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Effect of triclocarban on membrane potential of rat thymocytes: Assessment with *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol

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Abstract The effect of triclocarban (TCC), an environmental pollutant from household items and health care products, on membrane potential of rat thymocytes was examined by a flow cytometry with a fluorescent probe sensitive to membrane potential, *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol, because TCC changes intracellular ionic circumstance that may affect the membrane potential. TCC at 0.3 μM or more (up to 3 μM) depolarized the membranes. This TCC-induced phenomenon was against our prediction because TCC increases intracellular Ca^{2+} concentration that activates Ca^{2+}-dependent K^+ channels, resulting in a hyperpolarization. The depolarization was still observed under Ca^{2+}-free condition, but not under Na^+-free condition. Furthermore, TCC hyperpolarized the membranes under Ca^{2+}- and Na^+-free condition. To see if TCC inhibits Ca^{2+}-dependent hyperpolarization, the effect of A23187, a calcium ionophore, on the membrane potential was examined in the cells treated with TCC. A23187 induced large depolarization in the cells treated with 0.3–3 μM TCC. The A23187-induced depolarization in the presence of TCC was greatly attenuated under Na^+-free or Ca^{2+}-free condition whereas A23187 elicited hyperpolarization in the cells treated with 0.3–3 μM TCC under Ca^{2+}- and Na^+-free condition. Results suggest that 0.3–3 μM TCC increases membrane permeability of Na^+ and Ca^{2+}, resulting in the depolarization. Large depolarization induced by TCC in the presence of external Ca^{2+} and Na^+ may mask the hyperpolarization elicited *via* the increase in intracellular Ca^{2+} concentration by TCC. Thus, there is a possibility that TCC depolarizes membranes of lymphocytes, resulting in alteration of cellular functions of lymphocytes.

Keywords: triclocarban; thymocytes; membrane potential; flow cytometer

1. Introduction

Triclocarban (TCC) is widely used as an antibacterial agent in a number of common household items including pharmaceutical and personal care products (Perencevich et al., 2001). Significant amount of triclocarban is found in terrestrial and aquatic environment (Heidler and Halden, 2008). The widespread use of household items containing triclocarban has raised concerns regarding the compound's impacts on the environment and human health (Snyder et al., 2011; Snyder and O'Connor, 2013). Therefore, in the future, it may be necessary to examine the immune functions of humans or animals exposed to TCC in residential areas.

Cell membrane potential is regulated by membrane ion permeability and transmembrane ion gradient. The membrane potential would be changed if chemicals affect these factors. The change in membrane potential seems to be associated with some functions of lymphocytes because of following observations. The exposure of lymphocytes to mitogenic lectins or antibodies changes membrane potential (Gallin and Livengood, 1981; Tsien et al., 1982; DeCoursey et al., 1984). The alteration of membrane potential affects the activation process of lymphocytes (Oettgen et al., 1985; Gelfand et al., 1987).

In our study (Miura et al., In submitted), TCC increased intracellular Ca^{2+} concentration of rat

thymocytes of which the membranes possess Ca^{2+}-activated K^+ channels (Wilson and Chused, 1985). Therefore, the manipulation of lymphocyte membrane potential by TCC may be one of possible events in the immunotoxic action. However, there is no analysis on the effect of TCC on membrane potential of lymphocytes. In this study, we examined the effect of TCC on membrane potential of rat thymocytes using a flow cytometer with *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol (oxonol) and propidium iodide.

2. Materials and methods

2.1. Chemicals

TCC was purchased from Wako Pure Chemicals (Osaka, Japan). TCC (0.03–3 mM) was dissolved in distilled water and added to achieve final concentrations of 0.03–3 μM in the cell suspension. A23187 (Sigma Chemical Co., St. Louise, MO, USA), a calcium ionophore, was used to activate Ca^{2+}-dependent K^+ channels, resulting in hyperpolarization (Oyama et al., 1992). A23187 at 300 nM was reported to induce steady Ca^{2+}-dependent hyperpolarization in murine thymic lymphocytes (Oyama et al., 1992, Nishizaki et al., 2003). Charybdotoxin, a specific inhibitor of Ca^{2+}-activated K^+ channels, was obtained from Peptide Institute Inc., Protein Research Foundation (Osaka, Japan). Propidium iodide and *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol (oxonol) were purchased from Molecular Probe Inc. (Eugene, OR, USA).

