin. This molecular structure makes them capable of adsorbing onto and penetrating through biological membranes, hence they are potentially hazardous. Two such compounds, diphenyltin and triphenyltin, show different steric constraints when interacting with the lipid bilayer. It has been demonstrated that these compounds are positioned at different locations within model lipid bilayers, causing dissimilarity in their ability to affect membrane properties. In this paper we present a study regarding the ability of these two phenyltins to facilitate the transport of $S_2O_4^{-2}$ ions across the lipid bilayer, evaluated by a fluorescence quenching assay. In concentration range of up-to 60 μM those compounds do not affect lipid bilayer topology, when evaluated by vesicle size distribution. Both phenyltins facilitate the transfer of $S_2O_4^{-2}$ across the model lipid bilayer, but the dependence of dithionite transport on phenyltin concentration is different for both. In principle, above 20 μM triphenyltin is more efficient in transfering ions across the lipid bilayer than diphenyltin.

Introduction

Organotins are compounds that have one or more tin-carbon bonds and can be described with the general formula R_nSnX_{4-n}, where R is a hydrocarbon group, X are anionic species and n varies from 1 to 4 (Blunden and Evans, 1990). These compounds are widely applied in various industries. For example, dialkyltins are used as stabilizers for polyvinylchloride plastics and other materials containing chlorine (Craig, 1982), triphenyl- and tributyltin are used in agriculture and as antifouling paints, as they are toxic for microorganisms, insects, shellfish and seaweed (Blunden and Evans, 1990). Due to such a broad spectrum of application such compounds are common in the human environment. Their amphiphilic character makes them biologically potent, mainly because they are capable of penetrate physiological barriers (Gray *et al.*, 1987; Przestalski *et al.*, 2000). They affect the immunological system (Desantiago and Aquilarsantelises, 1999), cause systemic toxicity (WHO, 1980; Boyer, 1989; Colosio *et al.*, 1991; Lin and Hsuch; 1993, Ohhira *et al.*, 1999), phenyltins also induce hyperglycemia and hypertriglyceridemia (Ogino *et al.*, 1996; Ohhira *et al.*, 1999). Understanding the molecular mechanisms of this interaction with the lipid bilayer may prove useful to explain their influence on living organisms. In practical applications triphenyltins are mostly used. However, once the substance enters a living organism some transformations occur. In particular, the degradation from R_3SnX to R_2SnX_2 (but also to $RSnX_3$ and SnX_4) should be considered. Depending on concentration of the initial compound, time of experiment, and the kind of animal different contents of R_3SnX and R_2SnX_2 occur in various tissues of the animal (Ohhira *et al.*, 1997). The different distribution of the compounds in various tissues could be due to differential properties of cell membranes with respect to both organotins. For this and other reasons, we became interested in the effect of both compounds on liposome membranes. Only a limited number of reports concerning organotin biological potency is available, and studies on model membranes that address questions regarding the molecular basis of the biological effect of these compounds (Aldridge

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and Street, 1964; Selwyn *et al.,* 1970; Selwyn, 1976; Arkagawa and Wada, 1984; Ambrosini *et al.*, 1996; Cullen *et al.,* 1997; Sato *et al.,*1997). The objective of the study was to determine the difference between the effects of di- and triphenyltin on $S_2O_4^{-2}$ ion permeation across a phosphatidylcholine liposome membrane, and the concomitant difference in localization of the two phenyltins in the membrane. By photon correlation the influence of the two compounds on stability of the membrane structure of the liposomes used in $S_2O_4^{-2}$ permeabilization studies was also determined.

Materials and Methods

Materials

Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) was from Molecular Probes (Eugene, OR, USA). Diphenyltin chloride and triphenyltin chloride were purchased from Alfa Products (Karlsruhe, Germany) and sodium dithionite from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Vesicle size determination and fluorescence measurements.

