

Change in plasma membrane potential of rat thymocytes by *tert*-butylhydroquinone, a food additive: Possible risk on lymphocytes

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Highlights

- *tert*-Butylhydroquinone (TBHQ), a food additive, has therapeutic potential.
- TBHQ caused hyperpolarization that was followed by depolarization in rat thymocytes.
- The hyperpolarization was due to the activation of Ca²⁺-dependent K⁺ channels.
- The depolarization was due to a nonspecific increase in membrane ionic permeability.
- TBHQ-induced changes in membrane potential may be adverse for lymphocytes.

Abstract

Tertiary butylhydroquinone (TBHQ) is a food additive and has various beneficial actions under *in vitro* and *in vivo* experimental conditions. Therefore, it is necessary to collect additional data on the toxicity of TBHQ in order to avoid adverse effects during clinical applications. Changes in plasma membrane potential are associated with changes in physiological functions even in non-excitabile cells such as lymphocytes. Thus, compounds that affect membrane potential may modify some lymphocytic functions. The effect of TBHQ on plasma membrane potential was examined in rat thymocytes using flow cytometric techniques. Treatment of rat thymocytes with TBHQ caused hyperpolarization and then depolarization. The TBHQ-induced hyperpolarization was due to the activation of Ca^{2+} -dependent K^+ channels. TBHQ elevated intracellular Ca^{2+} levels. The depolarization by TBHQ was caused by a nonspecific increase in membrane ionic permeability. Both the sustained depolarization and elevation of intracellular Ca^{2+} level by TBHQ are thought to be adverse for thymocytes because such changes disturb membrane and intracellular signaling. The thymus is most active during neonatal and pre-adolescent periods. If TBHQ exerts adverse actions on thymocytes, it may result in an immunotoxic effect in neonates and adolescents.

Keywords: tert-butylhydroquinone; food additive; membrane potential; lymphocytes; calcium

1. Introduction

Tertiary butylhydroquinone (TBHQ, Chemical Abstracts 1948-33-0) is a food additive that has antioxidant activity (World Health Organization, 1999). The European Food Safety Authority has evaluated the toxicity of TBHQ and determined safe levels of TBHQ as a food additive (European Food Safety Authority, 2004). Antioxidative TBHQ exerts various beneficial actions under *in vitro* and *in vivo* experimental conditions (Jin et al, 2011; Rosanna and Salvatore, 2012; Mittal et al, 2014; Duan et al, 2016) because reactive oxygen species are involved in many diseases (Uttara et al., 2009; Brieger et al., 2012). Various major diseases such as atherosclerosis, arthritis, ischemia, Alzheimer's, Parkinson's, diabetes, sclerosis, gastritis, aging, liver diseases, cancer and AIDS are due to the generation of free radicals (Halliwell and Gutteridge, 1984). Since TBHQ has been already approved as a food additive, it is plausible that it will be used as a preventive and/or therapeutic medicinal agent. However, TBHQ also exhibits diverse toxic actions under experimental conditions (Imhoff and Hansen, 2010; Braeuning et al., 2012; Shibuya et al., 2012; Eskandani et al., 2014). For example, TBHQ induces mitochondrial oxidative stress causing Nrf2 activation. The oxidation of TBHQ produces more cytotoxic tert-butyl-p-benzoquinone. Therefore, it is necessary to collect additional data on the toxicity of TBHQ in order to avoid adverse actions during medical applications.

Plasma membrane potential is determined by membrane ionic permeability and transmembrane ionic gradient. Both are modulated by various physiological, pharmacological, and toxicological factors. The ionic gradient across membranes is maintained by the functional integration of cellular energy metabolism and a membrane with selective permeability. Changes in membrane potential are associated with altered physiological functions even in non-excitabile cells, such as lymphocytes (Tsien et al., 1982; Han and Kang, 2009; Varga et al., 2010; Lam and Wulff, 2011; Feske et al., 2012). Opening and closing of K^+ channels change membrane K^+ permeability, one of the important determinants of membrane potential. Charybdotoxin, a

specific blocker of Ca²⁺-dependent K⁺ channels (Miller et al., 1985), inhibits the proliferation of T lymphocytes (Price et al., 1989). Non-specific blockers of K⁺ channels inhibit the activation of B lymphocytes, resulting in an attenuation of lymphocytic progression through the cell cycle (Amigorena et al., 1990). Furthermore, the activity of phosphoinositide phosphatase is tuned within physiological range of plasma membrane potential (Murata et al., 2005). Thus, membrane potential can function beyond channel proteins. Based on the evidence, it is likely that the compounds that affect membrane potentials modify some functions in lymphocytes.

