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Hyperpolarization by N-(3-oxododecanoyl)-L-homoserine-lactone, a quorum sensing molecule, in rat thymic lymphocytes

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ABSTRACT

To study the adverse effects of N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL), a quorum sensing molecule, on mammalian host cells, its effect on membrane potential was examined in rat thymic lymphocytes using flow cytometric techniques with a voltage-sensitive fluorescent probe. As 3–300 μM ODHL elicited hyperpolarization, it is likely that it increases membrane K^+ permeability because hyperpolarization is directly linked to changing K^+ gradient across membranes, but not Na^+ and Cl^- gradients. ODHL did not increase intracellular Ca^{2+} concentration. ODHL also produced a response in the presence of an intracellular Zn^{2+} chelator. Thus, it is unlikely that intracellular Ca^{2+} and Zn^{2+} are attributed to the response. Quinine, a non-specific K^+ channel blocker, greatly reduced hyperpolarization. However, because charybdotoxin, tetraethylammonium chloride, 4-aminopyridine, and glibenclamide did not affect it, it is pharmacologically hypothesized that Ca^{2+}-activated K^+ channels, voltage-gated K^+ channels, and ATP-sensitive K^+ channels are not involved in ODHL-induced hyperpolarization. Although the K^+ channels responsible for ODHL-induced hyperpolarization have not been identified, it is suggested that ODHL can elicit hyperpolarization in mammalian host cells, disturbing cellular functions.

1. Introduction

Quorum sensing (QS) molecules are used as signal mediators in bacterial cell-to-cell communication and synchronize biological events in a group [1,2]. QS molecules affect host cells on which bacteria grow [3]. In the aspect of cytotoxicity of QS molecules, N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL) disrupts membrane integrity in epithelial Caco-2 cells [4,5] and enters membranes of Jurkat T-cell lines [6]. ODHL inhibits cell differentiation [7] and induces apoptosis in lymphocytes [8]. Furthermore, ODHL treatment releases intracellular Ca^{2+} in mast cells [9]. Thus, it is toxicologically interesting to examine the cellular actions of ODHL on mammalian host cells.

Membrane potentials are regulated by membrane permeability and transmembrane gradients of respective ions, such as Na^+, K^+, and Cl^-. An important determinant of membrane potential is membrane K^+ permeability that is defined mainly by the opening and closing of K^+ channels. Changes in membrane potentials are associated with cellular physiological and pathological events, although various exogenous substances modulate membrane potentials. Mitogens cause an early change in membrane potential associated with a transient increase in intracellular Ca^{2+} concentration in T lymphocytes [10]. The proliferation of T lymphocytes is inhibited by charybdotoxin [11], a specific blocker of Ca^{2+}-activated K^+ channels [12]. Non-specific K^+ channel blockers inhibit B lymphocyte activation, resulting in an attenuation of the cell cycle [13]. There are several actions on lymphocytes [14–17]. Furthermore, membrane potential seems to function beyond channel proteins as phosphoinositide phosphatase activity is regulated within the physiological membrane potential range [18]. Thus, it is likely that the compounds affecting membrane potentials modify some physiological functions in lymphocytes.

We examined the effect of ODHL on membrane potential using flow cytometric techniques with a voltage-sensitive fluorescent dye in rat thymocytes. One may argue that membrane potential is not a target for the pathological (or toxicological) actions of ODHL; however, in a previous study [19], we found that ODHL elevated intracellular Zn^{2+} levels in rat thymic lymphocytes. It is known that intracellular divalent metal cations such as Ca^{2+}, Pb^{2+}, and Zn^{2+} activate K^+ channels, resulting in hyperpolarization [20–22]. If ODHL affects membrane potential in lymphocytes, it would disturb physiological functions of lymphocytes. With concerns about the effects of bacterial bioactive

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Table 1
Reagents used in this study.

A. Fluorescent probes	
Excitation wavelength was 488 nm for all fluorescent probes.	
Probe [Manufacturer]	Emission wavelength (nm)
Propidium iodide [Molecular Probes, Inc., Eugene, OR, USA]	600 ± 20
bis-(1,3-Dibutylbarbituric acid)trimethine oxonol (Oxonol) [Molecular Probes]	530 ± 20
Fluo-3-AM [Dojin Chemical, Kumamoto, Japan]	530 ± 20

B. Specific reagents	
Reagent [Manufacturer]	Purpose
Dimethyl sulfoxide (DMSO) [Wako Pure Chemical, Osaka, Japan]	Solvent
Tetraethylammonium chloride (TEA) [Tokyo Chemical Industry, Tokyo, Japan]	Blocker of slow K^+ channels
4-Aminopyridine (4-AP) [Wako Pure Chemical]	Blocker of transient K^+ channels
Quinine [Tokyo Chemical Industry]	K^+ channel blocker
Charybdotoxin (CTX) [Peptide Institute, Osaka, Japan]	Blocker of Ca^{2+}-activated K^+ Channels
N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) [Dojin Chemical]	Intracellular Zn^{2+} chelator
Concanavalin A [Wako Pure Chemical]	Mitogen

substances on human health, the changes in membrane potential of rat thymic lymphocytes by ODHL, a bacterial QS molecule, may give new toxicological insights.

