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

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Highlights

- N-(3-Oxododecanoyl)-L-homoserine-lactone (ODHL) is a quorum sensing molecule.
- ODHL induced hyperpolarization in rat thymic lymphocytes.
- The hyperpolarization was inhibited by quinine, but not by other K^+ channel blockers.
- The hyperpolarization was due to an increase in membrane K^+ permeability.
- ODHL may disturb lymphocyte functions via changes in membrane potential.

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.

Keywords: N-(3-oxododecanoyl)-L-homoserine-lactone; lymphocytes; membrane potential; membrane K^+ permeability; K^+ channels

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 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).

(Table 1 near here)

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 sec. 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 (Figure 1A). Figure 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 Figure 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 (Figure 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 Figure 2C.

(Figures 1 and 2 near here)

As shown in Figure 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 Figure 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 (Figure 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 (Figure 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 Figure 3C. The strong hyperpolarizing action of ODHL was observed when the concentration of ODHL was 10–30 μM.

(Figure 3 near here)

3.2. Characteristics of ODHL-induced hyperpolarization

As shown in Figures 1–3, ODHL at lower concentrations reduced the intensity of Oxonol fluorescence (ODHL-induced hyperpolarization). Therefore, in this study, the property of ODHL-induced hyperpolarization was intensively examined. The cells were treated with 10 mM tetraethylammonium chloride (TEA), 5 mM 4-aminopyridine (4-AP), or 300 μM quinine for 30 min before the application of 30 μM ODHL to see if the ODHL-induced hyperpolarization is due to the activation of membrane K^+ channels; TEA did not affect the ODHL-induced hyperpolarization (Figure 4). ODHL-induced hyperpolarization was observed even in the presence of 4-AP. Quinine reduced hyperpolarization, although the treatment of quinine depolarized the membranes (Figure 4).

(Figure 4 near here)

To see if Ca^{2+}-activated K^+ channels are involved in ODHL-induced hyperpolarization, the effect of 300 nM charybdotoxin, a specific blocker of Ca^{2+}-activated K^+ channels, was examined. Charybdotoxin did not affect ODHL-induced hyperpolarization (Figure 5A) and ODHL did not elevate intracellular Ca^{2+} levels (Figure 5B). Glibenclamide (1–10 μM), a blocker of ATP-sensitive K^+ channels [25], did not affect the response. In a previous study [19], ODHL increased intracellular Zn^{2+} concentrations. Zn^{2+}-activated K^+ channels were reported in sea urchin spermatozoa [22]. To test the possibility that ODHL hyperpolarizes membranes via activation of Zn^{2+}-activated K^+ channels, the effect of ODHL was examined in the presence of TPEN. ODHL induced hyperpolarization even in the presence of 10 μM TPEN (Figure 5C).

(Figure 5 near here)

To confirm the involvement of K^+ channels, the effects of ODHL were examined under external ionic conditions. The addition of 50 mM KCl to the external solution depolarized the membranes and shifted ODHL-induced hyperpolarization, while the addition of 50 mM NaCl did not affect ODHL-induced hyperpolarization (Figure 5D).

Mitogens induce hyperpolarization by activating Ca^{2+}-activated K^+ channels in T cells [10]. The application of concanavalin A, a known mitogen, at 3–10 μM induced hyperpolarization at 10 min after application (Figure 6A), and then the membrane potential returned to regular levels within 60 min in the continued presence of concanavalin A (Figure 6B). Furthermore, there was no interaction between ODHL and concanavalin A.

(Figure 6 near here)

4. Discussion

It is likely that ODHL increases membrane K^+ permeability of rat thymic lymphocytes because of a variety of reasons. Changes in Na^+ and Cl^- gradients across the membranes did not affect ODHL-induced hyperpolarization, although the reduction of the K^+ gradient across the membranes depolarized the membranes and shifted ODHL-induced hyperpolarization (Figure 5D). Increases in external K^+ and Cl^- levels by adding 50 mM KCl shifted the equilibrium K^+ and Cl^- potentials to depolarizing and hyperpolarizing directions, respectively. The increase in external Na^+ level by adding 50 mM NaCl also moved the equilibrium potential to a depolarizing direction. Thus, it is concluded that K^+, but not Na^+ and Cl^-, is involved in ODHL-induced hyperpolarization.

