Tributyltin interacts with mitochondria and induces cytochrome c release

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Although triorganotins are potent inducers of apoptosis in various cell types, the critical targets of these compounds and the mechanisms by which they lead to cell death remain to be elucidated. There are two major pathways by which apoptotic cell death occurs: one is triggered by a cytokine mediator and the other is by a mitochondrion-dependent mechanism. To elucidate the mechanism of triorganotin-induced apoptosis, we studied the effect of tributyltin on mitochondrial function. We found that moderately low doses of tributyltin decrease mitochondrial membrane potential and induce cytochrome c release by a mechanism inhibited by cyclosporine A and bongkrekic acid. Tributyltin-induced cytochrome c release is also prevented by dithiols such as dithiothreitol and 2,3-dimercaptopropanol but not by monothiols such as GSH, N-acetyl-l-cysteine, l-cysteine and 2-mercaptoethanol. Further studies with phenylarsine oxide agarose revealed that tributyltin interacts with the adenine nucleotide translocator, a functional constituent of the mitochondrial permeability transition pore, which is selectively inhibited by dithiothreitol. These results suggest that, at low doses, tributyltin interacts selectively with critical thiol residues in the adenine nucleotide translocator and opens the permeability transition pore, thereby decreasing membrane potential and releasing cytochrome c from mitochondria, a series of events consistent with established mechanistic models of apoptosis.

Key words: adenine nucleotide translocator, apoptosis, permeability transition, swelling.

INTRODUCTION

Triorganotins such as tributyltin are toxic to many marine organisms and have therefore been used in animal-repellent paints on ships and other submerged structures. However, their use has resulted in the extensive contamination of aqueous environments with the cytotoxic agents [1,2]. Tributyltin is known to activate caspases and to induce the apoptosis of T-lymphocytes and the depletion of cortical thymocytes [3–8]. Because caspases are activated by several pathways in which the loss of mitochondrial membrane potential (ΔΨ) and the release of cytochrome c are important, tributyltin might exert its effect by impairing mitochondrial function directly [8,9].

In fact, tributyltin and related compounds are known to inhibit the F,F2-ATPase [10,11], anion channels [12] and the adenine nucleotide translocator (ANT) [13] in the mitochondrial inner membrane. Although tributyltin has been shown to decrease the proton gradient across the inner membrane and induce mitochondrial swelling [14], its precise molecular target and its involvement in cytochrome c release remain to be elucidated. We have therefore examined the effects of tributyltin on mitochondrial ΔΨ, swelling and cytochrome c localization. The present study demonstrates that tributyltin binds to critical cysteine residues of ANT, thereby opening the permeability transition pore, decreasing ΔΨ and releasing cytochrome c.

EXPERIMENTAL

Reagents

Bongkrekic acid and 3,3′-dipropylthiadicarbocyanine iodide [diS-C3-(5)] were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.) and Hayashibara Biochemical Laboratories (Okayama, Japan) respectively. Monoclonal antibody against cytochrome c (clone 7H8.2C12) was purchased from PharMingen (San Diego, CA, U.S.A.). Rabbit antiserum to ANT was a gift from Dr Hiroshi Terada (University of Tokushima, Tokushima, Japan). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Isolation of mitochondria from rat liver

Male Wistar rats (200–250 g) were obtained from SLC Co. (Shizuoka, Japan) and fed with a standard laboratory diet and water ad libitum. After the rats had been fasted overnight, mitochondria were isolated from the liver with a buffer containing 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, and 0.1 mM EDTA, as described previously [15]. The mitochondrial fractions were suspended in 0.25 M sucrose/10 mM Tris/HCl (pH 7.4) at 10–20 mg/ml protein.