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments in The University of Tokushima (No. 05279).

The procedure to prepare cell suspension was similar to that previously reported (Oyama et al., 1991; Chikahisa and Oyama, 1992). In brief, thymus glands dissected from ether-anesthetized Wistar rats were sliced at a thickness of 1 mm with razor under an ice-cold condition (1–4°C). The slices were triturated by gently shaking in normal Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3-7.4), Ca^{2+}-free Tyrode's solution (in mM: NaCl 150, $MgCl_2$ 1, EDTA 2), or Na^+-free Tyrode's solution (in mM: choline chloride 150, $CaCl_2$ 2, $MgCl_2$ 1) to dissociate thymocytes. Thereafter, Tyrode's solutions containing the cells were passed through a mesh to prepare the cell suspension (about 5×10^5 cells/ml). The cell suspension was incubated at 36°C for 60 min before any fluorescence measurements.

2.3. Fluorescence measurements of cellular and membrane parameters

Experimental method was similar to that previously described (Oyama et al., 1991, 1992, 1995; Chikahisa and Oyama, 1992). In brief, the measurement of membrane potential was made with oxonol (Rink et al., 1980; Wilson and Chused, 1985). Fluorescent measurements were performed with a flow cytometer equipped with an argon laser (Cyto-ACE 150, JASCO, Tokyo, Japan).

To monitor the change in membrane potential of living cells with intact membranes, oxonol was used in the combination with propidium iodide for staining dead cells and/or the cells with compromised membranes (Chikahisa and Oyama, 1992). Propidium and oxonol were respectively dissolved in distilled water and dimethyl sulfoxide (DMSO). These solutions were added into the cell suspension to achieve final concentrations of 5 μM for propidium iodide and 300 nM for oxonol. Excitation wavelength for propidium and oxonol was 488 nm and the emissions were detected at 600 ± 20 nm for propidium fluorescence and 530 ± 20 nm for oxonol fluorescence. The oxonol fluorescence was measured from the cells that were not stained with propidium (living cells with intact membranes). Shifts toward increased and decreased fluorescent intensities correspond to depolarization and hyperpolarization of membrane potential, respectively.

2.4. Numerical expression and statistics

Statistical analysis was performed with Tukey multivariate analysis. A P value of < 0.05 was considered significant. Values were expressed as the mean ± standard deviation of 4–8 experiments.

3. Results

3.1. TCC-induced change in oxonol fluorescence

TCC significantly increased the intensity of Fluo-3 fluorescence, an indicator of intracellular Ca^{2+}, at 2 h after the start of TCC application (Miura et al., In submitted). To see if TCC affects membrane

potential, the cells were treated with respective concentrations (0.03–3 μM) of TCC for 2 h. As shown in Fig. 1, TCC at 0.3–3 μM augmented oxonol fluorescence, indicating TCC-induced depolarization. The augmentation of oxonol fluorescence by TCC was statistically significant. This phenomenon was against our expectation because the increase in intracellular Ca^{2+} concentration activated Ca^{2+}-dependent K^+ channels (Wilson and Chused, 1985), resulting in hyperpolarization. Therefore, to test if TCC inhibits the hyperpolarization *via* the activation of Ca^{2+}-dependent K^+ channels, the effect of A23187 on oxonol fluorescence of the cells treated with and without TCC was examined.

