Cytotoxic Action of Triphenyltin on Mouse Thymocytes: A Flow-Cytometric Study Using Fluorescent Dyes for Membrane Potential and Intracellular \( \text{Ca}^{2+} \)

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ABSTRACT—Effects of triphenyltin on mouse thymocytes were examined using fluorescent dyes to monitor membrane potential and intracellular \( \text{Ca}^{2+} \). Triphenyltin at \( 3 \times 10^{-7} \text{M} \) to \( 1 \times 10^{-6} \text{M} \) hyperpolarized thymocytes and depolarized them at \( 3 \times 10^{-6} \text{M} \) or more, associated with increasing intracellular \( \text{Ca}^{2+} \). Hyperpolarization was suppressed by quinine, but not by tetaethylammonium and 4-aminopyridine, suggesting the involvement of \( \text{Ca}^{2+} \)-activated \( K^+ \) current. Triphenyltin failed to hyperpolarize thymocytes in \( \text{Ca}^{2+} \)-free solution. Results indicate that triphenyltin promotes \( \text{Ca}^{2+} \)-influx to thymocytes. Such an action of triphenyltin may be related to the immunotoxicity of organotins.

Organotins, one of possible environmental hazards, exert specific toxic actions on respective target organs and organ systems such as the central nervous system and the immune system (1). Among the organotin toxicities, immunotoxicity has been histologically studied. Reduction of thymus weight, associated with a depletion of cortical lymphocytes, was reported in rats fed a triorganotin such as tributyltin and triphenyltin at dietary levels as low as 15 to 25 mg/kg feed (2-4). Immune function studies with rats fed tributyltin or triphenyltin compounds revealed that the thymus-dependent immune responses were suppressed (5, 6). In contrast with our knowledge concerning the immunotoxicity of organotins in experimental animals fed these compounds, there is little information on the cellular mechanism for their immunotoxicity (1, 7).

In this study, therefore, we have examined the effect of triphenyltin on mouse thymocytes using a flow-cytometer with fluorescent dyes for membrane potential and intracellular \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]_i).

Experiments were performed on thymocytes dissociated from the thymus of 5-week-old ddY mice. Thymus minced with a pair of iris scissors was suspended in chilled phosphate-buffered saline. Phosphate-buffered saline containing thymocytes and residua of thymus was filtered through a mesh (diameter of 0.05 mm) to remove the residua. After removing red blood cells with 0.85% \( \text{NH}_4 \text{Cl} \) solution in some cases, the thymocytes were harvested by centrifugation at 600 rpm for 10 min and then resuspended at a density of \( 10^6 \text{cells/ml} \) in Hepes-buffered Tyrode’s solution. Thymocytes were incubated at 33°C for 60 min before use. Triphenyltin hydroxide was initially dissolved in dimethylsulfoxide, and the final concentrations of solvent was less than 0.1% where the solvent did not affect any measurements of fluorescence in the thymocytes. All chemicals except for fluorescent dyes were purchased.
The oxonol dyes have been used for indirect measurement of membrane potential in the cells with inside-negative potentials (5, 8). Increase in the oxonol fluorescence intensity indicates a depolarization of the cell, whereas the decrease shows a hyperpolarization. In this study, the photochemical measurement of membrane potential was made with oxanol dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (di-BA-C₄, Molecular Probe Inc., U.S.A.), since there is a linear relationship for oxonol fluorescence versus voltage over a wide range for lymphocytes (9). Experimental procedures used here were basically similar to those previously reported for lymphocytes (10). The oxonol dye di-BA-C₄ was added into the solution containing thymocytes to obtain a final concentration of 1 × 10⁻⁷ M a few minutes before the start of fluorescence measurement. The fluorescence intensity of di-BA-C₄ in the control was constant for 60 min or longer.

