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Triphenyltin pesticides in sea water as immunotoxins for tunicates

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Abstract

Triphenyltin derivatives (TPTs, Fentin) cause a severe immunotoxicity in tunicates, having significant inhibitory effects on yeast phagocytosis by hemocytes of the colonial ascidian *Botryllus schlosseri* — TPTC > TPTA > TPTH — in a concentration-dependent manner. The same order of inhibition is observed for Ca^{2+}-ATPase activity, suggesting that the inhibition of this enzyme and of phagocytosis may be closely linked. Addition of 20 μg/ml calmodulin is able to reverse the inhibition of Ca^{2+}-ATPase activity by TPTs, but not to restore the phagocytosis index. These results support the hypothesis that mitochondrial oxidative phosphorylation may also be involved, as O_2^- production is inhibited — TPTA > TPTC > TPTH — in a concentration-dependent manner, but not restored by calmodulin. Besides, TPTs cause some morphological changes without cytolysis, suggesting an interaction with cytoskeletal components, whereas viability is not affected up to 100 μM for TPTA and TPTC, and 1 mM for TPTH. © 1997 Elsevier Science B.V.

Keywords: triphenyltin compounds; immunotoxicity; Ca^{2+}-ATPase; O_2^- production; tunicates

1. Introduction

Among triphenyltin derivatives (TPTs), triphenyltin acetate or TPTA (Fentin acetate), chloride or TPTC (Fentin chloride) and hydroxide or TPTH (Fentin hydroxide) are used worldwide as agricultural nonsystemic fungicides, herbicides, molluscicides, insect and rodent repellents, or as cotoxicants to tributyltin compounds (TBTs) in antifouling boat paints. Severe contamination of both fresh and coastal marine waters may occur, through washing of adsorbed amounts in the soil (Schramel et al., 1973) and direct release from antifouling paints, respectively.

In Italy, only two TPTs, TPTA and TPTH, are used as agricultural fungicides against *Cercospora beticola* and as antifeedings against lepidopteran larvae of Nottuidae on sugar beet leaves (Ferrari, 1982; Muccinelli, 1993). Since 1920 in the Veneto Region production of this vegetable has been the second in Italy, and from 1967 to 1992 about 250 tons of organotins, corresponding to about 1390 tons of 18% commercial formulation (Annuario Statistico Italiano, 1967–1992), have been distributed for use. On the grounds of hydrography, hydrosolubility, adsorption in the soil, biotransformation (oxidation and

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methylation; Kimmel et al., 1977) and photochemical decomposition (Barnes et al., 1973), a progressive contamination of sediments and waters in the Lagoon of Venice over a period of 25 years may reasonably be inferred. Moreover, an excessive, uncontrolled and incorrect use of TPTs took place owing to the development of tolerant strains of *C. beticola* after several years of application (Giannopolitis, 1978). In order to avoid this risk, the Veneto Region has recently issued some practical directives to reduce the environmental use of these pesticides (Regione Veneto, 1994).

Considerable bioaccumulation of lipophilic TPTs has been observed in the fatty compartments of fish through a rapid dietary uptake from water (Yamada et al., 1994). Because of their very slow elimination, TPTs are considered slow-acting toxic compound with long-term effects (Tas et al., 1990).

Many studies show that TPTs have selective toxic effects on the immune system in chickens (Guta-Socaciu et al., 1986), rats (Vos et al., 1984; Snoeji et al., 1985), guinea pigs (Verschuuren et al., 1970), and mice (Dacasto et al., 1994): decreases in the numbers of lymphocytes and leukocytes, accompanied by atrophy of spleen and thymus, have been described. In humans, a specific decrease in the chemotactic activity of PMNs has recently been observed (Galli et al., 1993). Like the highly selective immunotoxicity in mammals, in teleosts decreased resistance to infections has been found even at the lowest-effect concentrations of TPTs as well as TBTs after continued exposure (Devries et al., 1991).

Our previous studies showed that butyltin derivatives are immunotoxic in tunicates, having inhibitory effects on yeast phagocytosis by hemocytes of the colonial ascidian *Botryllus schlosseri* (Cima et al., 1995). We therefore extended our study to TPTs, with the aim of investigating whether a similar toxic effect on the hemocytes of this filtering invertebrate species is observed also for these substances.

