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# Butyltins and calmodulin: which interaction?†

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**Tributyltin (TBT), widely used as an antifouling biocide, is the most abundant pesticide in coastal environments. One of its main toxic effects is immunosuppression in both vertebrates and invertebrates. At sublethal doses of TBT, phagocytes lose their ability to move towards and ingest foreign particles. For short-term cultures of haemocytes (60 min at 25 °C) of a marine invertebrate, the colonial ascidian *Botryllus schlosseri*, exposed to $10^{-5}$ M TBT, we previously reported dose- and time-dependent impairment of yeast phagocytosis and changes in cell morphology related to cytoskeleton disorganization. These effects are $Ca^{2+}$-dependent, since inactivation of $Ca^{2+}$-adenosine triphosphatase and a sustained increase in cytosolic $Ca^{2+}$ occurred. As TBT can antagonize the effect of chlorpromazine, a specific calmodulin (CaM) inhibitor, and the co-presence of exogenous CaM and TBT in the incubation medium resulted in the absence of effects, we hypothesized an interaction between TBT and CaM. TBT may remove endogenous CaM from cell proteins, thus inactivating them and causing alteration of $Ca^{2+}$ homeostasis. With the aim of confirming the hypothesis of a direct TBT–CaM interaction, we first studied the effects of co-incubation of TBT with other exogenous proteins on restoring the ability of phagocyte morphology. Although bovine serum albumin was never able to restore cell morphology, the effect of human spectrin was similar to that described for CaM, suggesting a common non-specific mechanism of action based on the interaction of TBT with exposed hydrophobic pouches. We also analysed the conformational changes of pure CaM ($10^{-5}$ M) in the presence of various concentrations ($10^{-4}$ to $10^{-3}$ M) of TBT and its degradation products, dibutyltin (DBT) and monobutyltin (MBT), by circular dichroism. Results indicate the dose- and time-dependent interaction of TBT with CaM. This interaction is a non-covalent interaction, probably hydrophobic in nature, between the aliphatic chains of TBT and the hydrophobic regions of $Ca^{2+}$-activated CaM. DBT and MBT turned out to be less active in inducing CaM conformational changes, without any significant differences between the two compounds.** 

**KEYWORDS:** tributyltin (TBT); dibutyltin (DBT); monobutyltin (MBT); calmodulin; circular dichroism; immunotoxicity; haemocytes; ascidians; *Botryllus*

## INTRODUCTION

Tributyltins (TBTs), mainly TBT oxide (TBTO) and TBT chloride (TBTC), are effective anthropogenic biocides that are especially used as antifouling agents in numerous formulations of marine paints, from which they are slowly released to sea water. They are extremely hazardous to many aquatic organisms at very low concentrations ($<3 \times 10^{-9}$ M). As a result, governmental restrictions by numerous countries worldwide have led to a decrease in the global use of TBTs in antifouling paints for small boats (<25 m in length).

In alkyl organotin compounds, the biocidal properties of TBTs are influenced strongly by the aliphatic groups and increase with alkyl chain length, whereas they are relatively independent of the anionic radical, which influences their solubility in water and non-polar solvents as well as their volatility.[1] Solubility data for organotin compounds are still incomplete. In general, their solubility in water at 25 °C

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ranges from $<10^{-6}$ to $>10^{-4}$ M at different temperatures and pH values, but they are very soluble in fats and many common organic solvents, such as ethanol, ethers, acetone, and halogenated hydrocarbons. Solubility depends on both the number and length of organic groups bound to the tin atom.[2] Therefore, trialkyltin compounds ($R_3SnX$) have the highest lipophilicity values: TBT has an octanol–water partition coefficient (log $P_{ow}$) between 3.19 and 3.84 for distilled water and 3.54 for sea water,[3,4] whereas dibutyltin (DBT) has a solubility in water of 1.5 M.[5] Owing to its high lipophilicity, TBT can easily cross biological membranes and interact with several intracellular targets,[6] thus irreversibly accumulating in both invertebrates and vertebrates. In particular, in mammals and fish, it preferably compartmentalizes in liver, kidney, brain and lymphoid organs.[7–11]

