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The effect of phenyltin chlorides on osmotically induced erythrocyte haemolysis

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The toxicity of many amphiphilic compounds may result from their effect on the lipid phase of biological membranes. Upon incorporation such compounds may change the properties of membranes in general and in particular alter the organization of membrane lipids. These changes should affect, among other things, the mechanical properties of membranes. We selected two amphiphilic compounds, diphenyltin dichloride (Ph_2SnCl_2) and triphenyltin chloride (Ph_3SnCl), which are known to be located at different regions of the lipid bilayer and to be toxic. As a model biological membrane the erythrocyte plasma membrane was used. Analysis of the haemolysis kinetics showed differences between the effect of the compound studied on mechanical properties at so-called non-lytic concentrations. Diphenyltin dichloride showed a limited effect on erythrocyte haemolysis, whereas triphenyltin chloride affected all the parameters measured (extent of initial haemolysis, extent of final haemolysis and membrane mechanical strength). We correlated these effects with the location of the investigated compounds in liposomes. The presented data show that triphenyltin chloride reduces the erythrocyte plasma membrane mechanical strength and increases the extent of haemolysis under osmotic stress conditions.

KEYWORDS: triphenyltin chloride; diphenyltin dichloride; erythrocytes; haemolysis; membrane

INTRODUCTION

Organotin derivatives are toxic compounds often occurring in the human environment.[1] Mostly, compounds of the organometallic group are present in the environment due to their use as fungicides,[2,3] biocides,[4,5] antifouling agents,[6,7] wood preservatives and plastics stabilizers.[8] Their interaction with a living organism may lead to diverse pathological changes.[9–13] The toxicity of these compounds depends on various factors. The most important seems to be their capability to enter biological membranes, which is correlated with the compound's lipophilicity.[14–18] The other simple and convenient measure of a compound's toxicity is its haemolytic activity.[19–23] This type of measure provides information not only on the compound's ability to enter biological membranes but also depends on its effect on biological membrane stability[24] and/or, crucial for cell functioning, metabolic processes inside the cell. The traditional haemolytic experiment, based on measurement of the quantity of lysed cells, provides very limited information regarding the effect of the compound on stability and organization of the plasma membrane. A variation of the haemolytic test, the osmotically induced haemolysis in the stopped-flow setup, enables the time dependence of the haemolytic process to be followed, which in turn can be used to estimate membrane parameters related to its mechanical properties. In addition, changes in the mechanical properties of biological membranes are a very sensitive parameter of various modifications caused by inclusions which are detectable at concentrations much lower than that used in lytic tests. We used this technique to evaluate the effect of two phenyltin compounds, namely diphenyltin dichloride and triphenyltin chloride, on stability of the biological membrane at non-lytic concentrations. The two compounds were selected because of their different modes of interaction with the model lipid bilayer and

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different dependence of their toxicity on concentration. Our previous studies[25–29] showed that, whereas diphenyltin dichloride intercalates into the hydrophobic region of the lipid bilayer, triphenytin chloride, due to sterical constrains, resides mainly in the membrane interphase. These results, along with preliminary data obtained with haemolytic tests, show that the two compounds' toxicity may result from differences in their location within the cell plasma membrane.[26]

MATERIALS AND METHODS

Materials

Organotin compounds, namely diphenyltin dichloride [$(C_6H_5)_2SnCl_2$ or Ph_2SnCl_2] and triphenyltin chloride [$(C_6H_5)_3SnCl$ or Ph_3SnCl], were purchased from Alfa Products (Karlsruhe, Germany).

Erythrocyte preparation

Erythrocytes of healthy individuals were isolated from the whole blood samples obtained from a Blood Bank. Erythrocytes were washed three times in 140 mM NaCl with phosphate buffer of pH 7.4 and diluted to obtain a cell suspension of 0.1% haematocrit.[30] Organotin compounds dissolved in methanol were added to cell suspensions for subsequent incubation at 36.6 °C. For each experiment an appropriate control sample with methanol alone was measured as a reference. The concentration of methanol in a sample with phenyltin compounds or in control samples (without phenyltin compounds but with the same amount of methanol as in the sample) did not exceeded 0.3% (v/v).