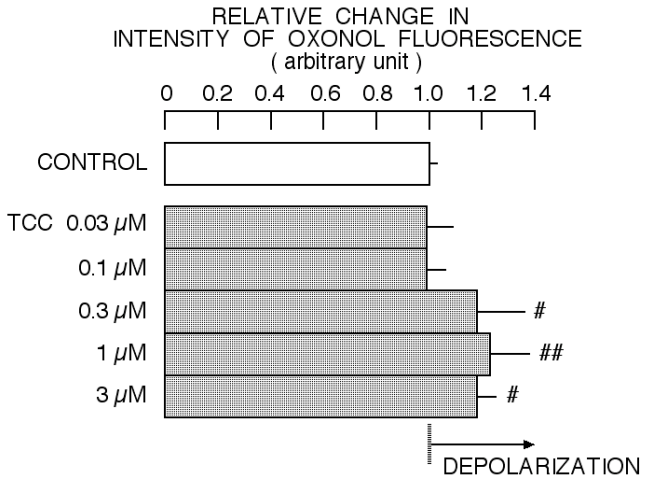

Fig. 1. Concentration-dependent change in intensity of oxonol fluorescence by TCC. Column and bar indicate mean and standard deviation of eight experiments. Symbols (#, ##) show significant difference ($P < 0.05$, 0.01) between control group (CONTROL) and the group of TCC-treated cells.

The application of 300 nM A23187 greatly attenuated oxonol fluorescence and the attenuation completely disappeared in the presence of 300 nM charybdotoxin, a specific inhibitor of Ca^{2+}-activated K^+ channels (Fig. 2). A23187 greatly augmented oxonol fluorescence of cells treated with 0.3–3 μM TCC (Fig. 3). There were two possibilities for A23187-induced increase in the intensity of oxonol fluorescence. One was that the depolarization by A23187 masked the hyperpolarization by A23187 in the presence of TCC. Another was that TCC completely suppressed the hyperpolarization by A23187 and TCC further depolarized membranes in the presence of A23187.

3.2. TCC-induced change in oxonol fluorescence under Ca^{2+}-free and/or Na^+-free condition

To test the possibilities described above, the effects of TCC and A23187 on oxonol fluorescence were examined under Ca^{2+}-free and/or Na^+-free condition. TCC increased the intensity of oxonol fluorescence under control condition and under Ca^{2+}-free condition while it was not the case under Na^+-free condition (Fig. 4), indicating the TCC-induced depolarization was Na^+-dependent. TCC decreased the intensity of oxonol fluorescence under Ca^{2+}-free and Na^+-free condition (Fig. 4). This result indicated that TCC hyperpolarized the membranes under Ca^{2+}-free and Na^+-free condition. A23187-induced attenuation of oxonol fluorescence in the absence of TCC was significantly reduced under Ca^{2+}-free condition and under Ca^{2+}-free and Na^+-free condition (Fig. 5).

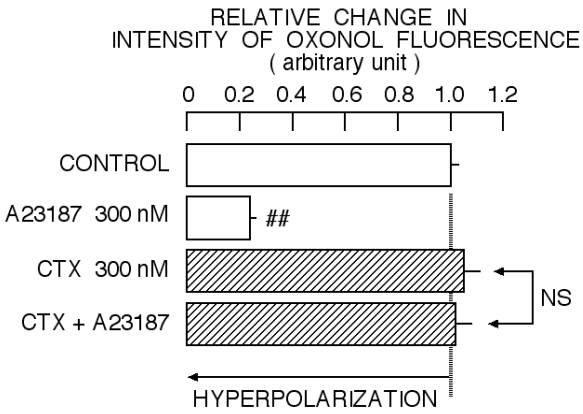

Fig. 2. Effect of charybdotoxin (CTX) on A23187-induced attenuation of oxonol fluorescence. The intensity of oxonol fluorescence attenuated by A23187 is assumed to be near an equilibrium potential for K^+ (Wilson and Chused, 1985). Column and bar indicate mean and standard deviation of four experiments. Symbol (##) indicates significant difference ($P < 0.01$) between control group (CONTROL) and test group. NS shows no significant difference between the groups arrowed.

A23187-induced augmentation of oxonol fluorescence in the presence of TCC was reduced under Ca^{2+}-free condition, under Na^+-free condition, and under Ca^{2+}-free and Na^+-free condition (Fig. 5), indicating possible involvement of both Ca^{2+} and Na^+.