In this study, we examined the effect of TBHQ on the plasma membrane potential in rat thymocytes. Voltages were assessed using a voltage-sensitive fluorescent dye and the cells were studied using flow-cytometry, which revealed the mechanisms of TBHQ-induced changes in plasma membrane potential. One may argue that membrane potential is not a target for the adverse effects of food additives, including preservatives. However, the depolarization modulates plasma membrane phospholipid dynamics and K-Ras signaling in fibroblasts, excitable neuroblastoma cells, and *Drosophila* neurons (Zhou et al., 2015). Thus, the changes in membrane potentials seem to affect membrane and cellular functions. Furthermore, the thymus is most active during neonatal and pre-adolescent periods. Since there is a major concern about the adverse effect of food additives on children's health, any adverse change in thymocytes may be clinically important.

2. Materials and methods

2.1. Chemicals

TBHQ (99.1% purity) was purchased from Tokyo Chemical Ind. (Tokyo, Japan). *bis*-(1,3-Dibutylbarbituric acid)trimethine oxonol (Oxonol), Fluo-3-AM, and propidium iodide (PI) were supplied by Invitrogen (Eugene, OR, USA). Chelators for extracellular Ca²⁺ and intracellular Zn²⁺ were, respectively, disodium salt of ethylenediamine-*N,N,N',N'*-tetraacetic acid and *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (EDTA and TPEN; Dojindo

Molecular Technologies, Inc., Kumamoto, Japan). Unless mentioned, all other reagents were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell preparation

Experiments were performed under the approval (No. 05279) of Tokushima University Committee for Animal Experiments. Thymus glands were obtained from 6–8 week old Wistar rats that were anesthetized with ether. To dissociate individual cells, the glands were triturated in chilled Tyrode's solution (2–4 °C). The solution containing dissociated cells (cell suspension) was incubated at 36–37 °C for 1 h before experiments. The cell suspension contained trace amounts (216.9 ± 14.4 nM) of zinc from the cell preparation.

Thymocytes were used for this study of chemical cytotoxicity due to the following experimental reasons. First, single dissociated cells with intact membranes are easily obtained since no enzymatic treatment is required to isolate individual cells. Second, several types of chemical and biological substances induce cell death (Corsini et al., 2013; Kuchler et al., 2014). Third, the process of cell death (apoptosis, necrosis, and autophagy) is well studied (Klein, Kyewski et al., 2014; Poon et al., 2014; Shimizu, et al., 2014). However, there is an experimental limitation for dissociated thymocytes. Thymocytes spontaneously undergo apoptosis during prolonged incubation. All experiments, therefore, including the preparation of the cell suspension, were completed within 8 h after the dissection of thymus from rats to avoid possible contribution of spontaneous apoptosis. Therefore, the time for TBHQ treatment should be less than 5 h. Cell shrinkage is one of parameters observed during an early stage of apoptosis. We employed forward scatter as a parameter of cell size. The intensity of forward scatter in control cells was not changed during the experiment. The cell viability of control cells was also unchanged during the experiment.

TBHQ (10–300 mM in 2 μ L dimethyl sulfoxide) was added to 2 mL of cell suspension to achieve final concentrations of 10–300 μ M, and the cells were treated with TBHQ for 1–4 h, depending on respective experimental procedures. A sample (100 μ L) from each cell suspension

was analyzed by flow cytometry. Data acquisition from 2000–2500 cells took approximately 10–15 s. The sheath flow rate was manually adjusted to measure 195–205 cells/s with an interval of 180 μ s between measurements of forward and side scatters. The measurement started after achieving a constant flow of cells. Cell lethality estimated from 2500 cells was quite similar to that estimated from 10000 cells. Therefore, 2500 cells were deemed sufficient to examine cellular actions of TBHQ.