The kinetics of haemolysis

The kinetics of erythrocyte lysis was measured in a home-made stopped-flow device (with dead time around 0.1 s), as presented elsewhere.[31] The cell suspension was mixed with an equal amount of distilled water, and light transmittance at 700 nm was continuously monitored. Each experiment was repeated at least five times. The intensity of transmitted light depends on the amount of light scattered and absorbed by the cell suspension. The initially scattering cell suspension became more transparent as haemolysis progressed, as illustrated in Fig. 1. We assumed that the absorption resulted exclusively from the presence of haemoglobin, the concentration of which remained constant during each experiment. Therefore, the amount of absorbed light was also constant. Consequently, the contribution of absorbance could be safely subtracted during data analysis. The effect of light scattering on transmittance is a complex function of cell shape and size and the geometry of the sample and detector.[32] We simplified our analysis by disregarding these factors. The simplification is based on the following assumptions: after an initial swelling period (a few seconds) all erythrocytes are spherical; therefore, there are no shape

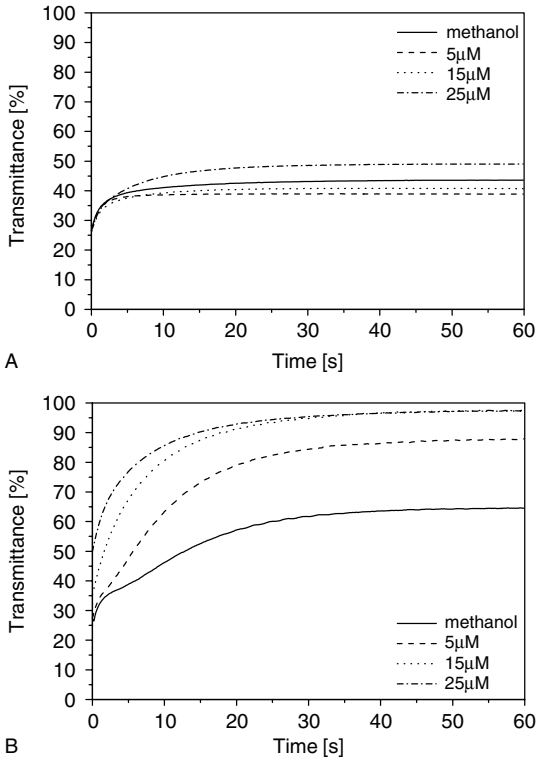

Figure 1. Examples of haemolytic curves for Ph_2SnCl_2 (A) and Ph_3SnCl (B) at concentrations 5, 15 or 25 μM, as indicated.

differences during the lysis period, which is used for the subsequent analysis. Differences in cell sizes alter the overall scattering process, but in a way that is proportional to the amount of cells present in the sample. Hence, its effect on haemolysis kinetics can be safely omitted. In addition, we have independently measured the level of haemolysis (data not shown), revealing a good correlation between the final transmittance and the quantity of surviving cells. Consequently, the final value of transmittance (after 45 s, diluted twice) indicates the extent of sample haemolysis, and can be used to evaluate size in the erythrocyte population that remained intact. The transmittance of the scattered sample depends on the experimental setup configuration, i.e. the angular acceptance of the detector. This in turn is a function of sample heterogeneity and the distance between the scattering medium and the detector. We assume that cells are uniformly distributed and that the solutions are well mixed in the chamber, ensuring constant geometry for the system. Therefore, the quantity of transmitted light depends solely on the number of intact cells present in the chamber during the progress of haemolysis.

Data analysis

Lysis kinetics was fitted with a single exponential.[31,33] The curve fitting was performed with standard libraries from the MathCad Professional 2001 software package. The function used for the subsequent analysis of transmittance as a function

of time has the following form:

$$T = y_0 + ae^{t/\tau} \quad (1)$$

where parameter y_0 can be correlated with the extent of final haemolysis, since not all erythrocytes are lysed in the experimental conditions. The value of transmittance at $t = 0$ equals $(y_0 + a) = B$ and was used to evaluate the initial extent of haemolysis. Time constant τ is associated with membrane mechanical strength. Selected experiments were carried out for at least three different blood samples and the representative data sets are presented. The standard deviations resulting from differences between individual blood samples for all parameters were calculated and were: $y_0 = 13\%$ (average value $65 \pm 13\%$), $a = 14\%$ (average values $35 \pm 14\%$) and $\tau = 4$ s (average values 12 ± 4 s). Owing to large variations between blood samples, each concentration and time dependence characteristic was performed on a blood sample from a single individual (average values from five repetitions). Despite individual differences, the trend of each characteristic was preserved. Because of that, representative changes are presented in all figures.