2. Experimental

2.1. Animals

Colonies of *B. schlosseri* from the Lagoon of Venice were reared in our laboratory, attached to glass slides immersed in aquaria, and fed with Liquifry Marine (Liquifry Co., Dorking) and algae.

2.2. Hemocyte collection

Blood cells were obtained by tearing the peripheral tunic vessels of colonies previously rinsed in filtered sea water (FSW), containing 10 mM L-cysteine, adjusted to pH 7.0 to prevent clotting. They were then washed by centrifugation at $780 \times g$ for 15 min and pellets were resuspended in FSW, for a final concentration of $8–10 \times 10^6$ cells/ml.

2.3. Phagocytosis assay

Culture chambers and phagocytosis assays were made following our previously described method (Ballarin et al., 1994).

Culture slides containing 50 μl of hemocyte suspension were kept upside-down for 30 min to allow cells to settle and adhere to the coverslips. After adhesion of the hemocytes, FSW was discarded and replaced with equal volumes of a suspension of autoclaved (15 min at 120°C) ordinary baker's yeast in FSW (yeast:hemocyte ratio = 10:1). Cultures were kept upside-down for 60 min at room temperature. Hemocyte monolayers were then washed several times in FSW, fixed in a solution of 1% glutaraldehyde and 1% sucrose in FSW at 4°C for 30 min, and stained with 10% Giemsa for 10 min. The coverslips were finally mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba) and cells were observed with a Leitz Dialux 22 light microscope.

Hemocytes were counted at a magnification of $1250 \times$ (at least 200 cells per coverslip in 10 fields) and the *phagocytosis index*, i.e., percentage of hemocytes with ingested particles, was determined.

2.4. Effects of triphenyltins

TPTA, TPTC and TPTH (Sigma) were first dissolved in dimethylsulfoxide (DMSO) at 10 mM and then diluted, at final concentrations of 0.1, 1 and 10 μM in the FSW used to suspend the yeast. 0.1% DMSO was added to FSW in controls. Viability was assessed by the trypan blue dye exclusion test (Dacasto et al., 1994), the dye being dissolved in

FSW. Parallel experiments were performed in the presence of 20 μg/ml calmodulin (CaM) (Sigma).

2.5. Assay for Ca^{2+}-ATPase

Histochemical assays for Ca^{2+}-ATPase activity were carried out on *B. schlosseri* blood smears according to Chayen et al. (1969). In controls for specificity, glycerophosphate was used in place of ATP. The *Ca^{2+}-ATPase* index, i.e., percentage of cells showing dark brown precipitates, was evaluated as a measure of Ca^{2+}-ATPase activity.

2.6. O_2^- assay

Intracellular superoxide anion was quantitatively assayed using nitroblue tetrazolium staining according to the method of Song and Hsieh (1994). The ratio of OD_{630} (optical densities of the dissolved cytoplasmic formazan at 630 nm) from the treated hemocytes to the OD_{630} of control hemocytes was drawn as an index, to compare the effects of the various treatments, i.e., yeast, 400 μg/ml superoxide dismutase (SOD; 2000 U/ml) (Sigma), 20 μg/ml CaM, and 0.1, 1, 10 μM TPTs on the generation of O_2^-. The same number of hemocytes was employed in both treated and control samples.

2.7. Statistical analysis

All experiments were repeated in triplicate with $5 \div 7$ independent cell samples. Data are expressed as means ± SD and were analysed using the X^2 test; Student's *t*-test was used for the O_2^- assay.

3. Results

3.1. Trypan blue dye exclusion test

The viability of *B. schlosseri* hemocytes, as assessed by trypan blue exclusion, exceeds 99.5% after 1 h of incubation in FSW plus DMSO not containing TPTs. A significant dose-dependent decrease in cell viability was observed only after 1 h exposure at concentrations higher than 100 μM for TPTA ($p < 0.05$) and TPTC ($p < 0.001$), and 1 mM for TPTH ($p < 0.001$), with mortality increasing up to 2.06% ± 0.19, 2.7% ± 0.007 and 2.45% ± 0.22, respectively. Two hours later, no further reduced viability was evident. Sublethal doses of TPTs, i.e., 0.1, 1 and 10 μM, were thereafter used in all experiments.