To estimate PC lipid bilayer permeability the fluorescence assay was used, allowing to measure the time course of dithionite penetration into the vesicle inner volume. Dithionite, a reducing agent, interacts with the fluorophore (covalently bound to the membrane surface) and quenches permanently its fluorescence. When preparing labeled liposomes, the PC was mixed with 1 mol% NBD-PE in chloroform, the sample was then evaporated under vacuum to dryness, resuspended in phosphate buffer of pH 7.4 (120 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.79 mM Na_2HPO_4 $2H_2O$, 1.80 mM Na_2H_2 PO_4 H_2O), vortexed and sonicated to obtain a small vesicles suspension. The vesicle suspension was prepared freshly prior to each experiment. Phenyltins were added from a concentrated ethanol stock solution (the final ethanol concentration never exceeded 2 mol% v/v) to the vesicle suspension and allowed to equilibrate. The final phenyltin concentration in the probe was changed from zero to 60 μM. Measurements were carried out when sample fluorescence intensity reached a stable value. The freshly prepared dithionite solution was than added to the vesicle suspension. The final PC and dithionite concentration in the sample was 130 μM and 8.3 mM, respectively. Fluorescence intensity as a function of time was recorded. All steady-state fluorescence experiments were performed with a SFM 25 Kontron Instrument fluorimeter (Switzerland). Vesicle suspension intended for size distribution measurements was prepared as described above, but without the fluorescent probe, and phenyltin was mixed with lipids prior to vesicle formation. Vesicle sizes were determined by a ZETASIZER 5000 analyzer (Malvern Instruments, UK). The photon correlation method is based on time-dependent intensity of light-scattered by liposomes. The measurements can be analysed using a proper correlation function and represented by the Gaussian normal distribution. Maximum in the distribution determines the liposome diameter and the half-width is equal to the double value of the diameter error.

Results and Discussion.

Diphenyltin and triphenyltin are compounds that interact with the lipid bilayer differently. The triphenyltin octanol-water partition coefficient was determined to be higher than that of diphenyltin, suggesting higher affinity toward biological membranes (De Young and Diu, 1990; Nagase *et al.*, 1991). The hemolytic potency of these compounds seem to follow the partition coefficient. However, determination of the location of these two molecules in the model lipid bilayers showed that the more hydrophobic triphenyltin is not able to penetrate the membrane hydrophobic interior as efficiently as diphenyltin. Consequently, both compounds are located in different membrane regions and affect the lipid bilayer differently. The surface charge introduced onto the membrane surface by triphenyltin was bigger but disturbance within the hydrophobic core was more eminent when diphenyltin was present (Langner *et al.,* 1998).

Since the addition of phenyltins modifies different lipid bilayer regions depending on the type of phenyltin added, the incorporation of the com-

pound may result in general membrane topology alteration resulting in drastic membrane property changes. To test this we studied sonicated vesicle size distribution when various amounts of phenyltins were added. Vesicle size distribution, as a function of diphenyltin and triphenyltin concentrations, when measured with photon correlation method, showed no vesicle size alteration when up to 30 μM of phenyltin was added (mean diameter of liposomes was 255,5 ± 187 nm). However, at a higher concentration of 60 μM the liposome diameter in the presence of diphenyltin did not change; whereas liposome with triphenyltin present changed their mean size by 28%. This experiment shows that phenyltin addition kept the lipid vesicles intact and does not aggregate.

Membrane permeabilization by phenyltins was measured with the quenching method, described elsewhere (McIntyre and Sleight, 1991). The location of the fluorescent probe on the membrane surface is affected by adsorbing compounds, hence the dependence of fluorescence intensity on the amount of phenyltin present is shown on Figure 1. The data presented show that phenyltins cause fluorescence intensity of NBD-PE to decrease, but the effect is small and can be neglected for all practical purposes (compare scales on Fig. 1 and Fig. 2).

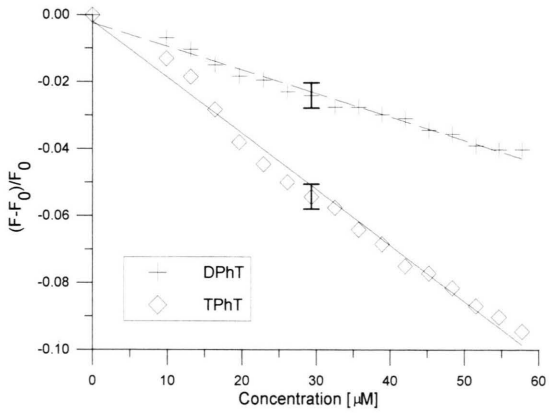

Fig. 1. The dependence of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) relative fluorescence intensity, $(F-F_0)/F_0$, on diphenyltin (DPhT) and triphenyltin (TPhT) concentrations. The fluorescence probe was incorporated into Egg-PC small unilamellar vesicles. The total lipid concentration used in samples was equal to 0.1 mg/1 ml (130 mM). Error bars represent deviation from the mean.

