Triphenyltin-Induced Increase in the Intracellular Ca^{2+} of Dissociated Mammalian CNS Neuron: Its Independence from Voltage-Dependent Ca^{2+} Channels

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ABSTRACT—To test the possibility that triphenyltin (TPT) increases the intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in neurons as found previously in thymocytes, the effect of TPT on $[\text{Ca}^{2+}]_i$ was examined in rat cerebellar neurons by a flow-cytometer with fluorescent dyes. TPT at concentrations ranging from 3×10^{-7} M to 1×10^{-5} M dose-dependently increased the $[\text{Ca}^{2+}]_i$. The TPT-induced increase in $[\text{Ca}^{2+}]_i$ was not attenuated by a Ca^{2+} channel blocker, suggesting that it was not dependent on voltage-dependent Ca^{2+} channels. As the concentration of external Ca^{2+} ($[\text{Ca}^{2+}]_e$) increased, TPT produced a more profound increase in the $[\text{Ca}^{2+}]_i$. However, the increase in the $[\text{Ca}^{2+}]_i$ by TPT was observed even in nominally $[\text{Ca}^{2+}]_e$ -free solution. These results suggest two possibilities. First, TPT may promote Ca^{2+} -influx to the neuron. Secondly, TPT may affect the intracellular Ca-store sites. This study is relevant to the neurotoxicity of organotins because it has become progressively clear that sustained increases in the $[\text{Ca}^{2+}]_i$ can activate various Ca^{2+} -dependent degradative processes.

We have suggested that triphenyltin (TPT) exerts an excitotoxic action on the mammalian central nervous system (CNS) by increasing the amplitude of the voltage-dependent Na^+ current (I_{Na}), associated with a prolonged decay phase, of mammalian CNS neurons (1). TPT was found to increase the intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) of mouse thymocytes in our previous study (2). If it is also the case for mammalian CNS neurons, it would further contribute to the toxicity of TPT in the mammalian CNS. To test this possibility, we studied the effects of TPT on the neurons dissociated from rat cerebellum, using a flow-cytometer with fluorescent dyes for $[\text{Ca}^{2+}]_i$ and membrane potential.

Experiments were performed on the cere-

bellar neurons dissociated from 1- to 2-week-old rats. The technique for dissociation was a modification of that previously reported (3). The brain slices (0.5 mm in width) of rat cerebellum were treated with dispase (10^3 PU/ml; Godo Shusei Co., Japan) for 60 min in Tyrode's solution at $31-32^\circ\text{C}$. After the slices were washed with Tyrode's solution to remove dispase, the cerebellar neurons were mechanically dispersed by gentle pipetting in Tyrode's solution containing 1% bovine serum. Tyrode's solution containing cerebellar neurons was filtered through a mesh (diameter of $53\ \mu\text{m}$) to remove larger neurons with dendrites and the residua. Since the neurons in the filtered cell suspension were characterized by their small size (diameter of about 10

μm) and spherical shape under microscopic observation, the cells were likely cerebellar granule neurons. The cell suspensions were incubated at the temperature of $31\text{--}32^\circ\text{C}$ for 60 min or longer before any fluorescent measurement.

For monitoring the $[\text{Ca}^{2+}]_i$, 1-[2-amino-5-(2,7-dichlor-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid (fluo-3) was used (4, 5). To load fluo-3 to the cells, the neurons were incubated with the pentaacetoxymethyl (AM) ester of fluo-3 (fluo-3-AM; Dojindo Laboratory, Japan) at a concentration of 3×10^{-7} M for 60 min at the temperature of $31\text{--}32^\circ\text{C}$. The photochemical measurement of membrane potential was made with *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol (di-BA- C_4 ; Molecular Probe Inc., U.S.A.) as previously reported (6, 7). The oxonol dye di-BA- C_4 was added into the cell suspension to achieve a final concentration of 1×10^{-7} M just before the fluorescence measurement. The fluorescence measurements for fluo-3, di-BA- C_4 and acridine orange were done on a flow-cytometer equipped with an argon laser (Cyto-ACE 150, Japan Spectroscopic Co., Ltd.) at room temperature ($24\text{--}25^\circ\text{C}$). The excitation wavelength used for these dyes was 488 nm, and the emission was detected at the wavelength of 530 ± 20 nm by a bandpass filter. Histograms representing the fluorescence distribution were obtained from a programmed number of neurons (1.5×10^3 to 3×10^3 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 an increase or a decrease in the $[\text{Ca}^{2+}]_i$ for fluo-3 and depolarization or hyperpolarization of membrane potential for di-BA- C_4 , respectively. The cytogram for the forward-angle light scatter versus the side-angle light scatter revealed a high density spot in the cell suspension. To examine the viability of dissociated neurons in the spot, acridine orange (Chroma-Gesellschaft, Ger-

many) was applied to the cell suspension at the concentration of 5×10^{-6} M (8). About 90% of the neurons in the spot seemed to be viable since they were stained with acridine orange.

