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Triclosan, an environmental pollutant from health care products, evokes charybdotoxin-sensitive hyperpolarization in rat thymocytes

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ABSTRACT

The effects of triclosan, an environmental pollutant from household items and health care products, on membrane potential and intracellular Ca^{2+} concentrations of rat thymocytes were examined by a flow cytometry with fluorescent probes, di-BA-C_4 and fluo-3-AM, because triclosan is often found in humans and wild animals. Triclosan at a concentration of 3 μM decreased the intensity of di-BA-C_4 fluorescence, indicating the triclosan-induced hyperpolarization. The application of charybdotoxin, a specific inhibitor of Ca^{2+}-dependent K^+ channels, and the removal of external Ca^{2+} eliminated the triclosan-attenuation of di-BA-C_4 fluorescence. Furthermore, triclosan augmented the fluo-3 fluorescence under normal Ca^{2+} condition, indicating that triclosan increased intracellular Ca^{2+} concentration. These results suggest that triclosan induces membrane hyperpolarization by increasing intracellular Ca^{2+} concentration that activates Ca^{2+}-dependent K^+ channels. Since the change in membrane potential of lymphocytes influence cellular immune functions, triclosan may exert adverse actions on immune system in human and wild animals.

1. Introduction

Triclosan is widely used as an antibacterial agent in a number of common household items including pharmaceutical and personal care products (Perencevich et al., 2001; Schweizer, 2001). Significant amount of triclosan is found in terrestrial and aquatic environment (Reiss et al., 2002; Heidler and Halden, 2008). The widespread use of household items containing triclosan has raised concerns regarding the compound's impacts on the environment and human health (Rodricks et al., 2010). Triclosan inhibits plant growth and soil respiration (Liu et al., 2009). This bactericidal agent increases the rate of metamorphosis and tail fin gene expression in North American bullfrog (Veldhoen et al., 2006). Furthermore, its structural resemblance with non-steroidal estrogens makes the compound a probable endocrine disruptor (Zorrilla et al., 2009; James et al., 2010). Although the metabolic activity of triclosan is not fully understood in humans, several studies have demonstrated probable biotransformation pathways (Fang et al., 2010). Recently, triclosan is considered to exert adverse actions on human immune functions (Clayton et al., 2011). Therefore, it is necessary to investigate the effect of triclosan on lymphocytes in order to reveal cellular basis of immunotoxicity of triclosan.

The membrane potential is controlled by membrane ion permeability and transmembrane ion gradient that are influenced by diverse factors. Therefore, the membrane potential would be changed if the chemicals including environmental pollutants affect one of diverse factors. The changes in membrane potential are associated with physiological functions of lymphocytes because of the following observations.

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The exposure of lymphocytes to mitogenic lectins or antibodies induces the change in membrane potential (Gallin and Livengood, 1981; Tsien et al., 1982; DeCoursey et al., 1984; Gallin, 1986; Gelfand et al., 1987). The alteration of membrane potential affects the activation process of lymphocytes and monocytes (Oettgen et al., 1985; Gelfand et al., 1987). Thus, the manipulation of membrane potential by triclosan may be one of events in the immunotoxic action. However, there is no analysis on the effect of triclosan on membrane potential of lymphocytes. In this study, we have examined the effect of triclosan on membrane potential of rat thymocytes using a flow cytometer with appropriate fluorescent indicators.

2. Materials and methods

2.1. Chemicals

Triclosan was purchased from Wako Pure Chemicals (Osaka, Japan). Triclosan (0.3–10 mM) was dissolved in distilled water and added to achieve final concentrations of 0.3–10 μM in the cell suspension. A23187, a calcium ionophore, was used as a reference agent to increase intracellular Ca^{2+}. A23187 induces a hyperpolarization in rat thymocytes by an activation of Ca^{2+}-dependent K^+ channels (Oyama et al., 1992). Charybdotoxin, a specific inhibitor of Ca^{2+}-dependent K^+ conductance, were products of Peptide Institution (Osaka, Japan). Other chemicals except for fluorescent probes were purchased from Wako Pure Chemicals (Osaka, Japan). Fluorescent probes were purchased from Molecular Probe Inc. (Eugene, Oregon, USA).