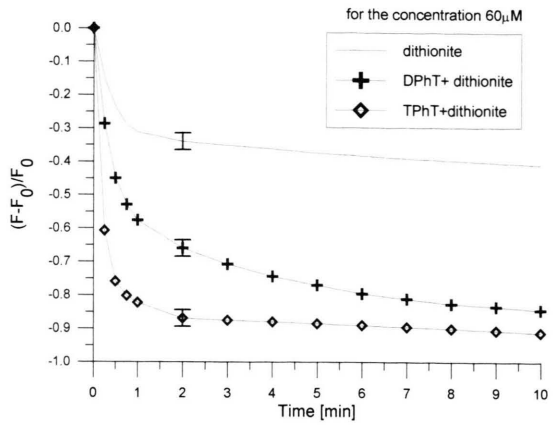

Fig. 2. Time dependence of the N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) fluorescence intensity, $(F-F_0)/F_0$, after a dithionite addition in the absence and presence of di- (DPhT) and triphenyltins (TPhT). The DPhT and TPhT concentrations were equal to 60 mM. Error bars represent deviation from the mean.

Positively charged phenyltins facilitate an electrically silent electric current by associating with a monovalent anion, as has been shown using black lipid membrane (BLM) model (Antonenko, 1990), erythrocytes and erythrocyte ghosts (Wieth and Tosteson, 1979). These electrically neutral pairs cross the lipid bilayer without net charge transfer. In order to measure phenyltin ability to carry large ions such as dithionite we adopted the fluorescence quenching assay enabling us to estimate dithionite transfer rate across the lipid bilayer (Langner and Hui, 1993). The fluorescent probe, NBD-PE, is symmetrically distributed between both membrane leaflets, hence the addition of a reducing agent such as dithionite into the labeled vesicle suspension causes time-dependent fluorescence change (Fig. 2). The time course of fluorescence intensity change induced by the dithionite addition shows that most probably two processes are taking place (Fig. 3). An initial fast fluorescence intensity drop corresponds to fluorescence depletion due to the permanent quenching of probes located on the outer leaflet of the lipid bilayer. The remaining fluorescence is therefore proportional to the amount of remaining probes located on the inner lipid leaflet. Water soluble dithionite does not penetrate the intact lipid bilayer, hence it interacts only with fluorescent dye located on the outer leaflet of the membrane. Fur-

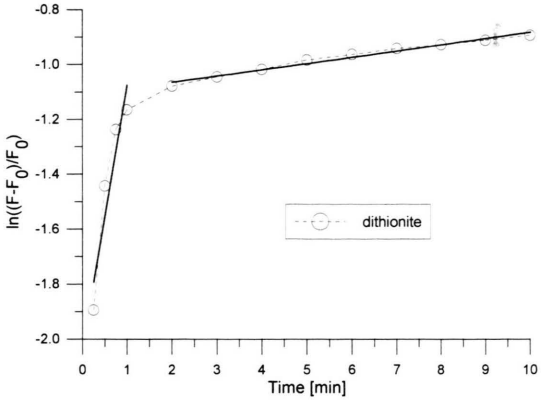

Fig. 3. Logarithm of the relative fluorescence intensity, $(F-F_0)/F_0$, as a function of time (after addition of dithionite). The time course shows that fluorescence quenching is a two-step process, consisting of a fast quenching of the externally located fluorescent probes and a slow quenching of the fluorescent probe on the inner leaflet of the lipid bilayer. These two processes are represented by straight lines.

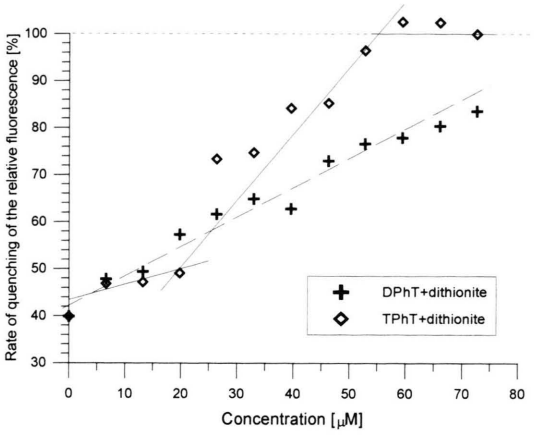

Fig. 4. The rate of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) fluorescence quenching efficiency, as a function of diphenyltin (DPhT) and triphenyltin (TPhT) concentration. The quencher (dithionite) was added after vesicle treatment with phenyltin.