TPT hydroxide (Kishida Chemical, Japan) was initially dissolved in dimethylsulfoxide, and the final concentrations of solvent was less than 0.1%. Solvent at such concentrations did not affect any measurement of oxonol and fluo-3 fluorescences. All chemicals except for fluorescent dyes and TPT were purchased from Wako Pure Chemical Industries, Japan.

TPT at concentrations less than 1×10^{-7} M did not exert any effect on the fluo-3 fluorescence profile of the neurons, indicating no change in the $[\text{Ca}^{2+}]_i$. Further increases in TPT concentration to 3×10^{-7} M or more (up to 1×10^{-5} M) augmented the fluo-3 fluorescence in a dose-dependent fashion (Fig. 1), indicating an increased $[\text{Ca}^{2+}]_i$ of neurons. The channel number was changed from 84.1 ± 31.0 to 91.9 ± 28.5 (mean $\pm$ S.D. of 3×10^3 cells) at 3×10^{-7} M (Fig. 1a), from 87.4 ± 31.3 to 104.8 ± 31.2 at 1×10^{-6} M (Fig. 1b), from 82.7 ± 31.2 to 112.0 ± 31.5 at 3×10^{-6} M (Fig. 1c) and from 84.1 ± 31.0 to 131.4 ± 28.6 at 1×10^{-5} M (Fig. 1d), Ionomycin, one of the Ca^{2+} ionophores, at the concentration of 2.5×10^{-6} M produced a further increase in the $[\text{Ca}^{2+}]_i$ by increasing the mean channel number to 146.6 ± 37.7 (Fig. 1e).

TPT was found to increase I_{Na} and decrease the transient and delayed K^+ currents of dissociated mammalian CNS neurons as shown in our previous study (1). In fact, the plateau of the action potential in the dissociated hippocampal pyramidal neurons was greatly prolonged in the presence of TPT (Y. Oyama, submitted for publication). Therefore, it is reasonable to suggest that TPT increases the $[\text{Ca}^{2+}]_i$ through the voltage-dependent Ca^{2+} channels during the depolarization. Therefore, the effect of TPT on the membrane potential (oxonol fluorescence) was studied (Fig. 2). TPT at the concentrations of 1×10^{-6} M or less did not affect the oxonol fluorescence pro-

Fig. 1. Effect of triphenyltin (TPT) on the fluo-3 fluorescence profile in 3×10^3 neurons. Histograms represent the fluorescence distribution in the neurons. Fluorescence intensity was resolved into 255 channels as shown in the abscissa. According to the tentative calibration method described by Kao et al. (5), the channel numbers of approximately 40, 100 and 135 correspond to 1×10^{-8} M, 1×10^{-7} M, and 1×10^{-6} M Ca^{2+} , respectively. Values at the ordinate correspond to the number of neurons at each channel. Changes to higher or lower channels correspond to an increase or a decrease in the $[\text{Ca}^{2+}]_i$, respectively. Thin and thick (with arrow) line histograms were obtained in the control and 10 min after the following applications: (a) 3×10^{-7} M TPT, (b) 1×10^{-6} M TPT, (c) 3×10^{-6} M TPT, (d) 1×10^{-5} M TPT, (e) 2.5×10^{-6} M ionomycin used as a reference, and (f) 3×10^{-6} M TPT in the presence of 1×10^{-5} M CD-349, a Ca^{2+} antagonist. Figure shows a representative one from three experiments.

Fig. 2. Effect of TPT on the oxonol fluorescence profile of neurons (3×10^3 cells) was examined at 10 min after the following applications: (a) 1×10^{-1} M KCl as a reference, (b) 1×10^{-6} M TPT, (c) 3×10^{-6} M TPT, and (d) 1×10^{-5} M TPT. Thin and thick (with arrow) line histograms were obtained, respectively, in the control and the presence of TPT or high K^+ concentration. Histograms represent the fluorescence distribution in the neurons. Fluorescence intensity was resolved into 255 channels as shown in the abscissa. Values at the ordinate correspond to the number of neurons at each channel. Changes to higher or lower channels correspond to depolarization or hyperpolarization, respectively. The depolarization induced by 3×10^{-6} M TPT was similar to that induced by 2×10^{-2} M KCl. The figure shows a representative one from three experiments.