Assay for cytochrome c

Mitochondria (1 mg/ml) were incubated at 25 °C for 5 min in 10 mM Tris/HCl buffer, pH 7.4, containing 0.15 M KCl, 5 mM succinate and other additives as described for each experiment. The incubated samples were centrifuged at 8000 g at 4 °C for 5 min. Aliquots of the supernatant (20 μl) were dissolved in SDS buffer [125 mM Tris/HCl (pH 7.4)/4 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/20 % (v/v) glycerol/200 μg/ml Bromophenol Blue], boiled at 100 °C for 5 min and subjected to SDS/PAGE followed by Western blot analysis.

Western blotting

Separated proteins were transferred electrophoretically from the gel to an Immobilon membrane (Millipore, Waltham, MA,

Abbreviations used: ANT, adenine nucleotide translocator; diS-C3-(5), 3,3′-dipropylthiadicarbocyanine iodide
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U.S.A.). The membrane was blocked in TBS [0.15 M NaCl/10 mM Tris/HCl (pH 7.4)] containing 5% (v/v) skimmed milk, then incubated for 1 h with primary antibodies diluted with TBS containing 0.05% (v/v) Tween 20 (TBST) at room temperature. After being washed three times in TBST, the membrane was incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) diluted in TBST. Immunoreactive bands were detected with an enhanced chemiluminescence system (ECL®; Amersham Pharmacia Biotech, Uppsala, Sweden).

Assay for mitochondrial membrane potential and swelling
The mitochondrial membrane potential was measured in 10 mM Tris/HCl, pH 7.4, containing 0.15 M KCl, 0.15 μg/ml diS-C3-(5) and intact mitochondria (0.1 mg/ml protein). Fluorescence at 672 nm was recorded in a fluorescence spectrophotometer (Hitachi 650-10LC) with excitation at 560 nm [16]. To measure mitochondrial swelling, mitochondria (0.1 mg/ml protein) were incubated at 25 °C in 10 mM Tris/HCl, pH 7.4, containing 0.15 M KCl and 5 mM succinate. Changes in A450 were recorded with a dual-beam spectrophotometer (Shimadzu UV-3000) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer [17].

Measurement of protein thiols
Mitochondria (0.5 mg) were suspended in ice-cold 10% (w/v) trichloroacetic acid containing 1 mM diethylenetriaminepentiacetic acid. The suspension was centrifuged for 5 min at 12000 g and 4 °C. The acid-insoluble precipitate was suspended in 400 μl of 0.5 M potassium phosphate buffer, pH 7.4, containing 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 5 mM EDTA, then incubated at 4 °C for 30 min. After the removal of insoluble materials by centrifugation for 5 min at 12000 g and 4 °C, the A412 of the supernatant was measured.

Binding of ANT to a phenylarsine oxide-conjugated agarose
Mitochondria (2 mg) treated with various concentrations of tributyltin were centrifuged for 5 min at 12000 g and 4 °C. Proteins in the precipitate were solubilized in 100 μl of 50 mM Hepes buffer, pH 7.4, containing 150 mM Na2SO4, 1 mM EDTA, 3% (v/v) Triton X-100, 1 mM PMSF and 1 μM leupeptin. After centrifugation of the lysate for 5 min at 15000 g and 4 °C, the supernatant fraction was incubated with 4-aminophenylarsine oxide agarose (ThioBond Resin; Invitrogen, Carlsbad, CA, U.S.A.) that had been equilibrated in 50 mM Hepes buffer, pH 7.4, containing 150 mM Na2SO4, 1 mM EDTA, 0.25% (v/v) Triton X-100, 1 mM PMSF and 1 μM leupeptin. After incubation at 4 °C for 15 min, the resin was washed three times with 0.5 ml of the equilibration buffer by centrifugation for 1 min at 15000 g and 4 °C. Proteins bound to the resin were eluted with 50 μl of the equilibration buffer plus 10 mM dithiothreitol and analysed by SDS/PAGE and Western blotting with anti-ANT antiserum.