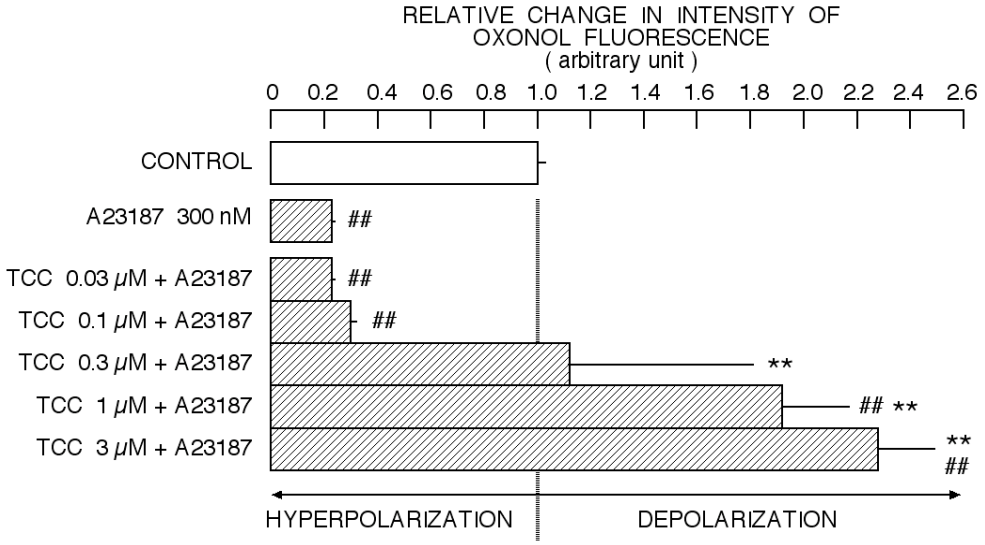

Fig. 3. Effect of A23187 on oxonol fluorescence monitored from the cells treated with 0.03–3 μM TCC. Column and bar show mean and standard deviation of eight experiments. Symbol (##) indicates significant difference ($P < 0.01$) between control group (CONTROL) and the group of TCC-treated cells. Asterisk (**) shows significant difference ($P < 0.01$) between the group of cells treated with A23187 alone and that of cells treated with TCC and A23187.