As the blockers of K^+ channels such as TEA (Figure 4B), 4-AP, and charybdotoxin (Figure 5A) did not affect the ODHL response, voltage-dependent and Ca^{2+}-activated K^+ channels are irrelevant. ATP-sensitive K^+ channels are ruled out because glibenclamide, a blocker of ATP-sensitive K^+ channels [25], did not affect the ODHL response. As ODHL induced hyperpolarization in the presence of TPEN (Figure 5C), Zn^{2+}-dependent K^+ channels

[22] can be also ruled out. Only quinine greatly reduced the ODHL-induced hyperpolarization (Figures 4A and B). It is hard to pharmacologically identify the K^+ channels involved in ODHL-induced hyperpolarization using quinine because it inhibits many types of K^+ channels. Voltage-gated K^+ channels (Kv1.1, Kv1.2, Kv1.3, and Kv1.6 channels) are sensitive to TEA and 4-AP. Therefore, voltage-gated K^+ channels are excluded. One may argue the possibility that ODHL is a K^+ ionophore, but not an activator of K^+ channels; though, it is unlikely because quinine cannot antagonize the action of valinomycin, a K^+ ionophore [26]. Resting membrane potential is regulated by membrane K^+ permeability in mammalian cells. Increase and decrease in membrane K^+ permeability by K^+ ionophores [27,28] and K^+ channel blockers [11,13], respectively, disturb cell functions in lymphocytes. Such examples were shown in other studies [14–17]. Changes in membrane potential due to concanavalin A were masked in the simultaneous presence of ODHL (Figure 6). Therefore, ODHL can modify cellular functions of lymphocytes.

5. Conclusion

The threshold concentration of ODHL to hyperpolarize membranes of rat thymic lymphocytes was observed to be 3 μM (Figure 2C). Although the information on local concentrations of ODHL released from bacterial biofilms is unavailable, micromolar concentrations of ODHL are expected to affect the membranes of mammalian host cells.

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Figure legends

Figure 1. ODHL-induced changes in histogram of Oxonol fluorescence. Each histogram was constructed using 2500 living cells. (A) Time-dependent changes induced by 300 μM ODHL. (B) Concentration-dependent changes induced by 10–300 μM ODHL. Histogram was obtained at 60 min after the start of ODHL application. (C) Portions of depolarized and hyperpolarized cells in the histogram.

Figure 2. ODHL-induced changes in Oxonol fluorescence intensity. Column and bar show the mean and standard deviation of 4 samples, respectively. (A) Time-dependent change induced by 30 μM ODHL. (B) Time-dependent change induced by 100 μM ODHL. (C) Concentration-dependent change induced by 3–300 μM ODHL. Effects of ODHL were examined at 60 min after the start of ODHL application. Asterisks (*, **) indicate significant difference ($P < 0.05$, 0.01) between control intensity (extreme left column and dotted line) and the intensity of fluorescence from the cells treated with ODHL.

Figure 3. ODHL-induced changes in population of hyperpolarized and depolarized cells. Column and bar show the mean and standard deviation of 4 samples, respectively. (A) Time-dependent changes in the population by 30 μM ODHL. (B) Time-dependent changes in the population by 100 μM ODHL. (C) Concentration-dependent changes in the population by 3–300 μM ODHL. Effects of ODHL were examined at 60 min after the start of ODHL application. Asterisks (*, **) indicate significant difference ($P < 0.05$, 0.01) between control intensity and the intensity of fluorescence from the cells treated with ODHL.

Figure 4. Effects of TEA and quinine on the ODHL-induced attenuation of Oxonol fluorescence. (A) ODHL-induced changes in histogram of Oxonol fluorescence in the presence of TEA and

quinine. Each histogram was constructed with 2500 living cells. Effects were examined at 10 min after the start of ODHL application. (B) ODHL-induced changes in the intensity of Oxonol fluorescence in the presence of TEA and quinine. Asterisk (**) indicates significant difference ($P < 0.01$) between control intensity (extreme left column and dotted line) and the intensity of fluorescence from the cells treated with TEA and quinine. Symbol (##) shows significant difference between control group and the group of cells treated with 30 μM ODHL.