RESULTS AND DISCUSSION

It has been shown previously[26] that the toxicities of the two phenyltins, when evaluated as the extent of erythrocyte lysis, are different, triphenyltin chloride being more toxic than diphenyltin dichloride. This toxicity was then correlated with compound's effect on model lipid bilayer properties.[28] These experiments showed that diphenyltin affects the lipid bilayer hydrophobic region, whereas the triphenyltin disturbs the membrane interphase. In addition, both compounds, being positively charged, change the membrane surface electrostatic potential.[34–36] The haemolytic experiments require high phenyltin concentrations (hundreds of μM) in order to destabilize and destroy the cell plasma membrane.[22,37,38] To detect biological membrane modification at lower compound concentrations, a more sensitive parameter needs to be measured. The membrane resistance to osmotic stress in a stopped-flow setup is a good indicator of the effect of membrane intrusions on mechanical strength.

Figure 1 shows selected examples of haemolytic curves obtained for erythrocytes treated with various amounts of Ph_2SnCl_2 (A) and Ph_3SnCl (B) after 1 h of incubation. There is an evident qualitative difference between those sets of curves, indicating that the two compounds act on the erythrocyte membrane differently. In order to quantify this difference, each haemolytic curve was fitted with a single exponential, despite the fact that for selected samples the two-exponential fit was better. We used a single exponential approximation in order to avoid the need for two-cell population analysis. This simplification is justified by the fact that, in the case when two-exponents fit was needed, the contribution of the second curve was always lower than 10%. In addition, in most samples the single-exponent fit was satisfactory.

Three parameters were obtained from the fitting procedure: the time constant τ, which can be associated with the membrane mechanical strength;[31,33] the final transmittance level, y_0, which indicates the final extent of haemolysis; and the initial haemolysis, $(y_0 + a) = B$.

The time constant for kinetics of haemolysis in the presence of Ph_2SnCl_2 as a function of its concentration is presented in Fig. 2(A). The time constant first decreases, to approximately 30% as compared with the sample treated with methanol only, and then progressively increases. Only when the Ph_2SnCl_2 concentration reaches 20 μM does the time constant rise above the level of a reference sample. This result shows at first glance that Ph_2SnCl_2 at low concentration (5–20 μM) makes the membrane more prone to lysis, whereas at higher concentrations it actually strengthens it. The other explanation is that the erythrocyte sample consists of two distinctly different cell populations, which respond to Ph_2SnCl_2 treatment differently. This explanation is supported by two additional observations: the dependence of the final

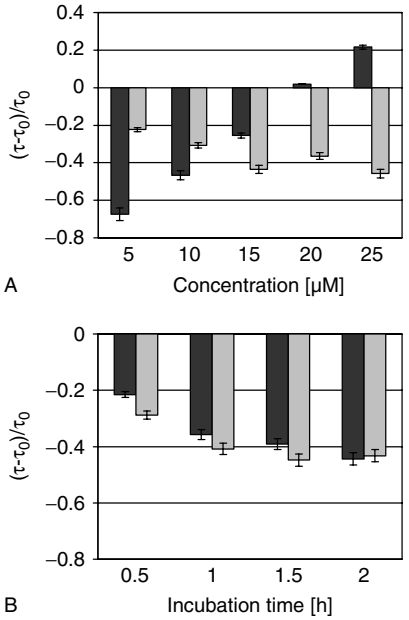

Figure 2. The effect of Ph_2SnCl_2 (dark bars) and Ph_3SnCl (light bars) on the relative change of the time constant τ calculated from kinetic curves. (A) Erythrocytes were incubated with various concentrations of phenyltins for 2 h at 36.6 °C prior to measurement. (B) The dependence of the relative changes of time constant for erythrocyte incubated with 20 μM phenyltins as a function of incubation time. Values represent the average of five repetitions from a single measurement. The standard deviation did not exceed 5%.