ther fluorescence intensity decrease results from fluorescent probe "flip-flop" across the membrane and residual dithionite transfer across the membrane. The slow rate of irreversible NBD-PE fluorescence quenching may be used as an indicator of dithionite transfer through the lipid bilayer (Langner and Hui, 1993). A typical dependence of fluorescence intensity on time, for an unmodified lipid bilayer, is shown by Figure 2. When the membrane is modified with phenyltin, fluorescence dependence on time changes (phenyltin concentration in this particular experiment was 60 μM). The presence of phenyltin on the lipid bilayer causes fluorescence quenching efficiency to increase. Altered quenching efficiency may be the result of enhanced dithionite transfer across the lipid bilayer or phenyltins forming a pore enabling the quencher to pass through the membrane. The latter can be excluded, as the chemical structure of phenyltin makes pore formation unlikely. The hypothesis of facilitated dithionite transport is supported by other studies that show organotins are associated with monovalent ions and that such neutral complexes can transfer the membrane (Antonenko, 1990). As it can be seen at Fig. 2 triphenyltin facilitates dithionite transfer more efficiently than diphenyltin. The dependence of quenching efficiency was measured as a function of phenyltin concentration and data are presented by Figure 4, which represents dithionite quenching efficiency time rate. Fluorescence depletion is calculated according to the equation $(F-F_0)/F_0$ where F_0 is the initial fluorescence intensity at zero time (before dithionite addition) and F fluorescence intensity at a fixed time interval after dithionite addition (2 minutes). Those data should therefore be understood as the quenching time rate. It can be concluded that triphenyltin is a more efficient membrane permeabilizer. In addition, concentration characteristics of triphenyltin and diphenyltin quenching rates differ in character. Rising diphenyltin concentration causes a linear increase of membrane permeability. Triphenyltin, on the other hand, requires a minimal concentration (about 20 μM) before facilitating dithionite permeability. After reaching this concentration quencher transport becomes very efficient, so that at a concentration of 60 μM the quenching rate caused by triphenyltin exceeds that caused by diphenyltin. This suggests that triphenyltin needs to form an aggregate on the membrane surface to become a dithionite carrier.

The two compounds are located in different lipid bilayer regions, namely diphenyltin penetrates the membrane hydrophobic core whereas triphenyltin apparently adsorbs within the membrane interface. Such compound locations seem to affect their ability to carry dithionite across the

membrane. Diphenyltin, penetrating the hydrophobic membrane core, after association with dithionite, seems capable of changing monolayers as a monomer. Triphenyltin does not have such capability. The quenching efficiency dependence on concentration suggests that triphenyltin needs to form an aggregate before efficient dithionite transfer can be effected. The assumption that the compounds Ph_3SnCl accumulates predominantly in the surface layer of the membrane, and Ph_2SnCl_2 in the hydrophobic layer allows to explain the dithionite permeabilization of the liposome membrane. The conflicting opinion of (Różycka-Roszak *et al.*, 2000) is based on quite different experiments (phase transitions) and other conditions, and thus do not apply to our conditions.

Triphenyltin potency has a stronger hemolytic potency than diphenyltin. Taking into account the lipid bilayer alterations caused by triphenyltin a cellular damage by a variety of mechanisms can be assumed: the compounds location within the interface enables it to interact electrostatically with surface – associated proteins and anionic lipids, and thus they interfere in various metabolic and regulatory processes (Langner and Kubica, 1999). In addition, as has been shown in this paper, triphenyltin can facilitate the transfer of anions, like $S_2O_4^{2-}$, across the plasma membrane disturbing the cellular ionic balance. Diphenyltin, on the other hand, introduces significantly smaller surface disturbance, but causes changes in the hydrocarbon chain region. Since the membrane hydrophobic core has an extensive ability to accommodate hydrophobic compounds, the amount of diphenyltin required to alter membrane properties is higher. A similar conclusion had been reached when the effect of cholesterol on phenyltin adsorption was measured (Langner *et al.*, 2000). Our previous experiments allow us to postulate that steric constraints cause diphenyltin to penetrate the lipid bilayer core more efficiently than triphenyltin.

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