2.3. Fluorescence measurement

Fluorescence was measured and analyzed using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) and JASCO software package (Chikahisa et al., 1996). Chemical reagents, except for fluorescent probes, exhibited no fluorescence under the experimental conditions used. PI (5 μ M), a fluorescent probe that stains the DNA of dead cells, was added to the cell suspension to assess cell lethality. Measurements of membrane potentials were made using 500 nM Oxonol. Cells were incubated with 1 μ M Fluo-3-AM for 50–60 min before fluorescence measurement in order to monitor the change in $[Ca^{2+}]_i$ (Kao et al., 1989). Fluo-3 fluorescence was monitored in the cells treated with 10 μ M TPEN to avoid the contribution of intracellular Zn^{2+} to Fluo-3 fluorescence. Oxonol and Fluo-3 fluorescence were monitored in cells that did not show PI fluorescence (living cells with intact membranes). The excitation wavelength for fluorescent probes was 488 nm, and emissions were detected at 530 ± 20 nm for Oxonol and Fluo-3 fluorescence, and at 600 ± 20 nm for PI fluorescence. Oxonol and Fluo-3-AM were dissolved in DMSO. Therefore, control cell suspension contained 0.1 % DMSO. The intensities of Oxonol and Fluo-3 fluorescence in control cells were similar to those observed in cell suspension containing 0.3 % DMSO. Cell viability of the suspension containing 0.1 % DMSO was also similar to that of cell suspension containing 0.3 % DMSO. Thus, DMSO (up to 0.3 %) did not affect cellular parameters examined in this study.

2.4. Statistical analysis and presentation

Statistical analysis was carried out using Tukey's multivariate analysis. *P*-values of < 0.05

indicate statistical significance. Values are expressed as mean and standard deviation of 4–8 samples. Each experimental series was conducted three times unless stated otherwise.

3. Results

3.1. Changes in oxonol fluorescence by TBHQ

TBHQ at a concentration of 300 μM decreased the intensity of Oxonol fluorescence immediately after application, which was maintained for 30–60 min (Fig. 1A). An augmentation was observed at 90 min after the start of application (Fig. 1A). Although the attenuation of Oxonol fluorescence always preceded the augmentation in the presence of 300 μM TBHQ, the time course of TBHQ-induced differs slightly between cells obtained from different animals. A similar attenuation of Oxonol fluorescence was also observed after the start of treatment with 100 nM A23187, a calcium ionophore (Fig. 1B). Results are summarized in Fig. 1C. The attenuation of Oxonol fluorescence by 100 μM TBHQ continued for 90 min after the application (not shown).

(Figure 1 near here)

3.2. Effect of CTX on TBHQ-induced changes in Oxonol fluorescence

Since the time course of TBHQ-induced change in Oxonol fluorescence varied between the cells obtained from different rats, the experiments described below were performed with the cells where the Oxonol fluorescence was significantly attenuated 60 min after the start of TBHQ application under normal Ca^{2+} conditions. This attenuation was dose-dependent, as can be seen in Figure 2A.

The attenuation of Oxonol fluorescence by 100–300 μM TBHQ and 100 nM A23187 was statistically significant. A23187 activates the Ca^{2+} -dependent K^+ channels found in the rat thymocytes (Wilson and Chused, 1985) by increasing $[\text{Ca}^{2+}]_i$, resulting in the attenuation of Oxonol fluorescence. CTX specifically inhibits Ca^{2+} -dependent K^+ channels (Miller et al., 1985). Therefore, the effect of 300 nM CTX on TBHQ-induced attenuation of Oxonol fluorescence

was examined to determine whether Ca^{2+} -dependent K^+ channels are involved in the response of TBHQ. CTX completely suppressed the attenuation of Oxonol fluorescence by 100 μM TBHQ (Fig. 2B). Indeed, in the presence of CTX, 300 μM TBHQ induced the augmentation of Oxonol fluorescence (Fig. 2B). Thus, CTX reversed the effect of 300 μM TBHQ, and significantly lessened the attenuation of Oxonol fluorescence induced by A23187 (Fig. 2B).