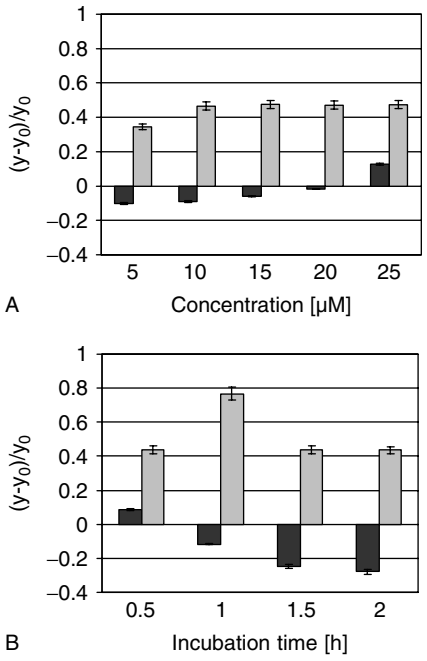

Figure 3. The effect of Ph_2SnCl_2 (dark bars) and Ph_3SnCl (light bars) on the relative change in the extent of final haemolysis, $(y - y_0)/y_0$, calculated from kinetic curves (single exponential fitting was used). (A) Erythrocytes were incubated with various concentrations of phenyltins for 2 h at 36.6 °C prior to measurement. (B) Dependence of the relative change of the extent of final haemolysis for erythrocytes incubated with 20 μM phenyltins as a function of the incubation time. Values represent the average of the five repetitions from a single measurement. The standard deviation did not exceed 5%.

extent of lysis on Ph_2SnCl_2 concentration [Fig. 3(A)]; and that, in this case, two-exponential fitting is more appropriate. The assumption of two different erythrocyte populations, for example, differing in age,[39,40] explains the limited changes in the final extent of haemolysis [Fig. 3(A)], which is accompanied by a decrease in the time constant [Fig. 2(A)]. This means that the plasma membrane mechanical strength of one population is reduced, whereas the second one is barely affected (the latter being resistant to haemolysis under the conditions used). The need for two-exponential fits confirms this supposition. The detailed explanation of this observation requires further study. At higher Ph_2SnCl_2 concentration (25 μM), the second erythrocyte population became affected, as indicated by the elevated final haemolysis [Fig. 3(A)]. Interestingly, at the same time the membrane resistance to osmotic stress (the time constant) increased monotonically with Ph_2SnCl_2 concentration up to 25 μM, showing the increased-extent of lysis in the second erythrocyte population [Fig. 2(A)]. The progressive reduction of the relative time constant as a function of erythrocyte incubation with Ph_2SnCl_2 at 20 μM concentration [Fig. 2(B)] indicates that there is a process associated with Ph_2SnCl_2 redistribution within the plasma membrane and/or cell volume or, if the two-cell population assumption is correct, an increased fraction of resistant cells undergoes lysis.

When similar experiments are performed in the presence of Ph_3SnCl, the picture is drastically different. Figure 1(B) shows examples of haemolysis kinetics of erythrocytes treated with triphenyltin at various concentrations. At first glance there is an evident difference when compared with the curves presented in Fig. 1(A) (when cells were incubated with Ph_2SnCl_2). The final haemolysis at all concentrations except 5 μM saturates at a level close to approximately 100%, showing that all cells are lysed under the osmotic stress, and indicating that the plasma membrane is substantially weakened by the compound in the whole cell sample. The time constant dependence on Ph_3SnCl concentration [Fig. 2(A)] confirms this. It decreases monotonically with the concentration down to 50% of its value for a cell treated with methanol alone. Furthermore, the dependence of final haemolysis [Fig. 3(B)] and time constant [Fig. 2(B)] on duration of the incubation confirms that observation, indicating that there is a small further change in those parameters, which means that the effect of Ph_3SnCl is rapid and non-reversible. This time dependence may indicate that Ph_3SnCl acts via mechanical disturbance of the plasma membrane in a detergent-like manner. A consistent picture emerges when the final and initial levels of haemolysis are analysed (*B* parameter). Ph_3SnCl causes little change in both time and concentration dependences (Fig. 4). The initial haemolysis starts to increase above 10 μM Ph_3SnCl, showing that the compound at those concentrations affects the membrane integrity to the extent that a fraction of the erythrocytes is lysed even before exposure to osmotic stress. The other informative observation is that the effect of incubation time on all the measured parameters in the case of Ph_3SnCl [Figs 2(B), 3(B) and 4(B)] shows little change in time, which proves that the effect of the compound is essentially completed, whereas, in the case of Ph_2SnCl_2, the time constant and the final extent of haemolysis change even after 2 h incubation [Figs 2(B) and 3(B)]. This may indicate that this compound acts not only in a detergent-like fashion but also interferes with other processes.