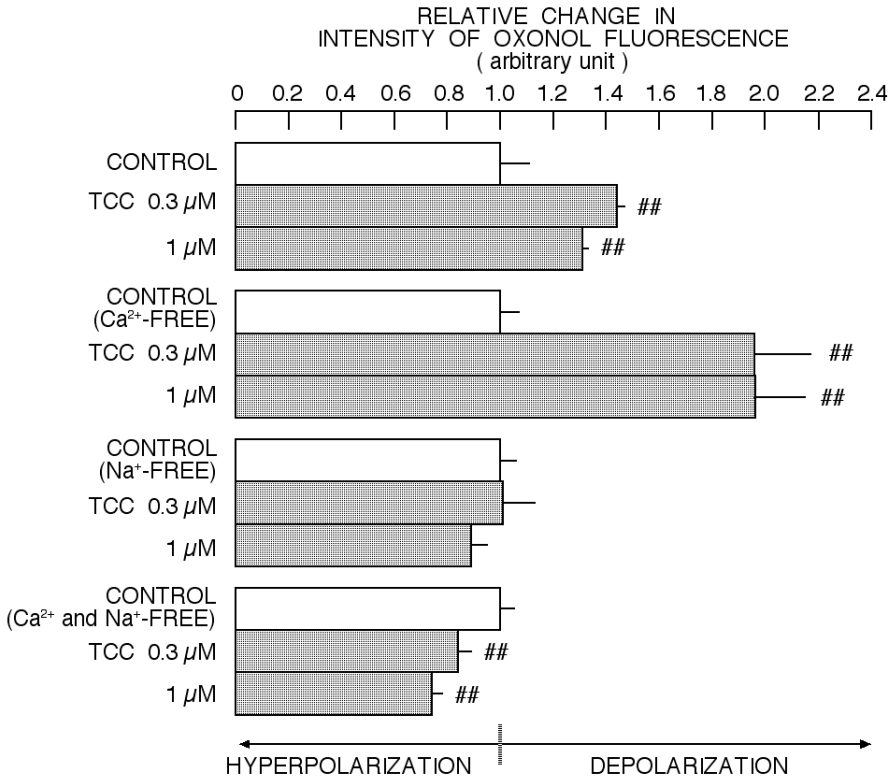

Fig. 4. TCC-induced change in oxonol fluorescence under Ca^{2+}-free and Na^{+}-free conditions. Column and bar indicate mean and standard deviation of four experiments. Symbol (##) indicates significant difference ($P < 0.01$) between control group (CONTROL) and the group of cells treated with TCC.

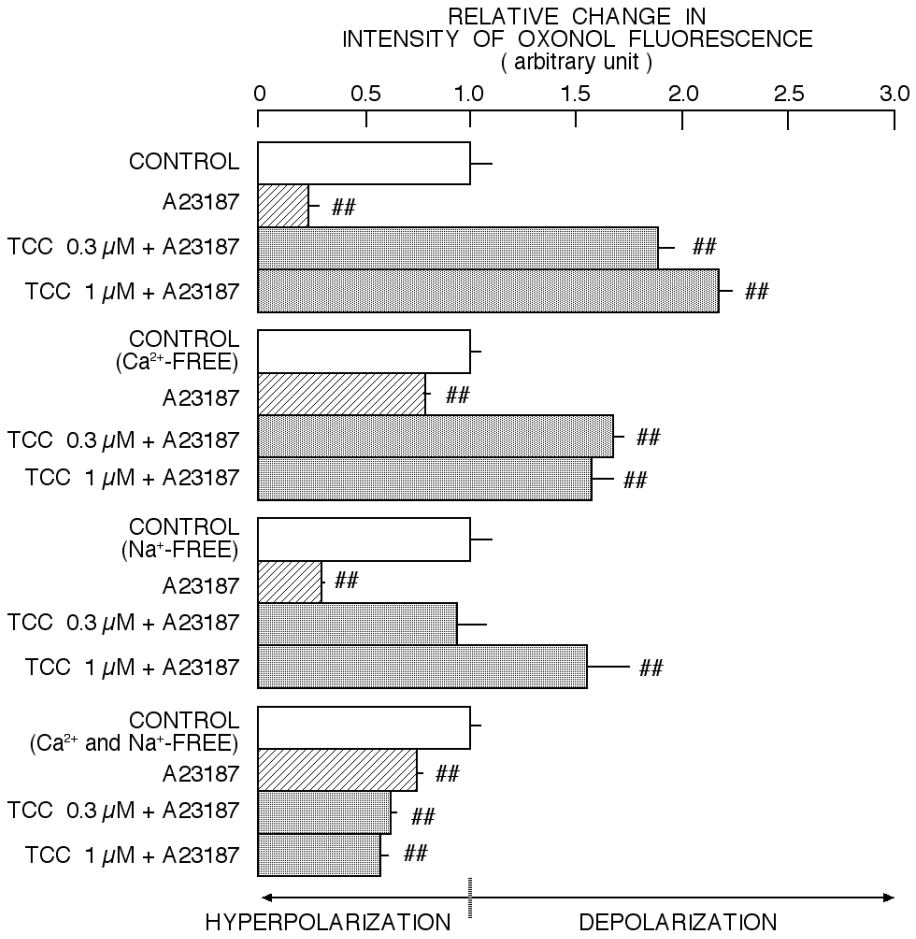

Fig. 5. Effect of A23187 on oxonol fluorescence monitored from the cells treated with TCC under Ca^{2+}-free and Na^{+}-free conditions. Column and bar show mean and standard deviation of eight experiments. Symbol (##) indicates significant difference ($P < 0.01$) between control group (CONTROL) and the groups of A23187-treated cells in absence and presence of TCC.