2. Materials and methods

2.1. Chemicals

N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL) was purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). Fluorescent probes used to measure cellular parameters and specific reagents with their abbreviations are listed in Table 1. Other chemical reagents were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell preparation

Experiments were performed under the approval (T29-52) of Tokushima University Committee for Animal Experiments.

The cell suspension was prepared as follows. Thymus glands were excised from 6–8-week-old Wistar rats (Charles River Japan, Shizuoka, Japan) that were anesthetized with thiopental (Ravonal 50–75 mg/kg via intraperitoneal injection). Sliced glands were gently triturated in Tyrode's solution (2–4 °C) and the solution was filtered with a mesh (56 μm diameter). The solution containing thymocytes (cell suspension) was stored at 36–37 °C for 50–60 min before experiments.

ODHL (3–300 mM in 2 μL DMSO) was applied to the cell suspension (1.998 mL) to make final concentrations (3–300 μM). The cells were incubated with ODHL at respective concentrations for 10–60 min. The cell suspension (100 μL) was cytometrically analyzed. Data acquisition (2000 cells or 2500 cells) took approximately 8–13 s. Sheath flow rate was adjusted to measure 200–250 cells/sec with an interval of 180 μsec between forward and side scatter measurements.

2.3. Fluorescence measurement

We examined cell fluorescence using a flow cytometer equipped with a software (CytoACE-150; JASCO, Tokyo, Japan) [23]. Propidium iodide (PI) at 5 μM was used to examine cell lethality. Membrane potentials were monitored using 500 nM *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol (Oxonol) [20]. Decrease and increase in Oxonol fluorescence intensity indicate hyperpolarization and depolarization, respectively. PI and Oxonol fluorescence from the cells were recorded in continued presence of PI and Oxonol. Cells were incubated with 1 μM Fluo-3-AM for 50–60 min before the experiment to examine the action of ODHL on intracellular Ca^{2+} levels [24]. Fluo-3 fluorescence was measured from the cells treated with 10 μM *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a chelator of intracellular Zn^{2+}, to remove the contribution of Zn^{2+} to Fluo-3 fluorescence. Decrease and increase in Fluo-3 fluorescence intensity show decrease and increase in intracellular Ca^{2+} level, respectively. Oxonol and Fluo-3 fluorescence were recorded in cells that did not show PI fluorescence (i.e., living cells with intact membranes). The excitation and emission wavelengths for fluorescent probes are listed in Table 1.

2.4. Statistical analysis and presentation

Statistical analysis was carried out using Tukey's multivariate analysis. P-values < 0.05 were considered statistically significant. Each experimental series was conducted thrice, unless stated otherwise.

3. Results

3.1. Effect of ODHL on Oxonol fluorescence (membrane potential) of rat thymocytes

ODHL at 300 μM initially shifted the histogram of Oxonol fluorescence to lower intensity (hyperpolarizing direction) within 10 min after application, and then gradually moved the histogram to higher intensity (depolarizing direction) during the next 50 min (Fig. 1A). Fig. 2B shows the concentration-dependent change of the histogram by 10–300 μM ODHL, when the cells were treated with ODHL for 60 min. ODHL at 10 μM continued to shift the histogram to a hyperpolarizing direction. As shown in Fig. 2A, the mean intensity of Oxonol fluorescence in the cells treated with 30 μM ODHL continued to be lower than the control level 60 min after application. On the other hand, 100 μM ODHL reduced the mean intensity of Oxonol fluorescence at 10 min after application, and then gradually increased the mean intensity during the next 50 min (Fig. 2B). Therefore, the effects of ODHL at 30 μM or less on membrane potential may be different from those at 100 μM or more. Concentration-dependent changes in the mean intensity of Oxonol fluorescence by ODHL are summarized in Fig. 2C.

As shown in Fig. 1B, there were hyperpolarizing and depolarizing peaks in the histogram of Oxonol fluorescence monitored from the cells treated with 30–100 μM ODHL. It may be inadequate to use the mean intensity to compare the effects of different concentrations of ODHL on membrane potential. Therefore, the changes in population of hyperpolarized and depolarized cells by ODHL were examined. As shown in Fig. 3A, ODHL at 30 μM significantly increased the population of hyperpolarized cells after application. The population of depolarized cells time-dependently increased in the presence of 30 μM ODHL and the increases at 45–60 min after the application were statistically significant (Fig. 3A). When the concentration of ODHL was 100 μM, the population of hyperpolarized cells initially increased, but the rate of increase significantly reduced in a time-dependent manner (Fig. 3B); the increases at 45–60 min were also not statistically significant. In the case of depolarized cells, the increases at 20–60 min were statistically significant. The population of depolarized cells increased while the population of hyperpolarized cells decreased in the continued presence of ODHL. Changes in the population of hyperpolarized and depolarized cells because of ODHL at concentrations ranging from 3 μM to 300 μM are shown in Fig. 3C. The strong hyperpolarizing action of ODHL was observed when the concentration of ODHL was 10–30 μM.