Figure 5. Ionic dependence of ODHL-induced attenuation of Oxonol fluorescence. Column and bar show the mean and standard deviation of 4 samples, respectively. (A) Effect of CTX on the ODHL-induced response. Asterisk (**) indicates significant difference ($P < 0.01$) between control cell group and ODHL-treated cell group. (B) Effect of ODHL on Fluo-3 fluorescence in the presence of TPEN. Pound (##) shows significant difference ($P > 0.01$) between the ODHL-treated cell groups without and with CTX. (C) The ODHL-induced response in the presence of TPEN. Asterisk (**) indicates significant difference ($P < 0.01$) between control cell group and ODHL-treated cell group. (D) Effects of NaCl and KCl on the ODHL-induced response. Asterisk (**) indicates significant difference ($P < 0.01$) between control cell group and ODHL-treated cell group. Pound (##) shows significant difference ($P > 0.01$) between the ODHL-treated cell groups.

Figure 6. Changes of Oxonol fluorescence intensity by ODHL, concanavalin, and their combination. Effects were examined at 10 min (A) and 60 min (B) after the drug application. Column and bar show mean and standard deviation of 4 samples, respectively. Asterisk (**) indicates significant difference ($P < 0.01$) between control intensity (leftmost column and dotted line) and the intensity of fluorescence from the cells treated with the agent(s).

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 voltage-gated 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