4. Discussion

4.1. Concentration of TCC

Shifts toward increased and decreased intensities in oxonol fluorescence correspond to depolarization and hyperpolarization of membrane potential, respectively. TCC at 0.3 μM started to depolarize the membranes of rat thymocytes (Fig. 1). TCC was detected in blood sampled from human subjects after showering with soap containing TCC (Schebb et al., 2012), with maximum blood concentrations ranging between 0.023 μM and 0.53 μM. Thus, the threshold concentration of TCC to change membrane potential in this study seems to be human relevant.

4.2. Effect of TCC on membrane potential and its mechanism

The depolarization induced by TCC was dependent on the increase in membrane Na^{+} permeability because of a following reason. As shown in Fig. 4, the depolarization by TCC was observed under control condition and Ca^{2+}-free condition, but not under Na^{+}-free condition and under Ca^{2+}-free and Na^{+}-free condition. Thus, Na^{+} was required for the TCC-induced depolarization. The influence of Ca^{2+} on TCC-induced depolarization seemed to be complicated. By removal of Ca^{2+}, TCC further depolarized the membranes in the presence of Na^{+} and further hyperpolarized the membranes in the absence of Na^{+} (Fig. 4). Ca^{2+} controls membrane Na^{+} permeability and the membrane Na^{+} permeability

is increased by the removal of external Ca^{2+} (Schulz and Heil, 1979; Skulskii et al., 1991). The TCC-induced increase in membrane Na^{+} permeability may be more profound under Ca^{2+}-free condition. Under Ca^{2+}-free and Na^{+}-free condition, the membrane ionic permeability to depolarize the membranes is largely inhibited. TCC hyperpolarized the membranes under Ca^{2+}-free and Na^{+}-free condition (Fig. 4). TCC released Ca^{2+} from intracellular calcium store of rat thymocytes, resulting in the increase in intracellular Ca^{2+} concentration (In submitted, Miura et al.). Murine thymocytes possess Ca^{2+}-activated K^{+} channels (Wilson and Chused, 1985). If TCC increases intracellular Ca^{2+} concentration of rat thymocytes, the membranes would hyperpolarize *via* the activation of K^{+} channels.

A23187 induced hyperpolarization under control condition and under Na^{+}-free condition more profoundly than that under Ca^{2+}-free condition and under Ca^{2+}-free and Na^{+}-free condition (Fig. 5). The increase in intracellular Ca^{2+} concentration by A23187 in the presence of external Ca^{2+} is much greater than that in the absence of external Ca^{2+} (Chikahisa and Oyama, 1992; Oyama et al., 1992). A23187 induced depolarization in the presence of TCC under control condition and under Ca^{2+}-free condition (Fig. 5). Under Na^{+}-free condition, the membrane potential of cells treated with TCC and A23187 was much more positive than that of cells treated with A23187 alone. Thus, A23187 induced depolarization in the cells treated with TCC under Na^{+}-free condition. This result suggests that A23187 may increase non-specific membrane permeability of cells treated with TCC under Na^{+}-free condition, resulting in the depolarization. Removal of external Ca^{2+} and Na^{+} also revealed the hyperpolarization elicited by A23187 in the presence of TCC (Fig. 5). The effects of TCC on membrane ionic permeability affecting membrane potential in lymphocytes may be summarized as follows. First, TCC increases membrane permeability of Na^{+} and Ca^{2+}, resulting in depolarization. Second, TCC also increases membrane K^{+} permeability *via* the activation of Ca^{2+}-dependent K+ channels. The increase in membrane Na^{+} and Ca^{2+} permeability by TCC is greater than that in membrane K^{+} permeability. Therefore, the depolarization by TCC conceals the hyperpolarization.

4.3. Toxicological implication

The concentration (0.3 μM) of TCC to affect membrane potential of rat thymocytes is among the concentrations detected in human blood samples (Schebb et al., 2012). The alteration of membrane potential affects the activation process of lymphocytes (Gallin and Livengood, 1981; Tsien et al., 1982; DeCoursey et al., 1984; Oettgen et al., 1985; Gelfand et al., 1987). Therefore, TCC may disturb physiological changes in lymphocyte membrane potential, possibly resulting in malfunction of immune reaction in human.

Conflict of interest

We have no conflicts of interests to declare.

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