3.2. Morphological changes in phagocytes

In the same experimental conditions as Section 3.1, none of the three TPTs ever caused cytolysis, but only some morphological changes beginning from 10 μM. Hemocytes withdrew their pseudopodia and became spherical (Fig. 1a and c).

3.3. Inhibition of phagocytosis index and effect of CaM

TPTs present in the incubation medium had inhibitory effects on in vitro phagocytosis of yeast cells. The phagocytic index ranged between 12 and 15 in controls and was significantly reduced in a concentration-dependent manner by 0.1 μM TPTC ($p < 0.05$), 1 μM TPTA ($p < 0.001$), and 10 μM TPTH ($p < 0.001$). The potency of inhibition was therefore TPTC > TPTA > TPTH (Fig. 2a).

CaM present in the incubation medium at 20 μg/ml did not affect the phagocytosis index in controls, whereas it restored the activity of hemocytes depressed by TPTs in a different concentration-dependent manner depending on the

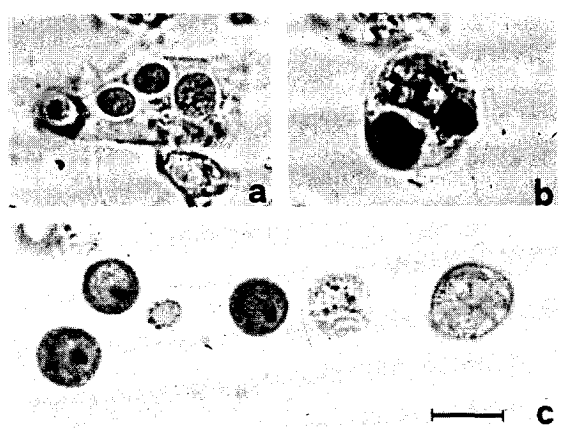

Fig. 1. Phagocytizing hemocytes of *B. schlosseri*, containing yeast cells (a), showing positivity for Ca^{2+}-ATPase (b), and becoming spherical in shape in presence of TPTs (c). Bar length: 10 μm.