Figure 1

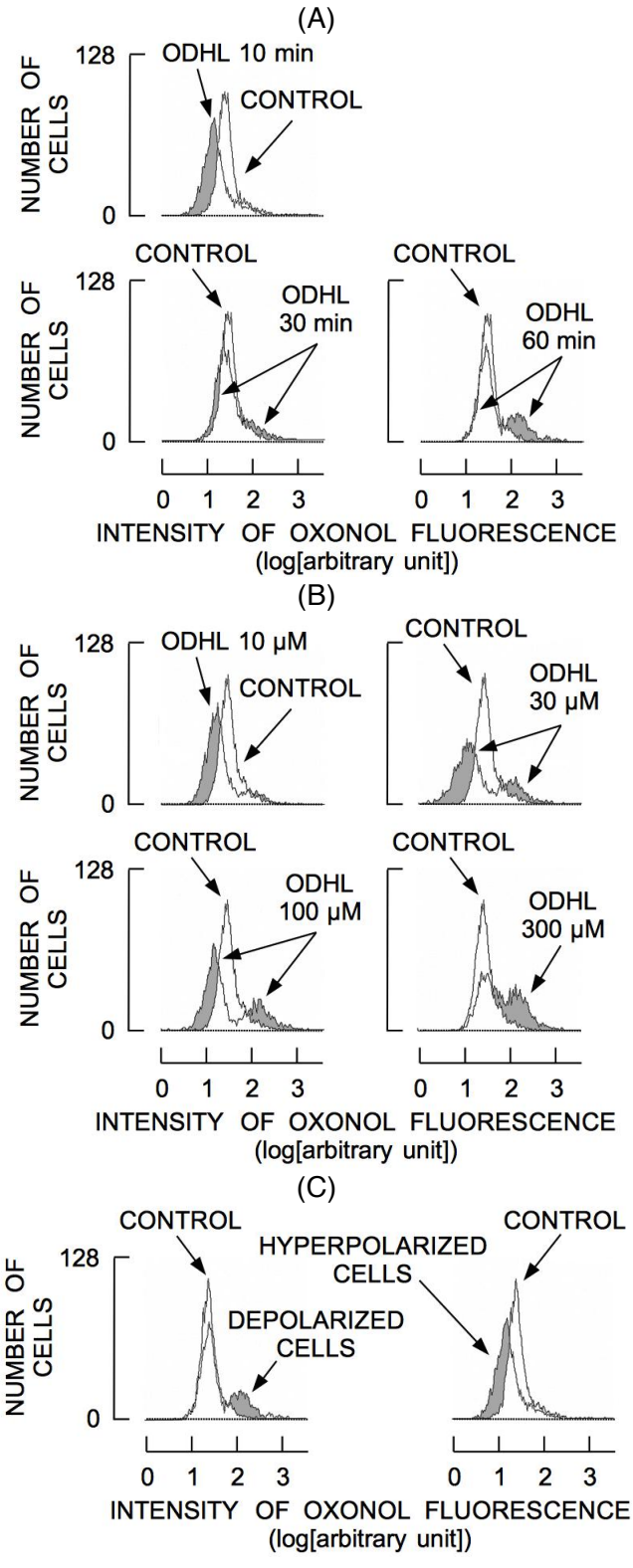

(A)
NUMBER OF CELLS
128
0
ODHL 10 min
CONTROL
CONTROL
ODHL 30 min
CONTROL
ODHL 60 min
0 1 2 3
0 1 2 3
INTENSITY OF OXONOL FLUORESCENCE
(log[arbitrary unit])
(B)
ODHL 10 μM
CONTROL
CONTROL
ODHL 30 μM
CONTROL
ODHL 100 μM
CONTROL
ODHL 300 μM
INTENSITY OF OXONOL FLUORESCENCE
(log[arbitrary unit])
(C)
CONTROL
HYPERPOLARIZED CELLS
CONTROL
DEPOLARIZED CELLS
INTENSITY OF OXONOL FLUORESCENCE
(log[arbitrary unit])

Figure 2

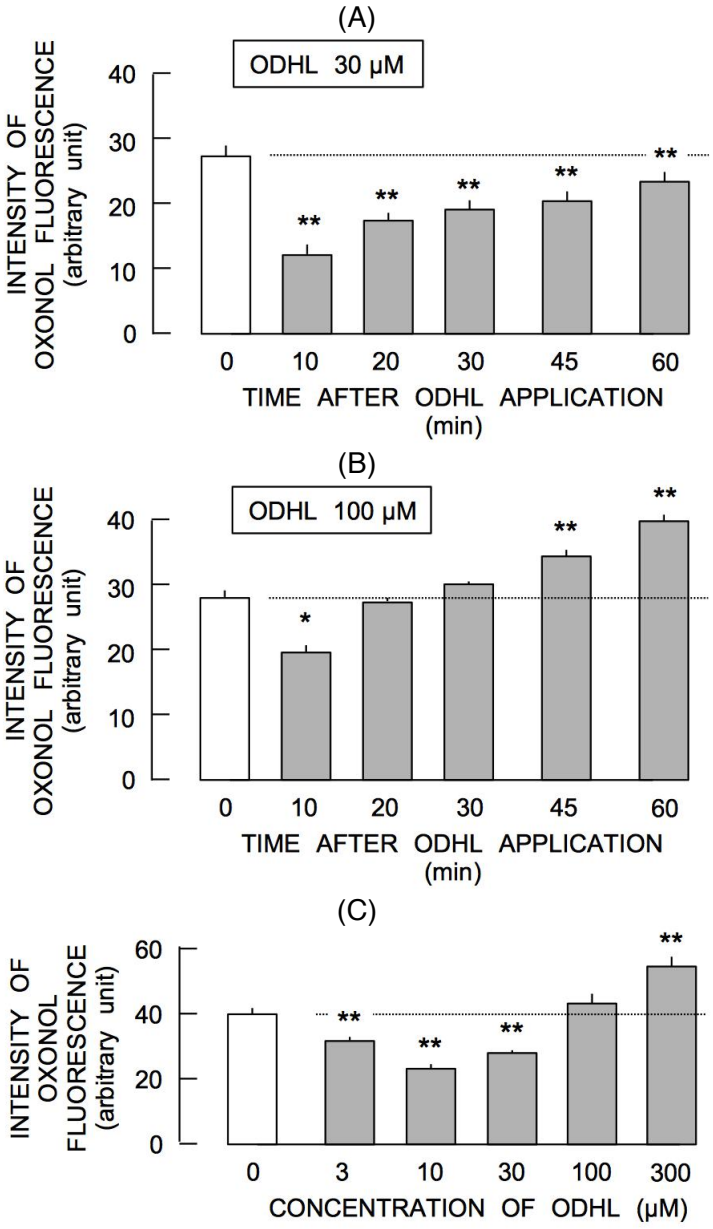

(A)
ODHL 30 μM
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
40
30
20
10
0
**
**
**
**
**
0
10
20
30
45
60
TIME AFTER ODHL APPLICATION (min)
(B)
ODHL 100 μM
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
40
30
20
10
0
*
**
**
0
10
20
30
45
60
TIME AFTER ODHL APPLICATION (min)
(C)
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
60
40
20
0
**
**
**
**
0
3
10
30
100
300
CONCENTRATION OF ODHL (μM)