TBT is degraded to DBT and monobutyltin (MBT) derivatives by metabolic processes in tissues, dealkylation having first been demonstrated in the liver of vertebrates.[12] This speciation may also occur in the environment through biotransformation, due to microbial action and photochemical decomposition by ultraviolet irradiation.[13,14]

One of the main toxic effects of organotin compounds is immunosuppression, in both vertebrates and invertebrates, through lymphocyte depletion and inhibition of chemotaxis and phagocytosis.[15] DBT and MBT appear to be less immunotoxic than TBT, since derivatives do not produce atrophy of lymphoid organs in mammals.[16] In cultured phagocytes of the ascidian *Botryllus schlosseri*, we previously demonstrated that the toxicity of butyltins is related to their lipophilicity, since they can significantly and irreversibly inhibit the ingestion of foreign particles (yeast cells) at sublethal doses ($10^{-7}$ to $10^{-5}$ M), in the order TBT $\geq$ DBT $>$ MBT.[17] This effect is concentration- and time-dependent and not associated with any cytolysis. Phagocytes of the above marine invertebrates represent a simple and valuable model in studying the mechanism of action of organotins, owns to their high sensitivity at low immunotoxin concentrations. As reported for mammals, TBT negatively affects cell activity and viability through both $Ca^{2+}$-dependent and -independent processes.[18,19] In *B. schlosseri* phagocytes, inhibition of phagocytosis by TBT is always associated with a diffuse, long-lasting, cytosolic $Ca^{2+}$ rise, indicating that most of the observed effects are consequences of organotin-mediated disruption of cellular calcium homeostasis, as reported for mouse thymocytes, in which TBT increases the membrane $Ca^{2+}$ permeability of cellular calcium stores and decreases $Ca^{2+}$-adenosine triphosphatase (ATPase) activity.[20] In the presence of butyltins, an order of inhibition of $Ca^{2+}$-ATPase activity similar to that reported for phagocytosis is observed, indicating that inhibition of both $Ca^{2+}$-ATPase and phagocytosis are closely linked.[17] After exposure to $10^{-5}$ M TBT, one remarkable consequence is a rapid, irreversible change in phagocyte morphology: cells lose their typical amoeboid shape, withdraw their cytoplasmic projections, and assume a spherical shape, indicating that TBT interferes strongly with cytoskeletal components. However, increasing concentrations ($10^{-6}$ to $10^{-5}$ M) of exogenous calmodulin (CaM) can totally prevent cytoskeleton disorganization, changes in cell shape and decrease of $Ca^{2+}$-ATPase activity when added together with $10^{-5}$ M TBT in the incubation medium, suggesting interaction between TBT and CaM, following a saturation pattern, which leads to the formation of a complex unable to cross the plasma membrane.[21,22] Since membrane-bound $Ca^{2+}$-ATPases are CaM-dependent enzymes, we considered CaM as one important $Ca^{2+}$-dependent intracellular target of organotin compounds. TBT may remove endogenous CaM from cell proteins, thus inactivating them and altering $Ca^{2+}$ homeostasis. Experiments with $ED_{50}$ isodynamic (i.e. having the same effect on cell morphology) mixtures of TBT and specific CaM inhibitors chlorpromazine and *N*-(6-amino-hexyl)5-chloro-l-naphthalenesulfonamide (W-7) reveal the synergistic effect of antagonism.[22]

We previously hypothesized that the severe damage to immunocytes in the presence of $10^{-5}$ M TBT was mediated mainly by direct interaction of TBT with endogenous CaM, which, in turn, prevents the regulative activity of CaM on $Ca^{2+}$-ATPases, with a consequent diffuse, delayed, cytosolic calcium rise upon cell stimulation.[22] Altered $Ca^{2+}$ homeostasis leads either to apoptosis in the case of prolonged exposure,[23] or to inhibition of the respiratory burst[17] and depolymerization of cytoskeletal components.[21] The latter effect is responsible for the remarkable changes in cell shape and loss of motility shown by TBT-treated phagocytes.