Data presented in the paper provide experimental evidence that, when the two compounds are applied in concentrations when there is no evident damage in cell integrity, their effects are qualitatively different. Previous experiments carried out at much higher phenyltin concentrations (above 0.1 mM)[26] show that their toxicities to erythrocytes have different concentration dependences, Ph_3SnCl being more toxic than Ph_2SnCl_2. The more detailed analysis of the process, presented in this paper, using kinetics of haemolysis, shows that the two compounds act on the plasma membrane differently. Ph_2SnCl_2 at low concentrations is relatively neutral, and only when it reaches 25 μM do the changes of the mechanical properties become apparent, whereas Ph_3SnCl, at the same concentrations, causes substantial membrane destabilization. The time scale of the process indicates that Ph_3SnCl acts via destabilization of the plasma membrane, whereas Ph_2SnCl_2

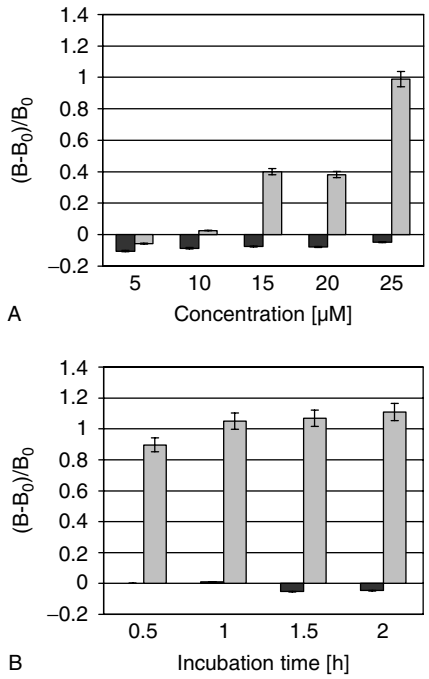

Figure 4. The effect of Ph_2SnCl_2 (dark bars) and Ph_3SnCl (light bars) on the relative change in the initial extent of haemolysis, $(B - B_0)/B_0$, extracted from kinetic curves. (A) Erythrocytes were incubated with various concentrations of phenyltins for 2 h at 36.6 °C prior to measurement. (B) The dependence of relative changes in the initial extent of haemolysis for erythrocyte incubated with 20 μM phenyltins as a function of incubation time. Values represent the average of the five repetitions from a single measurement. The standard deviation did not exceed 5%.

may in addition affect other processes since its effect on the plasma membrane is minimal at concentrations up to 25 μM (although there is a measurable change in the parameters with incubation time). The observed toxic effects may be correlated with the location on the two compounds within the lipid bilayer, meaning disturbance of different membrane regions. Ph_2SnCl_2 intercalates into the hydrophobic membrane interior, which has little effect on overall membrane properties, whereas Ph_3SnCl affects the interface at the vicinity of glycerol.[28,41] Such location–activity correlation agrees with experimental evidence and theoretical predictions that the disturbances imposed on the lipid bilayer at the glycerol level affect membrane stability the most.[42]

In conclusion, both compounds, Ph_3SnCl and Ph_2SnCl_2, influence the osmotically induced erythrocyte haemolysis, but triphenyltin chloride acts more intensively, reducing erythrocyte plasma membrane mechanical strength.

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