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Effect of econazole on membrane calcium transport in rat thymocytes

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ABSTRACT

Econazole, one of azole antifungals, is proven to exhibit an inhibitory action on *Mycobaterium tuberculosis* and its multidrug-resistant strains under *in vitro* and *ex vivo* conditions. However, econazole has been used as a pharmacological tool for inhibiting capacitative Ca^{2+} influx and exerts multiple effects on cellular Ca^{2+} circumstance. Therefore, to suggest the toxic effect of econazole at therapeutic concentrations, we have tested on the effect on membrane Ca^{2+} transport in rat thymocytes by using a flow cytometer with Fluo-3, an indicator of intracellular Ca^{2+}. Econazole at concentrations of 1-3 μM increased membrane Ca^{2+} permeability and inhibited capacitative Ca^{2+} influx without affecting passive Ca^{2+} influx, Ca^{2+} release from intracellular Ca^{2+} store sites, and membrane Ca^{2+} pump. Econazole at 0.3 μM, a therapeutic concentration against tuberculosis caused by multidrug-resistant and latent *M. tuberculosis*, did not affect membrane Ca^{2+} transport. It may be suggested that econazole at therapeutic concentrations exerts no side effect related to Ca^{2+}.

Keywords: econazole; calcium; cytotoxicity; lymphocyte

1. INTRODUCTION

Lanosterol-14a-methylase (CYP51) of *Mycobaterium tuberculosis* is similar in sequence to that of fungal CYP51 isozymes that are pharmacological targets for azole antifungals (Guardiola-Diaz *et al.*, 2001). Several azole antifungals bind CYP51 with high affinity, suggesting that CYP51 of M. tuberculosis is targeted for drug action (Guardiola-Diaz *et al.*, 2001). Econazole, one of azole antifungals, is proven to exhibit an inhibitory action on M. tuberculosis and its multidrug-resistant strains under *in vitro* and *ex vivo* conditions (Ahmad *et al.*, 2005, 2006, 2007, 2008). Thus, there is a chemotherapeutic potential of econazole against tuberculosis caused by multidrug-resistant and latent *M. tuberculosis*.

In pharmacological cellular studies, econazole has been used as an inhibitor of capacitative Ca^{2+} influx in many cells (Sargeant *et al.*, 1992; Mason *et al.*, 1993; Vostal *et al.*, 1993; Koch *et al.*, 1994). This type of Ca^{2+} influx is very important for refilling intracellular Ca^{2+} stores (Putney and Bird, 1993). Furthermore, econazole increases intracellular Ca^{2+} level by releasing Ca^{2+} from endoplasmic reticulum *via* inhibiting Ca^{2+}-ATPase (Mason *et al.*, 1993) and activates extracellular Ca^{2+} influx (Jan *et al.*, 1999). Thus, econazole seems to exert multiple effects on cellular calcium signaling. It is known that Ca^{2+} plays physiological and pathological roles in various types of cells. Therefore, econazole at therapeutic concentrations against tuberculosis may affect intracellular Ca^{2+} homeostasis, leading to harmful (or cytotoxic) effect. To test the possibility, the effect of econazole at concentrations of 3 μM or less on intracellular Ca^{2+} level under normal Ca^{2+} and Ca^{2+}-free conditions has been examined.

In present study, the effects of econazole have been examined on rat thymocytes by means of flow-cytometry with appropriate fluorescent probes. Rat thymocytes were used for the test because of following reasons. First, the cell membranes of thymocytes remain intact because single cells are prepared without an enzymatic treatment. Second, the Ca^{2+}-dependent process of cell death is extensively examined in murine thymocytes (McConkey *et al.*, 1994; Winoto, 1997). Third, many chemicals affect intracellular Ca^{2+} level *via* various mechanisms in thymocytes (Chikahisa and Oyama, 1992; Oyama *et al.*, 1994, 1995; Okazaki *et*

al., 1996; Hirama *et al*., 2004; Oyama *et al*., 2006).

2. MATERIALS AND METHODS

2.1. Chemicals

Econazole and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, USA). Econazole was initially dissolved in DMSO and then added to cell suspension. The final concentration of DMSO as a solvent was 0.1 % or less. The incubation with DMSO at 0.3 % or less did not affect the viability of rat thymocytes.

Fluorescent probes, propidium iodide and Fluo-3, were obtained from Molecular Probes (Eugene, USA). Other chemicals (NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and $ZnCl_2$) were purchased from Woko Pure Chemicals (Osaka, Japan).

2.2. Animals and cell preparation

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

Male rats (Wistar strain, Charles River Laboratories, Yokohama, Japan) were provided with water automatically and a commercial diet (MF, Oriental Yeast, Tokyo, Japan) *ad libitum*. The animal room was maintained at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 5 %, and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (08:00-20:00 h).