In further support of our hypothesis of direct TBT–CaM interaction, we studied conformational changes of pure CaM at various concentrations of both TBT and its degradation products, DBT and MBT, by circular dichroism (CD).

## MATERIAL AND METHODS

### Reagents

Bovine brain CaM (purity >99%; 30 μM $CaCl_2$) was purchased from Calbiochem. Butyltin compounds, i.e. TBT chloride (TBTC), DBT dichloride (DBTC) and MBT trichloride (MBTC) were all purchased from Aldrich; in all cases purity exceeded 96%. Bovine serum albumin (BSA) was purchased from Sigma. Human spectrin (HSP) was a kind gift from Dr A. Brunati, Department of Biochemistry, University of Padua.

### Amoebocytic index

Haemocytes from the colonial ascidian *B. schlosseri* were collected with a micropipette, after puncturing the marginal vessel, in 10 mM L-cysteine (Sigma) to prevent cell clotting. They were used to set up culture chambers, as described previously.[22] 60 μl of the haemocytes suspension was added to each culture chamber and left to adhere to the coverslips for 30 min. Then, they were co-incubated in $10^{-5}$ M TBT, obtained from a $10^{-2}$ M stock solution in 95% ethanol, and

increasing concentrations ($5 \times 10^{-6}$ to $10^{-5}$ M) of CaM, BSA and HSP in filtered sea water (FSW) at 25 °C for 60 min. In controls, the proteins were omitted and 0.1% of 95% ethanol was added. In all experiments, viability, assessed by the trypan blue assay, exceeded 95%. After exposure, the cells were fixed in 1% glutaraldehyde in FSW containing 1% saccharose at 4 °C for 30 min, stained with 1% Giemsa's solution for 5 min, and mounted with Acquovitrex (Carlo Erba) on glass slides. The *amoebocytic index*, i.e. the percentage of amoeboid-shaped haemocytes in ten fields (at least 500 cells) per glass slide, was evaluated under a Leitz Dialux 22 light microscope. Each experiment was repeated in triplicate. Comparisons between the amoebocytic indexes of controls and exposed haemocytes were analysed using the $\chi^2$ test with the FREQ procedure (SAS statistical package, SAS Institute Inc., Cary, NC).

**Table 1.** Effects on amoebocytic index of *B. schlosseri* haemocytes after exposure to TBT and co-exposure to TBT and various concentrations of CaM, BSA or HSP (incubation at 25 °C for 60 min). Control values reported as 100. Asterisks: significant differences with respect to controls: ** $P < 0.01$; *** $P < 0.001$

| Treatment | Amoebocytic index |
|---|---|
| FSW (controls) | 100.00 |
| $10^{-5}$ M TBT | $18.17 \pm 0.24$*** |
| $10^{-5}$ M TBT + $5 \times 10^{-6}$ M CaM | $51.43 \pm 0.53$*** |
| $10^{-5}$ M TBT + $10^{-5}$ M CaM | $100.00 \pm 2.87$ |
| $10^{-5}$ M TBT + $5 \times 10^{-6}$ M BSA | $24.18 \pm 2.31$*** |
| $10^{-5}$ M TBT + $10^{-5}$ M BSA | $51.93 \pm 0.71$*** |
| $10^{-5}$ M TBT + $5 \times 10^{-6}$ M HSP | $60.77 \pm 1.03$** |
| $10^{-5}$ M TBT + $10^{-5}$ M HSP | $83.35 \pm 0.21$ |

## CD analysis

CD measurements were carried out on a JASCO model J-715 spectropolarimeter interfaced with a PC. A 2 nm bandwidth was used, with a scan speed of 50 nm $min^{-1}$ and a time constant of 2 s. Six scans were accumulated in order to improve the signal-to-noise ratio. All measurements were carried out at 25 °C, using quartz cells with path-lengths of 0.1 cm.