Figure 3

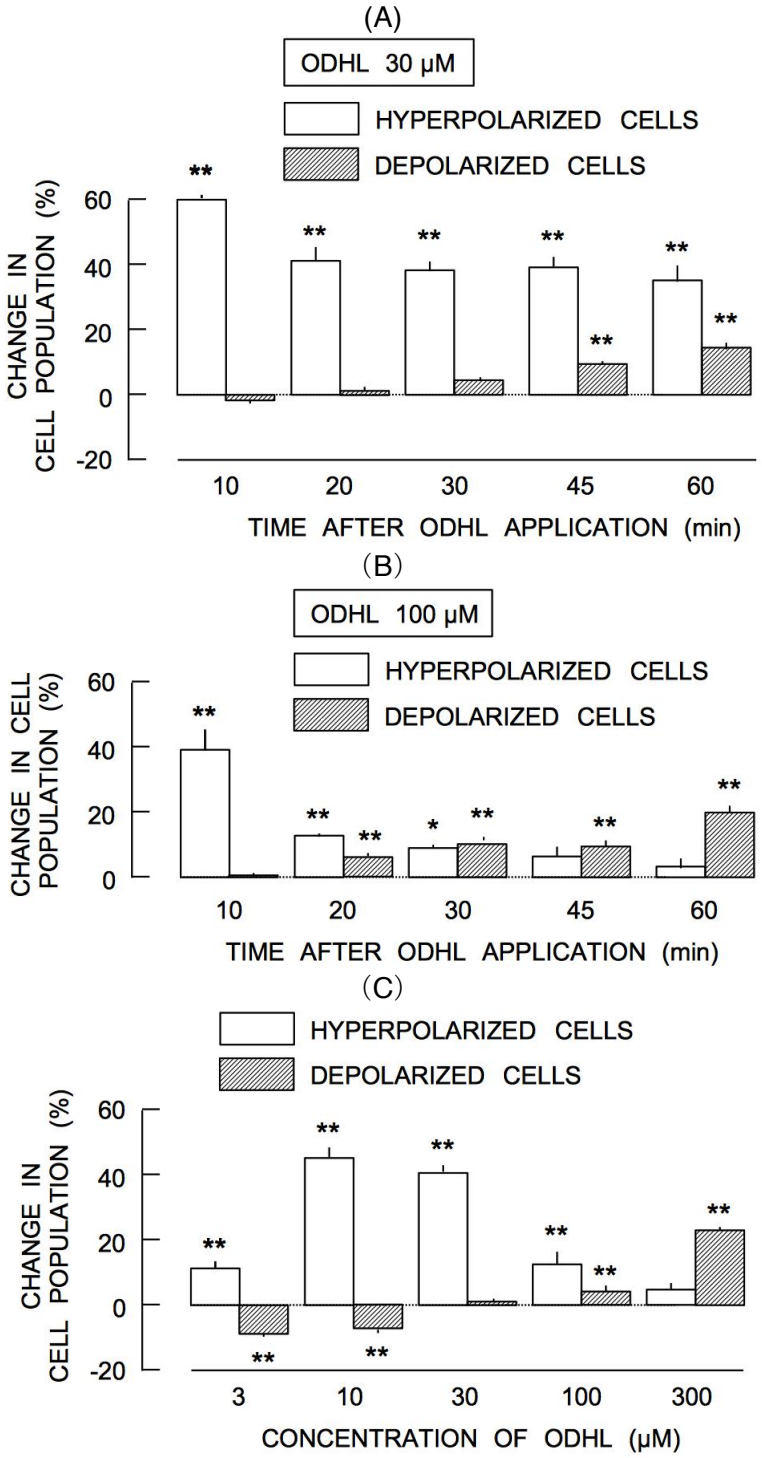

(A)
ODHL 30 μM
HYPERPOLARIZED CELLS
DEPOLARIZED CELLS
CHANGE IN CELL POPULATION (%)
60
40
20
0
-20
10
20
30
45
60
TIME AFTER ODHL APPLICATION (min)
(B)
ODHL 100 μM
HYPERPOLARIZED CELLS
DEPOLARIZED CELLS
CHANGE IN CELL POPULATION (%)
TIME AFTER ODHL APPLICATION (min)
(C)
HYPERPOLARIZED CELLS
DEPOLARIZED CELLS
CHANGE IN CELL POPULATION (%)
3
10
30
100
300
CONCENTRATION OF ODHL (μM)