(Figure 2 near here)

3.3. TBHQ-induced changes in Oxonol fluorescence under external Ca^{2+} -free conditions

From the result shown in Fig. 2B, TBHQ increased the $[\text{Ca}^{2+}]_i$ via Ca^{2+} influx, resulting in the activation of the Ca^{2+} -dependent K^+ channels. Therefore, the effects of TBHQ on Oxonol fluorescence were examined under external Ca^{2+} -free conditions. TBHQ at 100–300 μM significantly augmented the Oxonol fluorescence under external Ca^{2+} -free conditions (Fig. 2C). The removal of external Ca^{2+} reversed the TBHQ-induced responses. The attenuation of Oxonol fluorescence by A23187 under external Ca^{2+} -free conditions (Fig. 2C) was significantly smaller than that in the presence of external Ca^{2+} (Fig. 2A).

3.4. Changes in Fluo-3 fluorescence by TBHQ

The result shown in Fig. 2B and C suggested that Ca^{2+} was involved in the TBHQ-induced attenuation of Oxonol fluorescence. The effect of TBHQ on Fluo-3 fluorescence was examined to determine whether an increase in $[\text{Ca}^{2+}]_i$ was involved. The treatments of cells with 100–300 μM TBHQ for 60 min shifted the histogram of Fluo-3 fluorescence in a direction of higher intensity (Fig. 3A). The augmentation of Fluo-3 fluorescence by TBHQ at 30–300 μM was observed to be concentration-dependent (Fig. 3B). The augmentation of Fluo-3 fluorescence by 100–300 μM TBHQ was statistically significant. The augmentation by 100 nM A23187 was greater than that by TBHQ.

(Figure 3 near here)

3.5. TBHQ-induced changes in Fluo-3 fluorescence under external Ca^{2+} -free conditions

To determine whether external Ca^{2+} is involved in the TBHQ-induced augmentation of

Fluo-3 fluorescence, the effects of 100–300 μM TBHQ on Fluo-3 fluorescence were examined under external Ca^{2+} -free conditions. The cells were treated with TBHQ for 60 min under external Ca^{2+} -free conditions. The augmentation of Fluo-3 fluorescence by TBHQ was completely suppressed under external Ca^{2+} -free conditions (Fig. 4A), indicating an involvement of external Ca^{2+} . Removal of external Ca^{2+} also greatly diminished the A23187-induced augmentation of Fluo-3 fluorescence (Fig. 4A).

(Figure 4 near here)

3.6. Mn^{2+} -induced quenching of Fluo-3 fluorescence in the presence of TBHQ

The result shown in Fig. 4A suggested that TBHQ increased membrane permeability to Ca^{2+} , resulting in an increase in $[\text{Ca}^{2+}]_i$. To test this hypothesis, the cells were simultaneously treated with 300 μM TBHQ and 2 mM Mn^{2+} for 60 min under normal Ca^{2+} conditions. TBHQ itself augmented Fluo-3 fluorescence, while TBHQ and Mn^{2+} attenuated Fluo-3 fluorescence (Fig. 4B). Thus, we suggest that Mn^{2+} passes through membranes of cells treated with TBHQ, resulting in Mn^{2+} -induced quenching of Fluo-3 fluorescence.

4. Discussion

4.1. TBHQ-induced changes in membrane potential and $[\text{Ca}^{2+}]_i$

The attenuation and augmentation of Oxonol fluorescence indicate hyperpolarization and depolarization, respectively (Rink et al., 1980). Treatment of rat thymocytes with TBHQ initially induced hyperpolarization and thereafter depolarization. The fact that CTX inhibited TBHQ-induced hyperpolarization suggests that TBHQ activates Ca^{2+} -dependent K^+ channels, which increases membrane K^+ permeability. TBHQ (300 μM) depolarized the membranes in the presence of CTX and under external Ca^{2+} -free conditions. Thus, TBHQ is assumed to have a hyperpolarizing action *via* activation of Ca^{2+} -dependent K^+ channels and depolarizing action. Therefore, the time course of change in membrane potential of cells treated with TBHQ may be determined by the balance of hyperpolarizing and depolarizing actions. The depolarizing action

by TBHQ was caused by a nonspecific increase in membrane ionic permeability, as seen when Mn^{2+} passed through the membranes of cells treated with TBHQ. In the absence of TBHQ, however, treatment of cells with 2 mM Mn^{2+} for 60 min did not affect Fluo-3 fluorescence, suggesting that Mn^{2+} did not pass through the membranes (Fig4A). It is important to note that depolarization can also be caused by a decrease in resting membrane K^+ permeability. Therefore, it is possible that TBHQ possesses an inhibitory action on resting membrane K^+ permeability.