A23187 at concentrations ranging from 30 nM to 300 nM was reported to induce steady Ca^{2+}-dependent hyperpolarization in murine thymic lymphocytes (Oyama et al., 1992; Nishizaki et al., 2003). Therefore, 100 nM A23187 was used to elicit the hyperpolarization via activation of Ca^{2+}-dependent K^+ channel.

Charybdotoxin is a specific inhibitor for Ca^{2+}-dependent K^+ channels (Miller, 1995) and it was reported that the toxin at 30 nM or more strongly suppressed the hyperpolarization induced by ionomycin, a calcium ionophore, in rat thymocytes (Grinstein and Smith, 1989). Nishizaki et al. (2003) also showed the inhibition of A23187-induced attenuation of di-BA-C_4 fluorescence (hyperpolarization) by micromolar charybdotoxin. Therefore, 300 nM charybdotoxin was used in this study.

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 400–500 μm 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) or Ca^{2+}-free Tyrode's solution (in mM: NaCl 150, KCl 5, $MgCl_2$ 2, EDTA 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3–7.4) 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 45–60 min before any fluorescence measurements.

2.3. Fluorescence measurements of cellular and membrane parameters

Experimental methods were similar to those previously described (Oyama et al., 1991, 1992, 1995; Chikahisa and Oyama, 1992). In brief, the measurements of membrane potential and intracellular concentration of Ca^{2+} were made with *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol (di-BA-C_4) (Rink et al., 1980; Wilson and Chused, 1985) and pentaacetoxymethyl ester of fluo-3 (fluo-3-AM) (Kao et al., 1989; Minta et al., 1989), respectively. 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, di-BA-C_4 was used in the combination with propidium iodide for staining dead cells and/or the cells with compromised membranes (Chikahisa and Oyama, 1992). Di-BA-C_4 and propidium iodide were respectively dissolved in dimethyl sulfoxide (DMSO) and distilled water. These solutions were added into the cell suspension to achieve a final concentration of 300 nM for di-BA-C_4 and 5 μM for propidium iodide. The cells were incubated with di-BA-C_4 for 10 min and propidium iodide for 2 min before any fluorescence measurements. Excitation wavelength for di-BA-C_4 and propidium was 488 nm and the emissions were detected at 530 ± 20 nm for di-BA-C_4 fluorescence and 600 ± 20 nm for propidium fluorescence. Di-BA-C_4 fluorescence was measured from the cells that were not stained with propidium (living cells with intact membranes). To estimate the change in intracellular Ca^{2+} concentration of rat thymocytes, fluo-3-AM was used. Fluo-3-AM was also dissolved in DMSO. The cells were incubated with 500 nM fluo-3-AM for 60 min before any fluorescence measurements. Fluo-3 fluorescence was also measured from the cells that were not stained with 5 μM for propidium iodide. Excitation wavelength for fluo-3 was 488 nm and the emission was detected at 530 ± 20 nm. Shifts toward increased and decreased fluorescent intensities, correspond to depolarization and hyperpolarization of membrane potential for di-BA-C_4 fluorescence and increasing and decreasing intracellular Ca^{2+} concentration for fluo-3 fluorescence, respectively.

The fluorescence histogram was obtained from 2000 cells. The sheath flow rate was adjusted to set the measurement of 195–205 cells/s and the interval of 180 μs between measurements of forward and side scatters. The measurement started after achieving constant flow of cells. The time of about 10 s was required for data acquisition in the case of 2000 cells. The mean intensity of fluorescence obtained from 2000 cells was similar to that from 10,000 cells.

2.4. Numerical expression and statistics

Statistical analysis was performed with Tukey multivariate analysis. A P value of <0.05 was considered significant. Since there was no statistical difference in mean intensity of di-BA-C_4 and fluo-3 fluorescence between control groups (cells