To measure the level of [Ca²⁺], the tetracarboxylate fluorescent indicators quin-2, fura-2, and indo-1 are widely used, while such dyes require excitation at ultraviolet wavelengths which are potentially injurious to the cells. Therefore, in this study, 1-[2-amino-5-(2,7-dichlor-6-hydroxy-3-oxy-9-xanthenyl) phenoxy]-2-(2'-amino-5'-methylphenoxy) ethane-N,N',N'-tetraacetic acid (fluo-3) was used as an indicator of [Ca²⁺] (11), since its excitation wavelengths are in the visible range. For loading fluo-3 to thymocytes, the pentaacetoxy methyl ester of fluo-3 (fluo-3-AM, Dojindo Laboratory, Japan) was used. The thymocytes were incubated in Hepes-buffered Tyrode’s solution with fluo-3-AM at a concentration of 3 × 10⁻⁷ M for 60 min, as previously reported (12). The fluorescence intensity of fluo-3 was also constant for the next 60 min or more in the control.

The fluorescence measurements for di-BA-C₄ and fluo-3 were done in a flow-cytometer equipped with an argon laser (Cyto-ACE, Japan Spectroscopic Co., Ltd.). In both cases, the excitation wavelength used was 488 nm, and the emission was detected at the wavelengths of 530 ± 20 nm by a bandpass filter. Histograms representing the fluorescence distribution were obtained from a programmed number of thymocytes (4 × 10⁴ or 5 × 10⁴ cells in this study) by using software developed by Japan spectroscopic Co., Ltd. on a personal computer (PC9801RX, Nippon Electronics Company). Fluorescence intensity was resolved into 255 channels. Changes to higher or lower channels correspond to depolarization or hyperpolarization of membrane potential for di-BA-C₄ and an increase or a decrease in the [Ca²⁺], for fluo-3, respectively.

The effect of triphenyltin on the oxonol fluorescence profile of thymocytes in the dose-response titration is shown in Fig. 1A. Triphenyltin at the concentrations of 1 × 10⁻⁷ M or less did not affect the fluorescence profile, indicating no effect on the membrane potential of thymocytes (not shown). Thymocytes were found to hyperpolarize in the presence of 3 × 10⁻⁷ M (Fig. 1A-a) and 1 × 10⁻⁶ M (Fig. 1A-b) triphenyltin by a decreased intensity (number of channel) of the oxonol fluorescence from 158 ± 22 to 150 ± 25 channel (mean number ± S.D. in 5 × 10³ thymocytes) and from 157 ± 22 to 139 ± 29 channel, respectively. The hyperpolarization of thymocytes occurred in a time-dependent manner and reached a steady-state within 3 min after adding triphenyltin. Hyperpolarization remained at the same level for the next 2 min. Therefore, the effect of triphenyltin on the oxonol fluorescence was examined 5 min after adding triphenyltin in the following experiments. At 3 × 10⁻⁶ M (Fig. 1A-c), triphenyltin did not seem to exert significant action on the distribution of the fluorescent intensities of thymocytes. However, the effect of triphenyltin on the distribution at this concentration varied from thymocyte to thymocyte. In three of 6 cases, triphenyltin spread the distribution to both lower and higher channels (both polarities). However, the degree of hyperpolarization was always much less than that observed at lower concentrations of triphenyltin. Increasing the triphenyltin con-
centration resulted in a greatly-increased channel number (from 153 ± 22 to 227 ± 25 channel at 1 × 10⁻⁵ M), indicating clear depolarization of the thymocytes (Fig. 1A-d).

To characterize the hyperpolarization induced by triphenyltin, the following three types of K⁺ channel blockers were used: tetraethylammonium (TEA) for a delayed K⁺ channel, 4-aminopyridine (4-AP) for a transient outward K⁺ channel, and quinine for Ca²⁺-activated K⁺ channel (13). As shown in Fig. 1B, the hyperpolarization induced by 1 × 10⁻⁶ M triphenyltin was not suppressed by 1 × 10⁻² M TEA (Fig. 1B-c) or 5 × 10⁻³ M 4-AP (Fig. 1B-d), whereas in the presence of 3 × 10⁻⁴ M quinine, triphenyltin failed to hyperpolarize the thymocytes, but depolarized them (Fig. 1B-b). Since the results described above pharmacologically suggest the involvement of Ca²⁺-activated K⁺ current (I_KCa) in the hyperpolarization, the effect of triphenyltin on the level of [Ca²⁺]ᵢ was studied using fluo-3 fluorescence. Triphenyltin at 1 × 10⁻⁷ M or more increased dose-dependently the in-