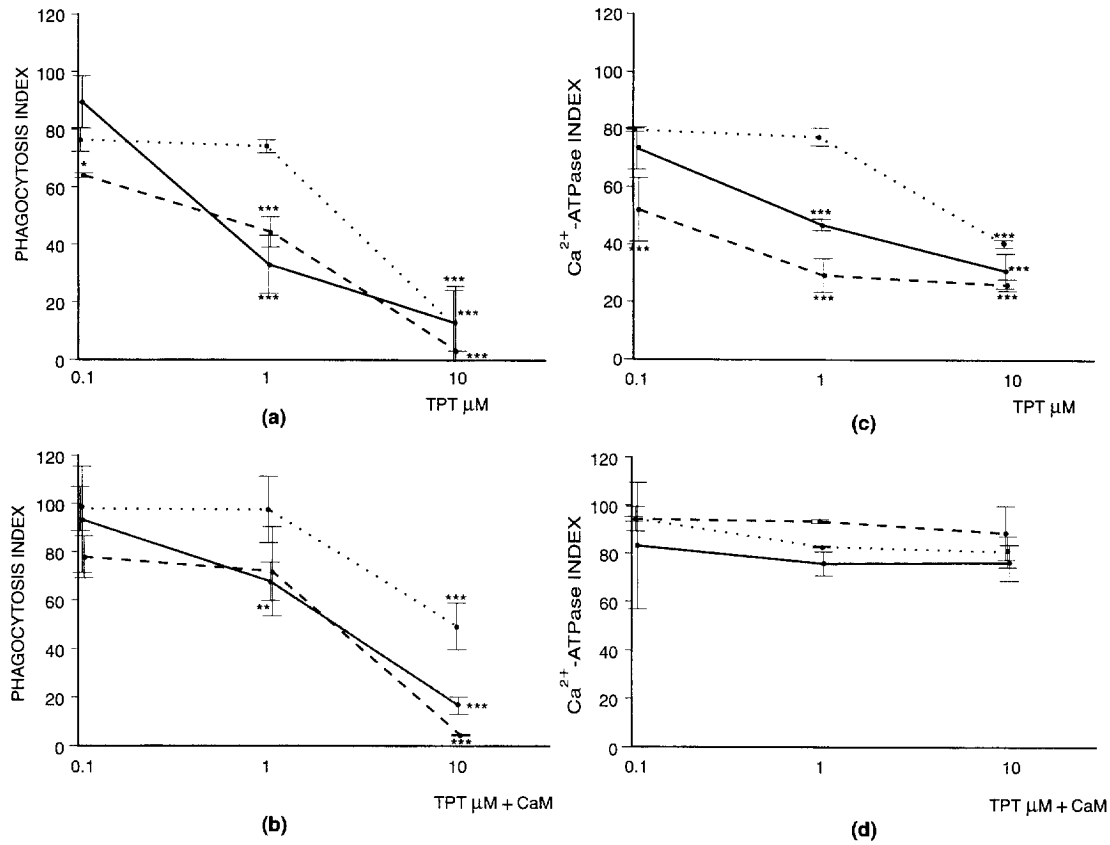

Fig. 2. Effects of TPTs on yeast phagocytosis (a) and Ca^{2+}-ATPase activity (c) of *B. schlosseri* hemocytes, and effects of co-presence of 20 µg/ml CaM on phagocytosis (b) and Ca^{2+}-ATPase (d) indexes. Asterisks: significant differences with respect to controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. TPTA (————); TPTC (— — — —); TPTH (- - -).

tested organotin derivative. Reversal of inhibition by CaM was highest for 0.1 and 1 µM TPTC, partial for 1 µM TPTA ($p < 0.01$), and absent for TPTH. The potency of restoration was therefore TPTC > TPTA > TPTH, but the inhibition by 10 µM TPTs was never reversed by CaM (Fig. 2b).

3.4. Inhibition of Ca^{2+}-ATPase index and effect of CaM

The Ca^{2+}-ATPase index, assayed histochemically in hemocytes (Fig. 1b), was increased in a highly significant manner ($p < 0.001$) during phagocytosis in the absence of TPTs. After TPT exposure, the enzyme activity showed the same order of inhibition found for the phagocytosis index (Section 3.3) (Fig. 2c).

The addition of 20 µg/ml CaM did not affect either basal or phagocytosis-stimulated activity, but was able to completely reverse the inhibition of Ca^{2+}-ATPase activity by TPTs at all tested concentrations (Fig. 2d).

3.5. Effects on respiratory burst

Phagocytosis was associated with respiratory burst, i.e., a rise in oxidative metabolism leading to increased production and excretion of highly reactive toxic oxygen metabolites such as O_2^-. This radical generation, expressed as the ratio of OD_{630} of treated hemocytes to the OD_{630} of control hemocytes, was significantly increased ($p < 0.01$) in hemocytes during phagocytosis. Using 400 µg/ml SOD as negative control, the ratio was significantly ($p < 0.05$) reduced.

Table 1
Relative production of O_2^- by hemocytes, expressed as OD_{630} of treated hemocytes/OD_{630} of control hemocytes. Asterisks: differences with respect to controls

Treatment	Concentration of TPT (μM)	Average relative production of O_2^-
FSW (control)	—	1.00 ± 1
CaM (20 μg/ml)	—	1.08 ± 0.071
Yeast	—	2.33 ± 0.127 **
SOD (400 μg/ml)	—	0.80 ± 0.021 *
TPTA	0.1	0.78 ± 0.014 *
TPTA	1	0.77 ± 0.091 *
TPTA	10	0.61 ± 0.007 **
TPTA + CaM	0.1	0.81 ± 0.042 *
TPTA + CaM	1	0.78 ± 0.091 *
TPTA + CaM	10	0.67 ± 0.007 **
TPTC	0.1	0.87 ± 0.106
TPTC	1	0.77 ± 0.071 *
TPTC	10	0.60 ± 0.021 **
TPTC + CaM	0.1	0.97 ± 0.035
TPTC + CaM	1	0.78 ± 0.028 *
TPTC + CaM	10	0.51 ± 0.049 **
TPTH	0.1	1.01 ± 0.007
TPTH	1	0.81 ± 0.162
TPTH	10	0.74 ± 0.106 *
TPTH + CaM	0.1	1.00 ± 0.136
TPTH + CaM	1	0.89 ± 0.169
TPTH + CaM	10	0.66 ± 0.077 *

* $p < 0.05$; ** $p < 0.01$.

