APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. **13**, 777–781 (1999)

Plasma Membrane Perturbation Induced by Tributyltin Chloride on Density-separated Trout Erythrocytes

A. M. Santroni,[1] D. Fadeli,[1] G. Zolese,[2] R. Gabbianelli[1] and G. Falcioni[1]*
[1]Dipartimento di Biologia M.C.A., Università di Camerino, Italy
[2]Istituto di Biochimica, Facoltà di Medicina e Chirurgia, Università di Ancona, Italy

By using a discontinuous Percoll gradient in the 45–65% range it is possible to separate nucleated *Salmo irideus* erythrocytes in three different density fractions that are related to aging of the cell. The stability to lysis of the erythrocytes obtained from the three layers was examined in the presence and in the absence of Bu_3SnCl_4 (TBTC). The rate of hemolysis in the absence of the organotin was nearly the same for the three erythrocyte fractions. The addition of TBTC to the suspension increased the hemolysis but the effect depended on the density of the fraction. The change in the hemolysis rate was in the order bottom (B) > middle (M) > top (T). The effect of TBTC on 1,6-diphenyl-1,3,5-hexatriene (DPH) steady-state fluorescence anisotropy was measured in liposomes formed by the lipids extracted from the different fractions. Compared with the controls, the DPH anisotropy decrease in the presence of TBTC. Steady-state fluorescence of 2-dimethylamino-6-laurylnaphthalene (Laurdan) was measured to evaluate membrane polarity. The presence of TBTC on the same liposomes decreased the generalized polarization (GP_{340}) in the top and middle fractions while it did not change in the bottom fraction. **Copyright © 1999 John Wiley & Sons, Ltd.**

Keywords: fish; erythrocyte; hemolysis; membrane fluidity; organotin

Received 25 September 1998; accepted 22 June 1999

* Correspondence to: Professor Giancarlo Falcioni, Dipartimento di Biologia M.C.A., Università di Camerino, Via Camerini 2, I-62032 Camerino (MC), Italy.
E-mail: falcioni@cambio.unicam.it
Contract/grant sponsor: CNR.

INTRODUCTION

Organotins are currently used by the paint industry in various formulations and also as agricultural biocides.[1] Their presence in water is principally due to their use in marine antifouling paint formulations and as a stabilizer for PVC.

The biological effect of organotin compounds is of some interest because many of them are known to exert a toxic action on mammals. The toxicity is probably linked to their incorporation into cells depending on their lipophilicity. Organotin derivatives of tin are much more toxic than their inorganic analogues; alkyltin compounds are generally more toxic than aryltin ones. In general, the toxicity decreases from tri- to mono-alkyltins.[2,3] Hemolytic activity of triorganotin derivatives has been extensively demonstrated and it can vary with different mammals.[4] Although various papers have been published on organotin-induced hemolytic action, the molecular mechanism of this process has not yet been clarified. Previously[5] we reported the effect of increasing concentrations (1–50 μM) of various organotins on plasma membranes of trout erythrocytes. Data showed that at each concentration used, tributyltin chloride (TBTC) and triphenyltin chloride (TPTC) increase (even if in an unequal manner) the hemolysis rate of trout red blood cells, while dibutyltin has a slight protective effect.

In order to obtain further information on the mechanism of action of organotins on the plasma membrane, we extended our investigation to density-separated trout erythrocytes obtained by using a discontinuous Percoll gradient in the 45–65% range.

Using this technique it is possible to separate the nucleated *Salmo irideus* erythrocytes in three fractions (top, middle and bottom) that could be related, similarly to human erythrocytes, to the age of the cell.[6,7] Previous studies showed a correlation between the density and the age of the cell; older

Copyright © 1999 John Wiley & Sons, Ltd. CCC 0268–2605/99/100777–05 $17.50

cells are characterized by an increased density.[8–10] These age-related populations of cells represent a good model to study the interaction of organotins with plasma membranes because they represent different cellular situations, where oxidative modifications correlated with the aging of the cell are present at different levels.