Fig. 1. Pharmacological property of triphenyltin-induced hyperpolarization in mouse thymocytes. (A) Effect of triphenyltin on the oxonol fluorescence profile of thymocytes. Histograms represent the fluorescence distribution in the thymocytes (5 × 10⁵ cells in this experiment). Fluorescence intensity was resolved into 255 channels as shown in the abscissa. Changes to higher or lower channels correspond to depolarization or hyperpolarization, respectively. Values at the ordinate correspond to the number of thymocytes at each channel. Thin and thick (with arrow) line histograms were obtained, respectively, in the control and 5 min after the addition of triphenyltin at the following concentrations: (a) 3 × 10⁻⁷ M, (b) 1 × 10⁻⁶ M, (c) 3 × 10⁻⁶ M, (d) 1 × 10⁻⁵ M. The figure shows a representative one from six experiments. (B) Effect of K⁺ channel blockers on the hyperpolarization induced by 1 × 10⁻⁶ M triphenyltin. Thymocytes were treated with the respective K⁺ channel blocker for 5 min before adding triphenyltin. Thin and thick (with arrow) line histograms were obtained, respectively, in the control and 5 min after the addition of triphenyltin in the presence of following agents: (a) control hyperpolarization induced by triphenyltin, (b) 3 × 10⁻⁴ M quinine, (c) tetraethylammonium (TEA) 1 × 10⁻² M, (d) 4-aminopyridine (4-AP) 5 × 10⁻³ M. The result shown is a representative one from four experiments. Tentative calibration of the oxonol fluorescence was made under the assumption that A23187-induced hyperpolarization reached the equilibrium potential for K⁺ at various external K⁺ concentrations and the intracellular K⁺ was 150 mM. In such a tentative calibration, the channels around 85, 105, 135, and 165 correspond to -90, -70, -50 and -30 mV, respectively.
tensity of Fluo-3 fluorescence of thymocytes from 103 ± 19 to 117 ± 22 channel (mean number ± S.D. in 4 × 10^3 thymocytes) at 1 × 10^{-7} M (Fig. 2A-b), from 102 ± 18 to 134 ± 22 channel at 3 × 10^{-7} M (Fig. 2A-c) and from 99 ± 19 to 164 ± 20 channel at 1 × 10^{-6} M (Fig. 2A-d), indicating an increase in the \([\text{Ca}^{2+}]_i\). Furthermore, quinine did not affect the triphenyltin-induced increase in \([\text{Ca}^{2+}]_i\), (not shown). Therefore, it can be suggested that the hyperpolarization is due to an activation of \(I_{\text{KCa}}\) by the \([\text{Ca}^{2+}]_i\), increased by triphenyltin when the \([\text{Ca}^{2+}]_i\) reaches the threshold concentration for the \(I_{\text{KCa}}\) at triphenyltin concentrations of 3 × 10^{-7} M or more.

The hyperpolarization of thymocytes was also produced by 3 × 10^{-9} M A23187, a \(\text{Ca}^{2+}\)-ionophore (10). This hyperpolarization could be also blocked by quinine, but not by TEA or 4-AP at the same concentrations as used for the triphenyltin experiment. A23187 at 3 × 10^{-9} M slightly depolarized the thymocytes that were exposed to 3 × 10^{-4} M quinine (not shown) as observed in the triphenyltin experiment (Fig. 1B). The steady-state of hyperpolarization was obtained within 1 min after adding A23187, and the hyperpolarization remained at the same level for the next 5 min. The time course for the hyperpo-