file, indicating no effect on the membrane potential of neurons. When the TPT concentration was increased to 3×10^{-6} M or more, the oxonol fluorescence increased in a time-dependent manner and reached a steady-state level within 20 min after application of TPT. The neurons were found to depolarize in a dose-dependent manner by an increased intensity of the oxonol fluorescence from 85.4 ± 26.7 to 111.4 ± 32.4 (mean number of channel $\pm$ S.D. of 3×10^3 cells) at 3×10^{-6} M (Fig. 2c) and from 87.5 ± 27.9 to 166.4 ± 19.7 at 1×10^{-5} M (Fig. 2d). Thus, TPT increased the $[Ca^{2+}]_i$ without affecting the membrane potential at the concentrations of 3×10^{-7} M and 1×10^{-6} M. Furthermore, the increase in $[Ca^{2+}]_i$ by TPT was not affected by 1×10^{-5} M CD-349, a Ca^{2+} -antagonist (9). In a preliminary study, TPT at the concentrations of 1×10^{-6} M or more decreased the voltage-dependent Ca^{2+} currents (Y. Oyama, unpublished observation). From these results, it can be concluded that the increase in $[Ca^{2+}]_i$ by TPT is not mediated through voltage-dependent Ca^{2+} channels.

In order to reveal the source of the increased $[Ca^{2+}]_i$ by TPT, the effect of 3×10^{-6} M TPT on the fluo-3 fluorescence profiles was examined in nominally external Ca^{2+} -free ($[Ca^{2+}]_e$ -free) solution where $CaCl_2$ was omitted from Tyrode's solution and 2×10^{-3} M EGTA was added. TPT also increased the fluo-3 fluorescence from 124.8 ± 32.7 to 139.7 ± 36.1 (mean channel number $\pm$ S.D. in 1.5×10^3 cells) under the nominally $[Ca^{2+}]_e$ -free condition, although the degree of increase in the channel number under the nominally $[Ca^{2+}]_e$ -free condition was less than those obtained at $[Ca^{2+}]_e$ of 2×10^{-3} M and 1×10^{-2} M, where the mean channel number changed from 132.0 ± 35.1 to 153.7 ± 36.0 and from 139.3 ± 35.1 to 197.0 ± 6.3 , respectively.

The present results reveal that TPT increases the $[Ca^{2+}]_i$ at concentrations ranging from 3×10^{-7} M to 1×10^{-5} M, as found in thymocytes (2). The level of resting $[Ca^{2+}]_i$ is maintained at concentrations below 1×10^{-7}

M by intracellular organelle and the cell membrane (10), since the $[Ca^{2+}]_i$ is well-known to be linked to a variety of cellular functions. Therefore, unless an increased $[Ca^{2+}]_i$ is restored to the resting level, it would initiate a pathological state and/or disturb the intracellular homeostasis. Organotins including TPT were reported to inhibit ATP formation in mitochondria at the hundred nanomolar range (11, 12), resulting in an increase in the mismatch between an increased $[Ca^{2+}]_i$ and the energy state for the active $[Ca^{2+}]_i$ -restoring system. If so, the abnormally-increased $[Ca^{2+}]_i$ by TPT would have important implications on the neurotoxicity of organotins (13).

The source of an increased $[Ca^{2+}]_i$ by TPT is another subject of concern in this study. It is plausible to suggest that TPT induces indirectly the release of Ca^{2+} from intracellular Ca^{2+} stores because of the suppression of ATP formation by TPT. This possibility is partly supported by the result that TPT increased the $[Ca^{2+}]_i$ under the nominally $[Ca^{2+}]_e$ -free condition. Even so, all of the increased $[Ca^{2+}]_i$ is not dependent on the intracellular Ca^{2+} stores in the presence of $[Ca^{2+}]_e$ because TPT produced a further increase in the $[Ca^{2+}]_i$ as the $[Ca^{2+}]_e$ increased. However, it is unlikely that an increased $[Ca^{2+}]_i$ by TPT was carried through the voltage-dependent Ca^{2+} channels, because the increase in the $[Ca^{2+}]_i$ was observed at the TPT concentrations where TPT did not affect the membrane potential (Fig. 2b). Furthermore, TPT could produce a sustained increase of the $[Ca^{2+}]_i$ in the presence of a dihydropyridine-type Ca^{2+} -antagonist, CD-349. Therefore, at present, the mechanism for the $[Ca^{2+}]_e$ -dependent increase in the $[Ca^{2+}]_i$ by TPT is not fully elucidated. It can be, however, speculated that TPT possesses an action similar to that of ionomycin, a Ca^{2+} ionophore.

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