Figure 4

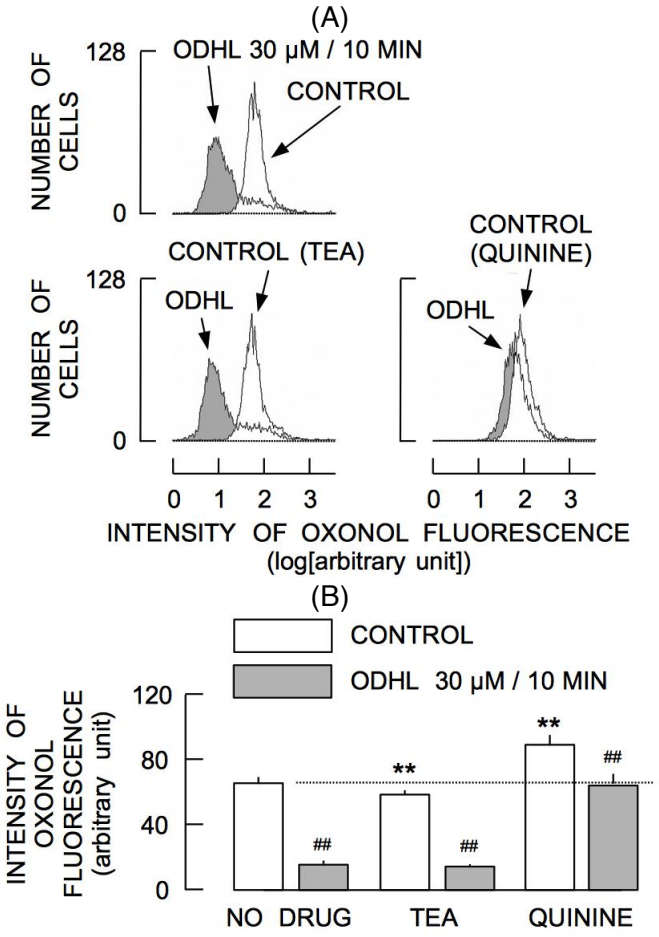

(A)
ODHL 30 μM / 10 MIN
CONTROL
128
0
NUMBER OF CELLS
CONTROL (TEA)
ODHL
CONTROL (QUININE)
ODHL
0 1 2 3
INTENSITY OF OXONOL FLUORESCENCE
(log[arbitrary unit])
(B)
CONTROL
ODHL 30 μM / 10 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
120
80
40
0
**
##
NO DRUG
TEA
QUININE