Here, we present data on the hemolytic activity of triorganotins on density-separated trout erythrocytes and on the modifications that they induce on the physico-chemical state of the membrane phospholipids. Our studies were carried out by steady-state fluorescence using two different probes, 1,6-diphenyl-1,3-5-hexatriene (DPH) and Laurdan (2-dimethylamino-6-lauroylnaphthalene), that give information on the changes of, respectively, membrane anisotropy and membrane polarity.

The results obtained indicate significant differences in the parameters measured for the three erythrocyte fractions.

MATERIALS AND METHODS

All reagents were of analytical grade. Percoll was obtained from Sigma; Laurdan and DPH were purchased from Molecular Probes (Eugene, OR, USA). Organotin compounds were obtained from Aldrich.

Sample preparation

Red blood cells from *Salmo irideus*, an inbred strain of trout, were used. Blood was obtained by puncturing the lateral tail vein of six to ten fish and withdrawing it into an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA; pH 7.8). After removal of plasma and buffy coat by centrifugation, the erythrocytes were washed three times and resuspended in the same isotonic buffer.

Erythrocytes were separated into subpopulations on a Percoll/BSA density gradient in the 45–65% range according to Rennie *et al.*[11] Three well-separated fractions (top, middle and bottom) were obtained. Hemoglobin and nucleus-free erythrocyte membranes were prepared according to Steer and Levitzki,[12] using a discontinuous sucrose gradient (25 and 35%, w/w). Lipids from membrane samples with the same content of proteins (measured by Lowry's method[13]) were extracted according to Folch *et al.*[14] and then dried under vacuum.

Hemolytic measurements

The degree of hemolysis was determined as $(100 \times A/10 \times A_{100\%})$, where A is the optical density at 540 nm of hemoglobin present in the supernatant of a red-cell suspension after centrifugation, and $A_{100\%}$ is the optical density of a red-cell suspension after complete lysis with 10 vol. distilled water at zero incubation time.

The three fractions were suspended in 0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA (pH 6.3) and incubated at 35 °C for the time necessary to obtain 100% hemolysis.

Organotin compounds at a final concentration of 20 μM dissolved in ethanol were added to these suspensions; controls were prepared at the same concentration of ethanol as used in the samples containing organotin.

Fluorescence measurements

Fluorescence measurements were performed using a Hitachi 4500 spectrofluorimeter with lipids extracted from the density-separated erythrocyte membranes.

Generalized polarization of Laurdan (GP_{340}) (λ = 340 nm) was calculated according to Parasassi *et al.*[15] using Eqn[1].

$$GP_{340} = (I_B - I_R)/(I_B + I_R) \qquad [1]$$

where I_B and I_R are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum and correspond to the fluorescence emission maxima in the gel and liquid-crystalline phases[16] of the bilayer, respectively. A solution of Laurdan dissolved in ethanol was added to the lipids with stirring, dried in a gentle stream of N_2 and rehydrated in the 0.1 M phosphate buffer, pH 8.0. The final probe and protein concentrations were, respectively, 1 μM and 0.4 mg ml^{-1}. Each organotin compound dissolved in ethanol at a final concentration of 30 μM was added to liposomes; the controls were prepared with the same concentration of ethanol.

DPH steady-state fluorescence anisotropy was calculated as described previously.[17] The excitation and emission wavelengths were, respectively, 360 and 430 nm. The final protein concentration in the sample was 0.8 mg ml^{-1}, while the probe concentration was 2 μM. The liposomes were incubated with probes and organotin for 2 h at 35 °C, in 0.1 M phosphate buffer at pH 8.0 in the dark. Measurements were performed at 35 °C.

Copyright © 1999 John Wiley & Sons, Ltd.

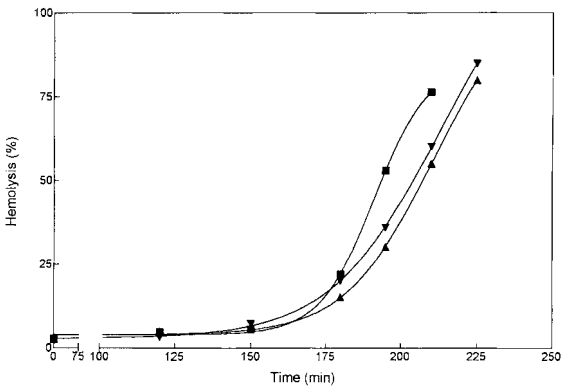

Figure 1 Time course of hemolysis in density-separated erythrocyte suspensions containing about 1.2×10^6 red blood cells/ml in isotonic medium at pH 6.3 incubated at 35 °C. ■, Top fraction; ▲, middle fraction; ▼ bottom fraction.