The attenuation and augmentation of Fluo-3 fluorescence indicate a decrease and an increase in the $[Ca^{2+}]_i$, respectively (Kao et al., 1989). TBHQ at concentrations of 100–300 μM significantly elevated the $[Ca^{2+}]_i$ in rat thymocytes by increasing the membrane permeability to Ca^{2+} , resulting in an influx of external Ca^{2+} along with transmembrane Ca^{2+} gradient. This mechanism is supported by the observation that the removal of external Ca^{2+} completely diminished the TBHQ-induced increase in $[Ca^{2+}]_i$. As described above, it was observed that Mn^{2+} passed through the membranes in the presence of TBHQ. Thus, the sustained treatment of cells with TBHQ may non-specifically increase membrane ionic permeability. The increase in $[Ca^{2+}]_i$ in the presence of TBHQ may be much adverseer than the TBHQ-induced change in membrane potential, since intracellular Ca^{2+} is involved in many physiological functions and the dyshomeostasis of intracellular Ca^{2+} disturbs many intracellular signaling pathways (Berridge et al., 1998). Thus, the sustained increase in $[Ca^{2+}]_i$ by TBHQ is linked to cell injury and cell death.

4.2. Implications

The change in membrane potential of lymphocytes is associated with physiological changes. Early studies showed that CTX, a specific inhibitor of Ca^{2+} -dependent K^+ channels, blocked the proliferation of T lymphocytes (Price et al., 1989). Selective immunomodulatory action is expected in the agents possessing inhibitory actions on voltage-dependent and Ca^{2+} -dependent K^+ channels in recent studies (Han and Kang, 2009; Varga et al., 2010; Lam and Wulff, 2011; Feske et al., 2012). Under control conditions in the presence of TBHQ, the original hyperpolarization was followed by the consistent depolarization. T-cell mitogens cause

changes in membrane potential and $[Ca^{2+}]_i$ (Tsien et al., 1982). Such changes can be disturbed by TBHQ. Thus, it may be unlikely that the mitogens cause cellular signalings of lymphocytes in the presence of TBHQ. Furthermore, as shown in the histogram of Figs. 1A and 3A, all cells responded to TBHQ. Therefore, the actions of TBHQ were independent from the cell types that were immunologically classified.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food was asked to advise on the safety in use of TBHQ in fats and oils used for human consumption (2004). TBHQ is used at a level of up to 200 mg/kg fat or oil. The Panel also considers the dog as the most sensitive species and allocated an acceptable daily intake of 0.7 mg/kg based on a no-observed-adverse-effect-level (NOAEL) of 72 mg/kg body weight per day in dogs to which a 100-fold safety factor was applied. If basal metabolic rate per one day in male adult (50 kg) is 3000 Kcal, 100 g of fat would be required when 30 % of basal metabolic rate is covered with fat intake. 100 g of edible oils contains 20 mg TBHQ at maximum. Even under the assumption that all BHT in edible oils (0.4 mg/kg body weight) is absorbed, it would be unlikely that the blood concentration of BHT reaches 30–100 μ M. Thus, the daily intake of BHT under FDA regulations is safe. The European Food Safety Authority (2016) estimates the 95% percentile exposure to TBHQ as a food additive to be 0.009–0.555 mg/kg body weight/day in infants (4–11 months), 0.084–0.798 mg/kg body weight/day in toddlers (12–35 months), 0.116–0.929 mg/kg body weight/day in children (3–9 years), and 0.040–0.420 mg/kg body weight/day in adolescents (10–17 years). It may be something unrealistic to predict blood concentration of TBHQ based only on oral ingestion. It is unlikely that TBHQ at concentrations used as a food additive affects thymocytes of infants and/or children since the minimum *in vitro* concentration of TBHQ needed to change the thymocyte membrane potential and $[Ca^{2+}]_i$ was 30–100 μ M (4.9–16.6 mg/L).