RESULTS
Tributyltin induces membrane permeability transition and cytochrome c release
Because tributyltin-induced apoptosis of Jurkat cells is associated with changes in mitochondrial membrane permeability, we examined the effect of tributyltin on mitochondrial membrane potential. Consistent with previous observations [8,9] was our observation that moderately low concentrations of tributyltin decreased ΔΨ across the inner mitochondrial membrane (Figure 1). Next we studied the release of cytochrome c from mitochondria, an event known to induce apoptosis in various cell types [18]. As shown in Figure 2, moderately low concentrations of tributyltin released cytochrome c from mitochondria. The amount of cytochrome c released was dependent on the dose of tributyltin administered, reaching a maximum at a tributyltin concentration of 5 nmol/mg of mitochondrial protein. A direct interaction of tributyltin with one or more mitochondrial components might trigger a reaction that releases cytochrome c from the intermembranous space. Although relatively low concentrations of tributyltin have been shown to induce mitochondrial swelling [14], in our study tributyltin induced mitochondrial

![Figure 1 Reduction of membrane potential by tributyltin](image1)

![Figure 2 Tributyltin induces cytochrome c release from mitochondria](image2)
Induction of cytochrome c release by tributyltin

Mitochondria (0.1 mg/ml) were incubated as described in the Experimental section. The swelling of mitochondria was monitored by measuring A540. Various concentrations of tributyltin (TBT) were added at the indicated time. Sodium succinate (Suc) (5 mM) was added as indicated.

swelling only above a ligand concentration of 60 nmol/mg of mitochondrial protein (Figure 3), which was 12-fold that required for the induction of Δψ loss and cytochrome c release.

The mechanism by which cytochrome c is released from mitochondria involves the Bcl-2 family proteins Bax, Bak and Bid [19,20]. Bax and Bak interact directly with a membrane channel known as the permeability transition pore, which consists of a voltage-dependent anion channel, ANT, cyclophilin D and other proteins [21,22]. Because this channel has a major role in the regulation of membrane permeability, we examined the effects of cyclosporine A and bongkrekic acid. Both compounds have been used as potent inhibitors of the permeability transition pore [23–25]. As shown in Figure 2, tributyltin-induced cytochrome c release was inhibited by both 1 μM of cyclosporine A and 10 μM of bongkrekic acid. In contrast, both cyclosporine A and bongkrekic acid failed to inhibit large-amplitude swelling induced by high concentrations of tributyltin (60–80 nmol/mg of mitochondrial protein).

Figure 3 Induction of mitochondrial swelling by tributyltin

Mitochondria (0.1 mg/ml) were incubated with various concentrations of tributyltin at 25 °C for 5 min. The mixtures were then treated with trichloroacetic acid and centrifuged for 5 min at 12000 g. Concentrations of free thiol groups in the acid-insoluble fractions were measured. Results are means ± S.E.M.

Effect of thiol compounds on tributyltin-induced cytochrome c release

Triorganotins have greater affinities for dithiols than for monothiols [26–28]. To test the possible involvement of one or more critical mitochondrial membrane thiol groups in the mechanism of tributyltin-induced cytochrome c release, the effects of various thiols were examined. Tributyltin reacted with free cysteine residues in mitochondria; the presence of thiol groups titratable with 5,5'-dithiobis-(2-nitrobenzoic acid) therefore decreased in a dose-dependent manner (Figure 4). However, membrane thiol groups did not decrease significantly at tributyltin concentrations lower than 20 nmol/mg of mitochondrial protein. Although dithiothreitol and 2,3-dimercaptopropanol strongly inhibited tributyltin-induced cytochrome c release from mitochondria, monothiols such as GSH, l-cysteine, N-acetyl-l-cysteine and 2-mercaptoethanol had no appreciable effect (Figure 5). In contrast, tributyltin-induced swelling of mitochondria was inhibited not only by dithiothreitol but also by other monothiols (Figure 6).