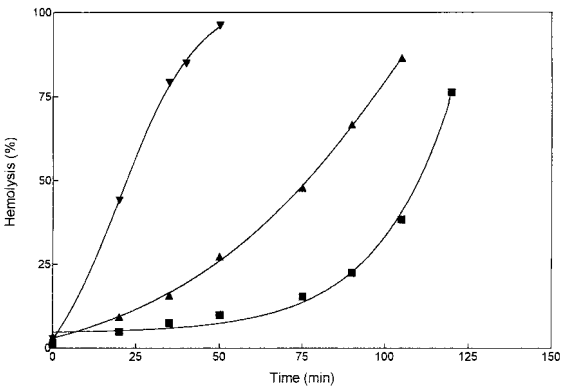

Figure 2 Time course of hemolysis. Conditions as in Fig. 1, but in the presence of 20 μM TBTC. ■, Top fraction; ▲, middle fraction; ▼, bottom fraction.

Statistical analysis

Statistical analysis was performed with Student's test. A value of $P < 0.05$ was considered statistically significant.

All data were obtained from five replicates within each experiment. Studies were repeated four times.

RESULTS

A discontinuous Percoll gradient in the 45–65% range produces three erythrocyte fractions in trout blood: a fraction of light cells between 52 and 58% Percoll (top); an intermediate cell fraction between 58 and 62% Percoll (middle) and a third fraction of the densest cells at 64% Percoll (bottom).

The stability to lysis of the erythrocytes obtained from the three layers was examined in the presence and in the absence of the triorganotin compounds. In trout red-blood cells, the hemolytic process depends strongly on the incubation temperature and medium pH; at 35 °C and pH 6.3 it can be followed in a relatively short time (a few hours) even when the cells are suspended in an isotonic medium. Figure 1 shows that the rate of hemolysis in the absence of organotin was nearly the same for the three erythrocyte fractions, although the older cells could withstand greater stress; the half-time ($t1/2$) of this process corresponds to an incubation of about 200 min. Addition of TBTC (20 μM) to the suspensions increased the hemolysis (Fig. 2) but the effect depended on the density of the fraction (TBTC increases hemolysis, starting from 5 μM). The change in the hemolysis rate was in the order bottom > middle > top and the half-times were, respectively, 110, 75 and 22 min. The effect of TPTC was similar to that of TBTC but less pronounced (data not shown).

Steady-state fluorescence was used to investigate the possible modifications induced by TBTC on the physicochemical state of erythrocyte membranes. Anisotropy measurements were performed using DPH as probe located in the hydrocarbon core of the bilayer. Steady-state fluorescence of Laurdan was measured to evaluate other physicochemical features. This probe, localized at the hydrophobic–hydrophilic interface of the lipid bilayer at the glycerol backbone level,[18] is sensitive to changes in the polarity of its microenvironment, and the parameter GP_{340} can be used to monitor these modifications.

DPH steady-state fluorescence anisotropy and Laurdan GP_{340} measured in liposomes formed by the lipids extracted from the different fractions are shown in Tables 1 and 2. The decrease in DPH anisotropy is in the order M > B > T, while Laurdan GP_{340} values change in the order T > M > B. Compared with the controls, the DPH anisotropy values are decreased in the presence of 30 μM TBTC (see Table 1); in the liposomes obtained from the top fraction the value reached was 95%, while from middle and bottom fractions it was 85 and 93% respectively.

Compared with the controls (Table 2), the GP_{340} values are decreased by the presence of 30 μM TBTC, by about 22 and 25% in the liposomes

Copyright © 1999 John Wiley & Sons, Ltd.