Spectra are reported in terms of mean residue molar ellipticity $[\Theta]_R$ (deg $cm^2$ $dmol^{-1}$). An aqueous stock solution of $10^{-5}$ M CaM was freshly prepared for each experiment. The protein was dissolved in Milli-Q water, obtained by treating deionized water with a Millipore Reagent Grade Water System. Butyltin chlorides (TBTC, DBTC, MBTC) were added to the CaM solution to final concentrations ranging from $10^{-4}$ to $10^{-3}$ M. They were prepared by dilution in Milli-Q water of a $10^{-1}$ M stock solution in 95% ethanol. Control measurements were carried out with both Milli-Q water and butyltin solutions alone.

## RESULTS AND DISCUSSION

Cultured phagocytes of *B. schlosseri* exposed for 60 min to $10^{-5}$ M TBT underwent severe morphological changes, with withdrawal of their long pseudopodia and the assumption of a spherical shape. This particular behaviour was evaluated as inhibition of the amoebocytic index. Ethanol, added in controls, had no effect on cell morphology. TBT, at concentrations ranging from $10^{-7}$ to $10^{-5}$ M, reduced this index significantly. However, according to our previous studies,[21,22] the amoebocytic index was progressively restored to control values when phagocytes were exposed to $10^{-5}$ M TBT in the co-presence of exogenous CaM at increasing concentrations ($5 \times 10^{-6}$ to $10^{-5}$ M). This suggests that direct interaction of TBT with exogenous CaM occurs, preventing the organotin compound from entering the cells. Analogously, the interaction of TBT and endogenous CaM in the cytosol probably results in the conformational changes of various important molecules, such as cytoskeletal proteins and $Ca^{2+}$-ATPases, the latter causing the previously reported alteration in $Ca^{2+}$ homeostasis.[17] Direct TBT–CaM interaction is also supported by empirical observations of a white, floccular precipitate in the culture medium containing TBT and the highest concentration of CaM. This phenomenon may be due to (i) direct interaction of the tin atom of TBT with CaM through its positive charge, as observed with certain heavy metals like vanadium, cadmium, mercury, aluminium, lead and manganese;[24] (ii) interaction of the hydrophobic region of TBT with the hydrophobic core of $Ca^{2+}$-activated CaM. This second hypothesis fits our observation of antagonism between TBT and chlorpromazine or W-7, which appear to bind to a common receptor.[22] These compounds, although belonging to different chemical classes, are known to bind CaM directly in a $Ca^{2+}$-dependent, irreversible manner,[25] and, owing to their similar tridimensional conformations,[26] they compete for the same binding site on CaM.[27] Interaction with CaM primarily involves non-specific hydrophobic bonding,[28] and also ionic attraction between a positively charged amino group on the drug and a negatively charged residue on CaM.[29]

In order to verify whether the TBT–protein interaction is hydrophobic in nature, we used increasing concentrations ($5 \times 10^{-6}$ to $10^{-5}$ M) of BSA and HSP to evaluate their ability to antagonize the effect of organotins on cell morphology when co-incubated with TBT. These proteins differ in the quantity of exposed hydrophobic domains, being few and scattered in BSA and many and regularly distributed in HSP. As shown in Table 1, both exogenous CaM and HSP, but not BSA, can restore the amoebocytic index to values not significantly different from those of controls.