Effect of tributyltin on the thiol status of ANT

Because low doses of tributyltin induce cytochrome c release by a mechanism that is inhibited by dithiothreitol and 2,3-dimercaptopropanol, one or more thiol groups might be involved in the opening of the membrane permeability transition pore, leading to the release of cytochrome c. To test this possibility, the effect of tributyltin on the thiol status of ANT was examined.
Mitochondria (1.0 mg/ml) were incubated at 25 °C for 5 min with various concentrations of tributyltin. The samples were lysed with Triton X-100 and incubated at 4 °C for 15 min with phenylarsine oxide agarose (25 l/mg of protein). Proteins bound to the resin were eluted with 10 mM dithiothreitol and subjected to Western blot analysis with anti-ANT antibody.

Mitochondria (1.0 mg/ml) were incubated with tributyltin (5 nmol/mg of mitochondrial protein) in the absence (−) or presence (+) of 0.2 mM EGTA (A) or 10 μM oligomycin (B). After centrifugation, aliquots of the supernatants were subjected to Western blot analysis for cytochrome c.

Figure 8 Tributyltin-induced cytochrome c release is not affected by EGTA and oligomycin

Mitochondria (1.0 mg/ml) were incubated with tributyltin (5 nmol/mg of mitochondrial protein) in the absence (−) or presence (+) of 0.2 mM EGTA (A) or 10 μM oligomycin (B). After centrifugation, aliquots of the supernatants were subjected to Western blot analysis for cytochrome c.

with the use of phenylarsine oxide agarose, an affinity matrix that interacts selectively with vicinal diithiols in some proteins and critical cysteine residues in ANT [29,30]. Figure 7 shows that the binding of ANT to phenylarsine oxide agarose was inhibited by pretreating mitochondria with low concentrations of tributyltin (not more than 5 nmol/mg of mitochondrial protein).

**Tributyltin-induced cytochrome c release is not affected by oligomycin and EGTA**

Both tributyltin and oligomycin interact directly with the F₀ subunit of FₐFₕ-ATPase to inhibit ATP generation [10,11]. To confirm whether inhibition of FₐFₕ-ATPase causes cytochrome c release, we incubated mitochondria with oligomycin. Oligomycin failed to induce the release of cytochrome c (Figure 8A). Thus tributyltin induced cytochrome c release from mitochondria by some mechanism which is different from its oligomycin-like activity.

Ca²⁺ has been suggested to play an essential role in membrane permeability transition and release of cytochrome c. However, induction of cytochrome c release by tributyltin was not affected in the presence of EGTA, indicating that Ca²⁺ is not required for tributyltin-induced cytochrome c release (Figure 8B).

**DISCUSSION**

The present study demonstrates that moderately low concentrations of tributyltin react with mitochondria to induce a change in membrane permeability, allowing the release of cytochrome c. Two mechanisms have been proposed for Bcl-2 family-dependent cytochrome c release from mitochondria [19,20]. First, Bax and Bik activate the permeability transition pores, decreasing Δψ across mitochondrial membranes and allowing cytochrome c release. Conversely, Bid releases cytochrome c without modulating the permeability transition pores and Δψ. Low concentrations of tributyltin decrease mitochondrial Δψ and induce cytochrome c release by a mechanism that is inhibited by either cyclosporine A or bongkrekic acid, indicating that it might act in a Bax-like manner, triggering the opening of permeability transition pores.

The present study also demonstrates that the release of cytochrome c is inhibited selectively by dithiothreitol and 2,3-dimercaptopropanol but not by monothiols. Therefore some vicinal diithiols and/or two proximal thiol groups in a mitochondrial protein might underlie the mechanism for the tributyltin-induced release of cytochrome c. The tertiary structure of proteins, particularly with regard to cysteine residues, is important for the formation of stable complexes with triorganotins [31–33]. For example, triethyltin forms stable complexes with two cysteine residues or between cysteine and histidine residues in cat haemoglobin [31,32]. It also binds to specific cysteine and histidine residues in hexokinase [34]. A candidate for tributyltin binding is ANT, a component of the permeability transition pore; the modification of two opposed thiol groups in an ANT dimer (Cys-56 in each ANT monomer) triggers the opening of the channel [29,30,35]. Indeed, the treatment of mitochondria with tributyltin (5 nmol/mg of protein) decreased the binding of ANT to phenylarsine oxide (see Figure 7), an assay which reliably reports the modification of critical thiol groups [29,30,35].