Conflict of interest

All authors affirm that there are no conflicts of interest to declare.

Acknowledgements

This study was supported by Grant-in-Aids for Scientific Research (C26340039) from the Japan Society for the Promotion of Science.

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Figure legends

Figure 1. Changes in Oxonol fluorescence after the addition of 300 μM *Tert*-butylhydroquinone (TBHQ) and 100 nM A23187. (A) TBHQ-induced shift of Oxonol fluorescence histogram. Histograms, consisting of 2500 mouse thymocyte cells, were obtained 60 min and 90 min after the start of TBHQ application. (B) The shift of the fluorescence histogram by TBHQ and A23187. The histogram was obtained at 60 min after the start of application. (C) Changes in the mean Oxonol fluorescence intensity in cells treated with TBHQ or A23187. Column and bar indicate mean and standard deviation of four samples. Dotted line indicates control level. Asterisks (**) show significant difference between control group and TBHQ or A23187 groups.

Figure 2. *Tert*-butylhydroquinone (TBHQ)-induced attenuation of Oxonol fluorescence in mouse thymocytes. (A) A concentration-dependent decrease in the mean intensity of Oxonol fluorescence is seen after the application of 30–300 μM TBHQ. A23187 was used as a reference drug. (B) The effects of TBHQ and A23187 in presence of 300 nM charybdotoxin. (C) The effects of TBHQ and A23187 under external Ca^{2+} -free conditions. The results of (A)–(C) were obtained from the samples dispensed from same cell suspensions. Effects were examined at 60 min after the start of drug application. The dotted line shows respective control level. The column and bar indicate mean and standard deviation of four samples. Asterisks (**) show significant differences between the control group and groups of cells treated with TBHQ or A23187 under respective experimental conditions.

Figure 3. Changes in Fluo-3 fluorescence in rat thymocytes after the application of *Tert*-butylhydroquinone (TBHQ) and A23187. (A) Shift of Fluo-3 fluorescence histogram due to application of 100–300 μM TBHQ. Histogram consisted of 2500 cells. The effect was examined 60 min after the start of drug application. (B) A concentration-dependent increase in the mean

intensity of Fluo-3 fluorescence was observed after the application of 30–300 μM TBHQ. A23187 was used as a reference drug, and the dotted line shows the fluorescence level of the control group. The column and bar indicate the mean and standard deviation of four samples. Asterisks (**) show a significant difference between the control group and TBHQ or A23187 groups under respective experimental conditions.

Figure 4. *Tert*-butylhydroquinone (TBHQ)-induced augmentation of Fluo-3 fluorescence. (A) The effect of 100–300 μM TBHQ and 100 nM A23187 under external Ca^{2+} -free conditions. (B) The effect of 300 μM TBHQ in the presence of 2 mM MnCl_2 . Effects were examined 60 min after the start of the drug application. The dotted line shows the fluorescence of the control sample. The column and bar indicate the mean and standard deviation of four samples. Asterisks (**) show a significant difference between the control group and TBHQ or A23187 groups under respective experimental conditions.