Conformational studies by CD confirm the hypothesis of direct interaction between CaM and butyltins. This interaction is time-dependent (it was evident after only 30 min incubation) and dose-dependent (Figs 1–3). Of the butyltins

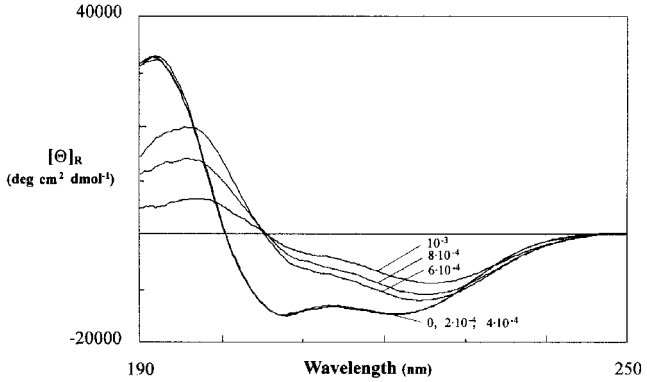


**Figure 1.** CD spectra of CaM in aqueous solution containing various molar concentrations of TBTC (indicated in spectra). Protein concentration $10^{-5}$ M.

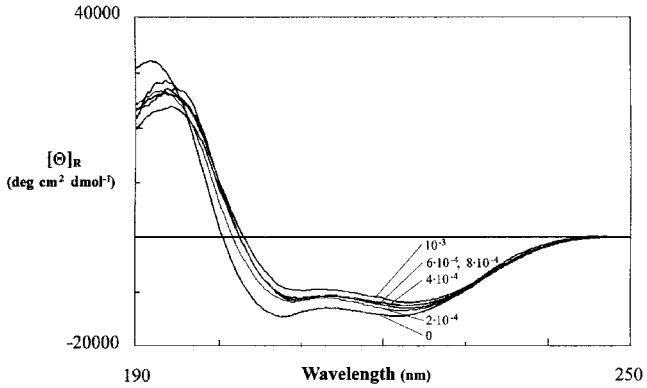


**Figure 2.** CD spectra of CaM in aqueous solution containing various molar concentrations of DBTC (indicated in spectra). Protein concentration $10^{-5}$ M.

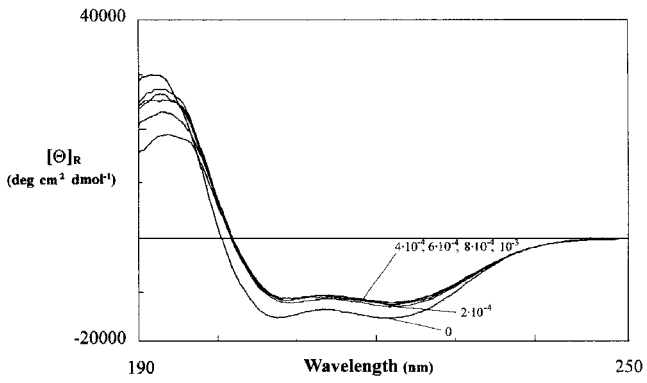


**Figure 3.** CD spectra of CaM in aqueous solution containing various molar concentrations of MBTC (indicated in spectra). Protein concentration $10^{-5}$ M.

assayed, TBT produces greater conformational changes in CaM than DBT and MBT, without any significant differences between the two latter compounds (Figs 2 and 3). The interaction seems to be non-covalent, since it was not possible to derive any dissociation constant. Our observations on cultured cells, indicating a similar behaviour of HSP and CaM with respect to TBT, suggest a hydrophobic interaction between the aliphatic chains of butyltins and hydrophobic pouches of $Ca^{2+}$-activated CaM.

It has previously been suggested that organotin structures are different in different aqueous media, their size and stereochemistry influencing the number of water molecules displaced in solution. In particular, triorganotin is probably a trigonal bipyramid, in which the organic ligands form a plane around the equator of the tin, whereas diorganotin has an octahedral structure.[30] TBT in aqueous solutions dissociates, with the formation of a hydrated tributyltin cation, modifying its net charge and polarity and probably undergoing reactions with anions present, although data on the equilibrium constants for these reactions are not available.[4] Our observations do not rule out the possibility of additional interactions, of an electrostatic nature, with biological molecules involving the positive charge on the metal atom of butyltins; this kind of interaction cannot be underrated, since a combination of both electrostatic and hydrophobic interaction probably occurs, as demonstrated with artificial lipid membranes.[5]

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