Figure 5

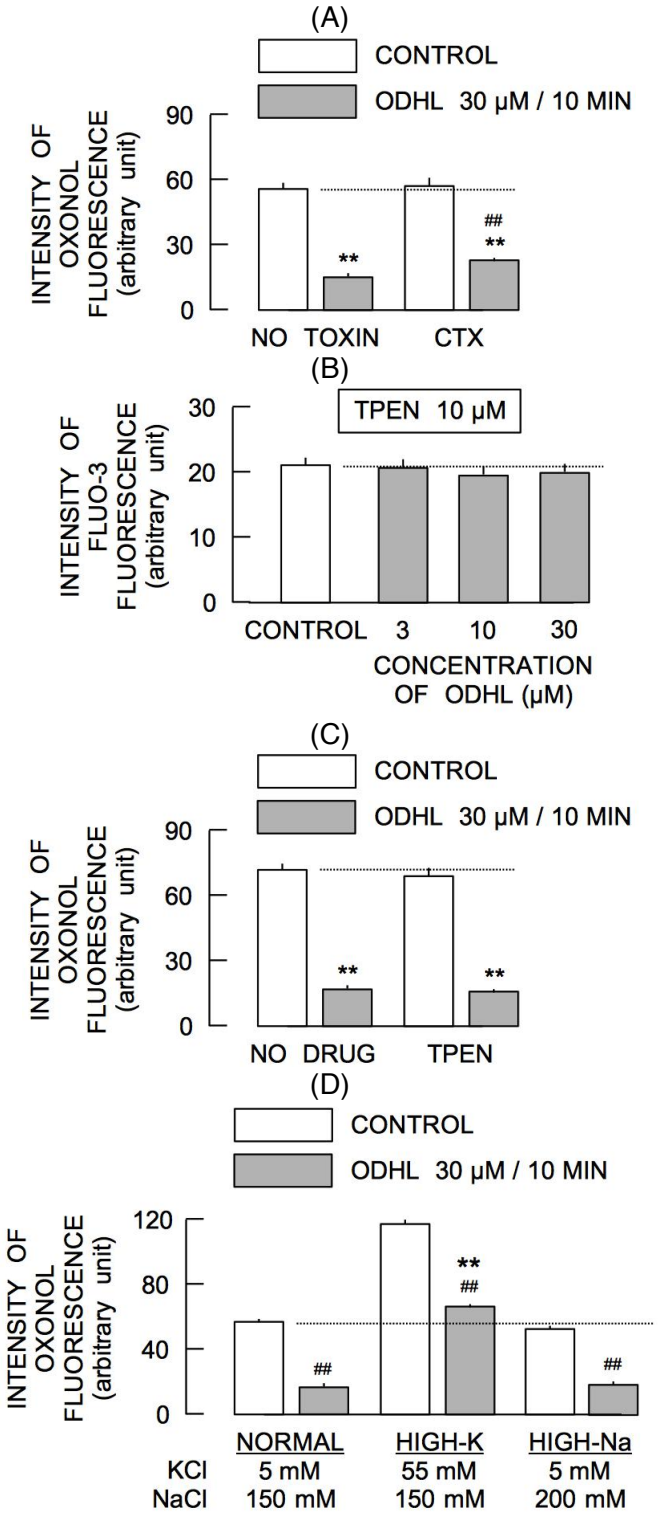
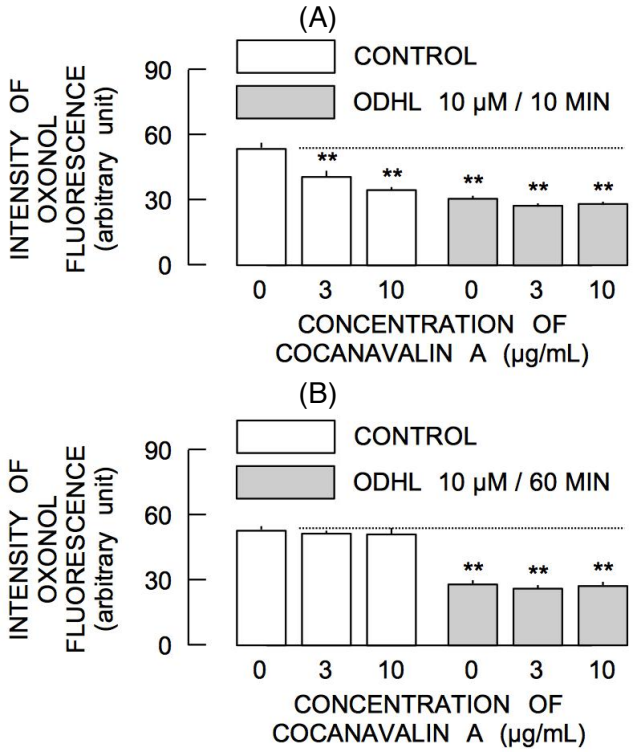

(A)
CONTROL
ODHL 30 μM / 10 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
90
60
30
0
**
##
**
NO TOXIN
CTX
(B)
TPEN 10 μM
INTENSITY OF FLUO-3 FLUORESCENCE (arbitrary unit)
30
20
10
0
CONTROL
3
10
30
CONCENTRATION OF ODHL (μM)
(C)
CONTROL
ODHL 30 μM / 10 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
90
60
30
0
**
**
NO DRUG
TPEN
(D)
CONTROL
ODHL 30 μM / 10 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
120
80
40
0
##
**
##
##
NORMAL
HIGH-K
HIGH-Na
KCl
5 mM
55 mM
5 mM
NaCl
150 mM
150 mM
200 mM

Figure 6


(A)
CONTROL
ODHL 10 μM / 10 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
90
60
30
0
**
0 3 10 0 3 10
CONCENTRATION OF COCANAVALIN A (μg/mL)
(B)
CONTROL
ODHL 10 μM / 60 MIN
INTENSITY OF OXONOL FLUORESCENCE (arbitrary unit)
90
60
30
0
**
0 3 10 0 3 10
CONCENTRATION OF COCANAVALIN A (μg/mL)