Figure 1

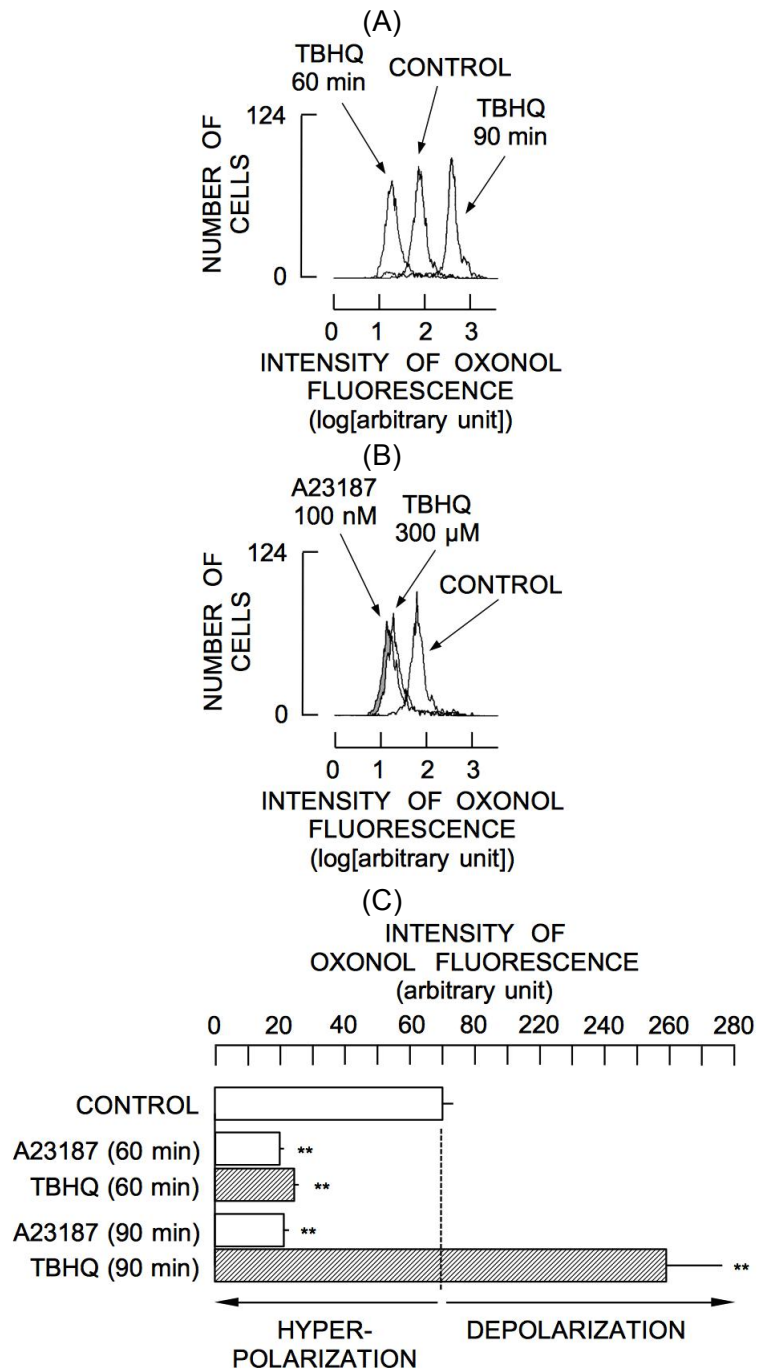


Figure 2

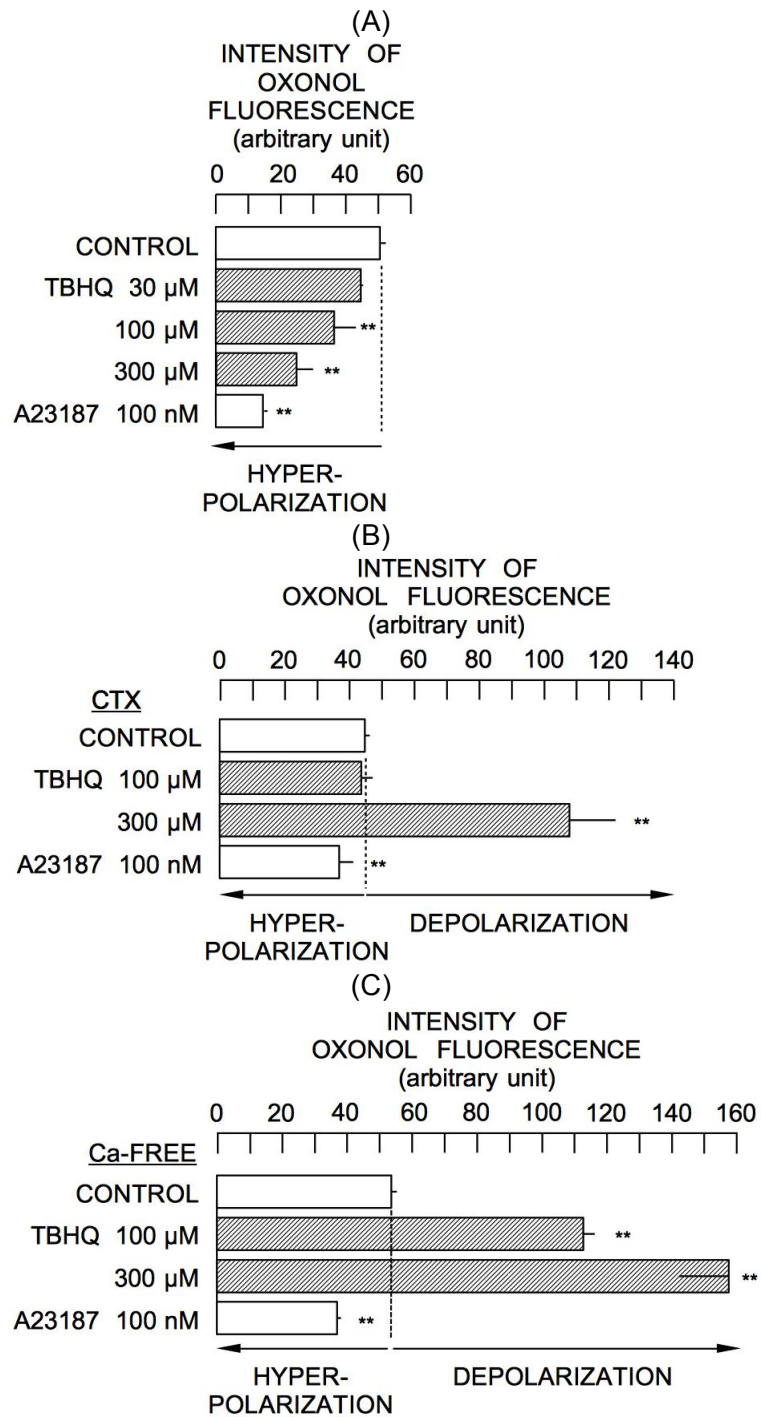