Table 1 Effect of 30 μM TBTC on steady-state fluorescence anisotropy of DPH in lipid extracted from density-separated trout erythrocytes[a]

Fraction	Control	+ TBTC
Top	0.171 ± 0.012	0.163 ± 0.005*
Middle	0.183 ± 0.016	0.157 ± 0.005**
Bottom	0.165 ± 0.011	0.154 ± 0.008**

[a] Data are represented as means $\pm$ SD.
* $P < 0.05$ compared with control.
** $P < 0.001$ compared with control.

Table 2 Effect of 30 μM TBTC on generalized polarization (GP_{340}) ($\lambda_{ex} = 340$ nm) for Laurdan, measured in lipid extracted from density-separated trout erythrocytes[a]

Fraction	Control	+ TBTC
Top	0.309 ± 0.024	0.241 ± 0.006*
Middle	0.280 ± 0.034	0.209 ± 0.041*
Bottom	0.133 ± 0.031	0.132 ± 0.033

[a] Data are represented as means $\pm$ SD.
* $P < 0.001$ compared with control.

formed by phospholipids extracted from, respectively, the top and middle fractions. In contrast, the presence of TBTC does not change the GP_{340} value when the liposomes are from the bottom fraction.

DISCUSSION

Triorganotin compounds (R_3SnX), the most widely studied tin compounds, are important pollutants in aquatic ecosystems.[19,20] The majority of biological effects produced by organotins concern interactions with plasma membranes.[21] One factor known to govern organotin-mediated membrane effects is the lipophilicity of the compound.[22,23] It is clear that compounds capable of entering membrane lipid domains fulfill a prerequisite for being membrane effectors. Organotins induce inhibition of sodium–potassium and calcium pumps and may destabilize the membrane and deplete cellular ATP.[24,25] Anion transport across erythrocyte membranes mediated by organotins could also contribute to membrane instability.[26,27]

The three different density-separated trout erythrocyte fractions used in this study produced, in our experimental conditions, the same hemolysis rate as when the process was followed in the absence of the organotin compound, even although the three different erythrocyte fractions are characterized at the membrane level by significant differences, such as lipid composition, lipid peroxidation, fluidity, polarity and Na^+/K^+ ATPase activity.[6] Clearly, molecular adaptation mechanisms, probably due also to membrane lipid composition, will be developed in these nucleated cells to mantain the same hemolysis stability. The compositional heterogeneity of PLs in a biological membrane can modify fluidity, membrane permeability, and water organization at the membrane surface, which can affect the structure and the activity of membrane proteins. The hemolytic effect of TBTC was evident in all the three erythrocyte fractions but it increased in the densest cells. In other words, the effect of TBTC on the hemolysis event was more marked in older cells.

To evaluate the effect of TBTC on the structural and physicochemical characteristics of density-separated trout erythrocytes, we studied the fluorescence of probes embedded in the lipid bilayer of liposomes from extracted lipids. Steady-state fluorescence anisotropy of DPH has been widely used to investigate the molecular order of the hydrophobic part of the membrane where this fluorescent probe is located.[28] In our samples the steady-state anisotropy of DPH (Table 1) showed different values (they were previously reported by us to be in a different order[7] and we believe that this difference may be attributed to animal variability and/or to a different seasonal period) that were decreased by the presence of TBTC. This compound hence increased plasma membrane fluidity and the modification was more marked in the M fraction.

The character of the polarity of the lipid bilayer as monitored by Laurdan, using the GP parameter, related to water penetration and to the dynamics of the solvent molecules surrounding the probe, was found to be quite different for the three density-separated fractions (Table 2); the presence of TBTC decreased the GP parameter in the T and M fractions, while no effect was observed in the B fraction (the oldest fraction).

The general conclusion formulated on the basis of our experiments is that TBTC is more efficient in increasing the hemolytic rate in those erythrocytes (oldest cells) where the physicochemical properties evaluated by fluorescence studies were nearly unchanged.

These results could indicate that oxidative modifications due to cell aging play an important role in the hemolytic process induced by TBTC, even if other factors have to be considered.

Copyright © 1999 John Wiley & Sons, Ltd.