Fig. 2. \(\text{Ca}^{2+}\)-dependence of triphenyltin-induced hyperpolarization. (A) Effect of triphenyltin on the Fluo-3 fluorescence profile of thymocytes. Histograms were obtained from 4 × 10^3 thymocytes. Fluorescence intensity was also resolved into 255 channels as shown in the abscissa. Changes to higher or lower channels correspond to a decrease or increase in the \([\text{Ca}^{2+}]_i\), respectively. Values at the ordinate correspond to the number of thymocytes at each channel. Thin and thick (with arrow) line histograms were obtained in the control and 5 min after the following respective drug applications: (a) 3 × 10^{-9} M A23187 as a reference drug, (b) 1 × 10^{-7} M triphenyltin, (c) 3 × 10^{-7} M triphenyltin, (d) 1 × 10^{-6} M triphenyltin. Figure is a representative one from four experiments. (B) Dependence of hyperpolarizations induced by triphenyltin and A23187 on extracellular \(\text{Ca}^{2+}\) ([\(\text{Ca}^{2+}\)]_e). The effect of (a) 3 × 10^{-9} M A23187 and (c) 1 × 10^{-6} M triphenyltin on membrane potential was examined in nominal [\(\text{Ca}^{2+}\)]_e-free solution. Thin and thick (with arrow) line histograms were respectively obtained in the control and in the presence of respective agent. Reintroduction of [\(\text{Ca}^{2+}\)]_e (1 × 10^{-7} M) produced the hyperpolarization in the continued presence of 3 × 10^{-9} M A23187 (b) and 1 × 10^{-6} M triphenyltin (d). The result shown is a representative one from four experiments.
larization induced by triphenyltin seemed to be slower than that by A23187. It was reminiscent of the possibility that there were some differences in the source of increased \([Ca^{2+}]_i\) between triphenyltin and A23187. Therefore, the effects of \(3 \times 10^{-9} \text{M A23187 (Fig. 2B-a)}\) and \(1 \times 10^{-6} \text{M triphenyltin (Fig. 2B-c)}\) on the oxonol fluorescence profiles were examined in nominal \(Ca^{2+}\)-free Tyrode’s solution where \(CaCl_2\) was omitted from the solution and \(5 \times 10^{-3} \text{M EGTA was added. Both agents failed to hyperpolarize thymocytes in extracellular Ca^{2+}-free ([Ca^{2+}]_e-free) solution 5 min after adding the respective agent. Triphenyltin at } 1 \times 10^{-6} \text{M slightly depolarized the thymocytes. However, the reintroduction of } 1 \times 10^{-2} \text{M CaCl}_2 \text{ into the [Ca^{2+}]_e-free solution in the presence of A23187 (Fig. 2B-b) or triphenyltin (Fig. 2B-d) caused the hyperpolarization of thymocytes. The results described above suggest not only that triphenyltin may promote Ca^{2+}-influx to thymocytes, resulting in increased \([Ca^{2+}]_i\), which opens the channel for } I_{KCa}, \text{ but also that triphenyltin at higher concentration may directly block the } I_{KCa} \text{ channels, resulting in thymocyte depolarization.}

The present study provides data with important implications about the toxicity of organotins, although it only provides qualitative information about the effect of triphenyltin on \([Ca^{2+}]_i\). The resting concentration of \([Ca^{2+}]_i\) is generally maintained at a level lower than \(1 \times 10^{-7} \text{M by the mitochondria, endoplasmic reticulum, membrane Ca^{2+}-pump, and Na^{+}-Ca^{2+} exchange system, since an increased [Ca^{2+}]_i is linked to a variety of cellular functions. Therefore, an abnormal increase in the [Ca^{2+}]_i would initiate pathological states and disturb the homeostasis unless an increased [Ca^{2+}]_i is restored to basal levels. Mitochondria have a tremendous ability to buffer against changes in \([Ca^{2+}]_i\) primarily when there is a high \([Ca^{2+}]_i\) loading (14). Triorganotins at concentrations higher than \(1 \times 10^{-7} \text{M are suggested to inhibit ATP formation by binding to the ATP synthase complex in the mitochondria of rat thymocytes (1, 7). If so, it would increase the mismatch between the abnormally-increased [Ca^{2+}]_i and the cellular ability to buffer against changes in \([Ca^{2+}]_i\) in organotin-exposed thymocytes. Therefore, further systematic study on the effect of triphenyltin on the mobilization of [Ca^{2+}]_i will be necessary to elucidate the cellular basis of the organotin toxicity.}

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