Although Ca²⁺ has been suggested to be essential for the changes in membrane permeability, tributyltin induces cytochrome c release even in the presence of a high concentration of EGTA. In this context, however, Ca²⁺ overload is known not to be essential for the loss of Δψ and the release of cytochrome c in the course of tributyltin-induced apoptosis of Jurkat cells [9]. Some reagents that cross-link thiol groups also exhibit a similar effect to that of Ca²⁺ in altering the conformation of ANT, forming a non-specific pore [30,36]. Because tributyltin interacts directly with such critical thiol groups of ANT, it might open the permeability transition pore independently of Ca²⁺.

We also found that the induction of mitochondrial swelling required higher concentrations of tributyltin (at least 60 nmol/mg of mitochondrial protein) than was necessary for the loss of Δψ and the release of cytochrome c. Under such conditions, a significant fraction of membrane thiol groups in mitochondria were modified by tributyltin. Because the swelling of mitochondria induced by high concentrations of tributyltin was inhibited not only by diithiols but also by monothiols, which did not inhibit tributyltin-induced cytochrome c release, non-specific modification of thiol groups seems to inhibit the function of ion transporters in mitochondrial membranes. Consistent with this notion is the report [37] that high concentrations of mercurial compounds react non-specifically with thiol groups to inhibit mitochondrial transporter function. These observations suggest that tributyltin-induced cytochrome c release and swelling of mitochondria are triggered by independent mechanisms. Studies
on the binding of triorganotins to rat liver mitochondria indicate the presence of high-affinity and low-affinity binding sites on the membranes [38]. Interestingly, the dissociation constant of the low-affinity site for triethyltin (70 nmol/mg of mitochondrial protein) is in good agreement with concentrations of tributyltin required for the swelling of mitochondria (see Figure 3). Mitochondrial swelling therefore seems to be induced by the binding of tributyltin to the low-affinity membrane sites.

Triorganotin compounds catalyse Cl-/OH- exchange, thereby inducing mitochondrial swelling [14]. C1-/OH- -exchange-related swelling was observed at a ligand concentration of 1.75 nmol/mg of protein as measured by light scattering. It should be noted that the sensitivity of the light-scattering method is fairly high and can detect a moderate level of swelling, whereas the absorbance method employed in the present study is relatively insensitive and can detect only large-amplitude swelling. Because the concentration of tributyltin required for the induction of large-amplitude swelling was significantly higher than that required for the catalysis of Cl-/OH- exchange, the large-amplitude swelling observed in the present experiment might occur independently of Cl-/OH- exchange.

It is well recognized that organotins interact directly with the F1F0-ATPase to inhibit ATP production [10,11]. However, inhibition of the F1F0-ATPase does not account for the tributyltin-induced release of cytochrome c because cytochrome c was not released with fairly high concentrations of oligomycin, a potent inhibitor of the F1F0-ATPase. Note that maximal inhibition of the F1F0-ATPase was observed at tributyltin concentrations (0.75 nmol/mg of mitochondrial protein) lower than that required for the release of cytochrome c [11]. Thus tributyltin-induced cytochrome c release seems to involve the opening of the permeability transition pores independently of the inhibition of the F1F0-ATPase.

Although mitochondrial swelling can be induced upon the opening of the permeability transition pores [29,30,39], the present study demonstrates that a low concentration of tributyltin induces the opening of permeability transition pores and the release of cytochrome c without inducing large-amplitude swelling. It has been reported that, in the presence of oligomycin, changes in permeability can be generated without inducing large-amplitude swelling [40]. Because tributyltin binds to the F0 subunit of the F1F0-ATPase, the oligomycin-like activity of tributyltin might inhibit large-amplitude swelling induced by the permeability change. Large-amplitude swelling was not inhibited by cyclosporine A or bongkrekic acid, indicating that the swelling was induced by the formation of membrane pores, independently of the opening of permeability transition pores.

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