Figure 3

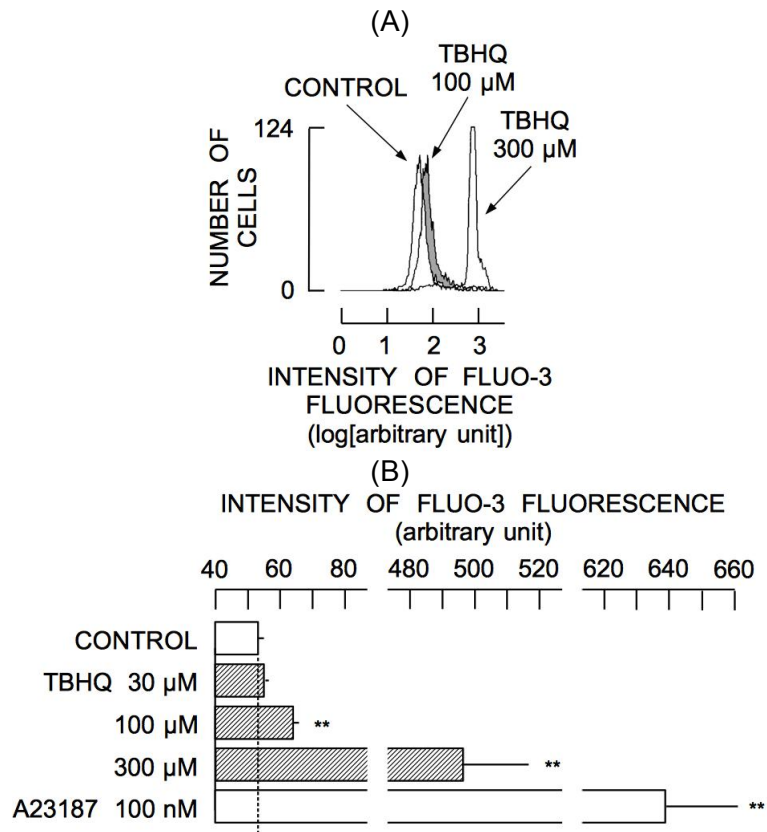


Figure 4