O_2^- production was inhibited in a concentration-dependent manner by 0.1 μM TPTA ($p < 0.01$), 1 μM TPTC ($p < 0.05$), and 10 μM TPTH ($p < 0.05$), so that the potency of inhibition was TPTA > TPTC > TPTH. CaM at 20 μg/ml did not affect the respiratory burst (Table 1).

4. Discussion

The immunotoxic properties of butyltin derivatives in tunicates, previously demonstrated in *B. schlosseri* (Cima et al., 1995), have also been shown for phenyltins. In fact, some fundamental responses are common to both classes of organotins, such as the co-inhibition of phagocytosis and Ca^{2+}-ATPase activity associated with some morphological changes, without reduction of viability.

A correlation between the two classes of organotins seems to be unsuitable, in spite of their apparent chemical analogy, because of the strong influence of alkyl or aryl groups on the physical and biological properties of these compounds. However, a singly charged anion (chloride in mono-, di-, tributyltin and triphenyltin) or an anionic group (acetate and hydroxide in triphenyltin) greatly influences solubility and volatility (WHO, 1980). In an attempt to explain the results obtained with TPTs, it appears noteworthy that the order of inhibition either of phagocytosis or Ca^{2+}-ATPase activity is the same, very probably depending on the lipophilic property, which is lowest in TPTH. It is therefore conceivable that TPTH crosses the membranes to a lesser extent in comparison with TPTC and TPTA. TPTH thus turned out to be the least active derivative in all the assays; conversely, TPTC was more toxic than TPTA in inhibiting both phagocytosis and Ca^{2+}-ATPase activity, but less active in inhibiting the respiratory burst. It may be inferred that the higher lipophilic property of TPTA explains the target effect on mitochondrial dehydrogenase activity, in agreement with activities observed in rodent thymocytes (Dacasto et al., 1994). Like other organotin pesticides, TPTA also inhibits the synthesis of ATP, through the reaction of its phenyl groups with thiol groups such as lipoic acid, with the consequent enzymatic inhibition of lipoic acid acetyl transferase and lipoamide dehydrogenase. Moreover, TPTA causes an oligomycin-like inhibition of coupled respiration (Aldridge and Cremer, 1955; Ascher and Nissim, 1964).

On the grounds of the typical correlation between phagocytosis and Ca^{2+}-ATPase activity in *B. schlosseri* hemocytes (Ballarin et al., 1994; Cima et al., 1995), TPTC appears to act on membrane Ca^{2+} permeability, decreasing Ca^{2+} pump activity and increasing the intracellular Ca^{2+} concentration, like TBTC in mouse thymocytes (Oyama et al., 1994). It is possible that metallic tin in those compounds interacts with CaM, altering its conformation, as has been observed with other metals, and causing the inhibition of CaM-stimulated Ca^{2+}-ATPase, a membrane-bound enzyme (Yallapragada et al., 1990). This hypothesis is supported by the reversal of Ca^{2+}-ATPase inhibition induced by TPTs, which we observed after excess addition of CaM.

Organotin compounds have been shown to be immunotoxic in laboratory animals (Boyer, 1989), and we too have observed that TPTs impair cell-

mediated immunity compromising phagocytosis. This situation is different from that in rat in which, for example, TPTH exposure suppresses cell-mediated immunity without compromising the mononuclear phagocyte system (Vos et al., 1984).

Lastly, we observed some morphological changes in phagocytes in the presence of active doses of TPTs, suggesting a strong interaction with cytoskeletal constituents, as already proven with the G-actin of PMN microfilaments in mammals (Galli et al., 1993). No cytolytic response was observed in the hemocytes of our species, differently from the PMNs of rat (FAO/WHO, 1971, pp. 337–338) and rabbit (Elferink et al., 1986).

In conclusion, we confirm the role of tunicates as effective candidates as sentinels of organotin pollutants in sea water. Other morphological and biochemical studies are in progress to get further insight into the TPT immunotoxicity in hemocytes of *B. schlosseri* aimed to verify the effects on (i) shape and internal organization, (ii) calcium homeostasis, and (iii) oxidative phosphorylation.

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