Acknowledgements This work was supported by a grant from the CNR fund to G.F.

REFERENCES

1. I. C. Boyer, *Toxicology* **55**, 253 (1989).
2. N. J. Snoeij, A. H. Penninks and W. Seinen, *Environ. Res.* **44**, 335 (1985).
3. P. T. S. Wong, Y. K. Chan, O. Kramar and A. Bergert, *Can. J. Fish Acquat. Sci.* **39**, 483 (1992).
4. K. H. Byington, R. Y. Yed and L. R. Forte, *Toxicol. Appl. Pharmacol*, **27**, 230 (1974)
5. G. Falcioni, R. Gabbianelli, A. M. Santroni, G. Zolese, D. E. Griffiths and E. Bertoli, *Appl. Organomet. Chem.* **10**, 451 (1996).
6. G. Falcioni, F. Grelloni, A. R. Bonfigli and E. Bertoli, *Biochem. Int.* **28**, 379 (1992).
7. R. Gabbianelli, A. M. Santroni, G. Falcioni, E. Bertoli, G. Curatola and G. Zolese, *Arch. Biochem. Biophys.* **336**, 157 (1996).
8. S. Piomelli, G. Lurinsky and L. R. Wasserman, *J. Lab. Clin. Med.* **69**, 674 (1967).
9. M. Rocchigiani, M. Pescaglini, S. Sestili, V. Micheli and C. Ricci, *J. Int. Med. Res.* **17**, 461 (1989).
10. M. Moretti, M. Villarini, G. Scassellati-Sforzolini, A. M. Santroni, D. Fedeli and G. Falcioni, *Mut. Res.* **397**, 353 (1998).
11. C. M. Rennie, S. Thompson, A. C. Parker and A. Maddy, *Clin. Chim. Acta* **98**, 119 (1979)
12. M. L. Steer and A. Levitzki, *J. Biol. Chem.* **250**, 2080 (1975).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)
14. J. Folch, M. Less and G. H. Sloane-Stanley, *J. Biol. Chem.* **226**, 466 (1975).
15. T. Parasassi, G. De Stasio, A. D'Ubaldo and E. Gratton, *Biophys. J.* **57**, 1179 (1990).
16. T. Parasassi, G. De Stasio, G. Ravagnan, R. M. Rush and E. Gratton, *Biophys. J.* **60**, 179 (1991)
17. G. Zolese, R. Gabbianelli, E. Bertoli, M. Wołniak and G. Falcioni, *Chem. Phys. Lipids* **75**, 97 (1995).
18. T. Parasassi, F. Conti and E. Gratton, *Cell. Mol. Biol.* **32**, 103 (1986).
19. C. Alzieu, P. Michel, I. Tolosa, E. Bacci and L. D. Meeand Readman, *J. W. Mar. Environ. Res.* **32**, 261 (1991).
20. A. Fargašová, *Ecotoxicol. Environ. Safety* **36**, 38 (1997).
21. J. Gabrielska, J. Sarapuk and S. Z. Przestalski, *Z. Naturforsch, Teil C* **52**, 209 (1997).
22. B. H. Gray, M. Porvaznik, C. Flemming and L. H. Lee, *J. Appl. Toxicol.* **6**, 363 (1986).
23. R. B. Laughlin Jr, R. B. Johannesen, W. French, H. Guard and F. E. Brinckman, *Environ. Toxicol. Chem.* **4**, 343 (1986).
24. J. S. Morrow and R. A. Anderson, *Lab. Invest.* **54**, 237 (1986).
25. M. J. Selwyn, in: *Organotin Compounds: New Chemistry and Applications* Zuckerman, J. J. (ed.), American Chemical Society, Washington, DC, 1976, pp. 204–226.
26. R. A. Anderson and V. T. Marchesi, *Nature (London)* **318**, 295 (1985).
27. Y. Arakawa and O. Wada, *Biochem. Biophys. Res. Commun.* **123**, 543 (1984).
28. M. Shinitzky and Y. Barenholz, *Biochem. Biophys. Acta* **515**, 367 (1978).

Copyright © 1999 John Wiley & Sons, Ltd.