The procedure to prepare cell suspension was similar to that previously reported (Chikahisa *et al*., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400-500 μm with razor under ice-cold condition (1-4 °C). The slices were gently triturated by shaking in chilled 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) to dissociate thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (a diameter of 10 μm) to prepare the cell suspension (about 10^6 cells/ml). The beaker containing the cell suspension was water-bathed at 36 °C for 1 h before the experiment.

Some thymocytes spontaneously undergo apoptosis during a prolonged incubation for 6 h or more. Therefore, we used thymocytes freshly isolated from thymus glands for this study. The total number of 8-12 week old rats sacrificed under ether inhalation anesthesia was 10. Each experiment was completed within 6 h after the isolation of cells from thymus glands.

2.3. Fluorescence measurements of cellular and membrane parameters

The methods for measurements of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa *et al*., 1996). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition.

To assess membrane integrity, propidium iodide was added to the cell suspension to achieve a final concentration of 5 μM. Since propidium stains the cells with compromised membranes, the measurement of propidium fluorescence from the cells provides a clue to select the cells with intact membranes. Propidium fluorescence was measured at 2 min after the application by a flow cytometer. Excitation wavelength for propidium was 488 nm and emission was detected at 600 ± 20 nm.

To estimate the change in intracellular Ca^{2+} concentration of rat thymocytes, Fluo-3-AM (Kao *et al*., 1989) was used with propidium iodide. The dyes were added to the cell suspension to achieve a final concentration of 500 nM for Fluo-3-AM and 5 μM for propidium iodide. The cells were incubated with Fluo-3-AM for 60 min and propidium iodide for 2 min before any fluorescence measurements, respectively. Fluo-3 fluorescence was measured only from the cells that were not stained with propidium. Excitation wavelength for Fluo-3 was 488 nm and emission was detected at 530 ± 15 nm.

2.4. Statistics

Statistical analysis was performed by using Tukey multivariate analysis. A P value of < 0.05 was considered significant. Values are mean ± standard deviation (SD) of 4 experiments.

3. RESULTS

3.1. Effect of econazole on Fluo-3 fluorescence of rat thymocytes in absence and presence of external Ca^{2+}

Under external Ca^{2+}-free condition where Ca^{2+} was replaced with equimolar Mg^{2+}, the incubation of cells with econazole at concentrations ranging from 0.3 μM to 3 μM for 1 hr did not increased the mean intensity of Fluo-3 fluorescence (Fig. 1). Results suggest that econazole at therapeutic concentrations does not induce the release of Ca^{2+} from intracellular Ca^{2+} store sites. In the presence of 2 mM Ca^{2+}, econazole at 0.3 μM did not affect the intensity of Fluo-3 fluorescence. However, the incubation with 1 μM econazole for 1 hr increased the intensity of Fluo-3 fluorescence. Further augmentation of Fluo-3 fluorescence was observed in the case of 3 μM

econazole (Fig. 1). It is suggested that econazole at concentrations of 1 μM or more increases intracellular Ca^{2+} concentration. Thus, since the increase by econazole is dependent on external Ca^{2+}, econazole at micromolar concentrations may increase membrane Ca^{2+} permeability.

3.2. Effects of econazole on Fluo-3 fluorescence augmented by reintroduction of Ca^{2+} in absence and presence of thapsigargin

Under Ca^{2+}-free condition, the reintroduction of external Ca^{2+} increased the mean intensity of Fluo-3 fluorescence (Fig. 2). The augmentation of Fluo-3 fluorescence by the reintroduction of external Ca^{2+} may be due to passive Ca^{2+} influx. Even in the presence of econazole at concentrations ranging from 0.3 μM to 3 μM, the augmentation of Fluo-3 fluorescence by the reintroduction of external Ca^{2+} similarly occurred. Thus, it is suggested that passive Ca^{2+} influx is not affected by econazole at therapeutic concentrations.

Under external Ca^{2+}-free condition, the incubation of cells with 100 nM thapsigargin depletes Ca^{2+} from endoplasmic reticulum, leading to an activation of capacitative Ca^{2+} influx (Oyama *et al.*, 1994). Therefore, the augmentation of Fluo-3 fluorescence by the reintroduction of external Ca^{2+} is due to the combination of passive and capacitative Ca^{2+} influx in the presence of thapsigargin. As shown in Fig. 2, while the incubation with 0.3 μM econazole did not affect the augmentation of Fluo-3 fluorescence by the reintroduction, the augmentation was attenuated by 1 μM and 3 μM econazole. Since econazole did not affect the passive Ca^{2+} influx, it is likely that the attenuation of Fluo-3 fluorescence by econazole is due to the decrease in capacitative Ca^{2+} influx.

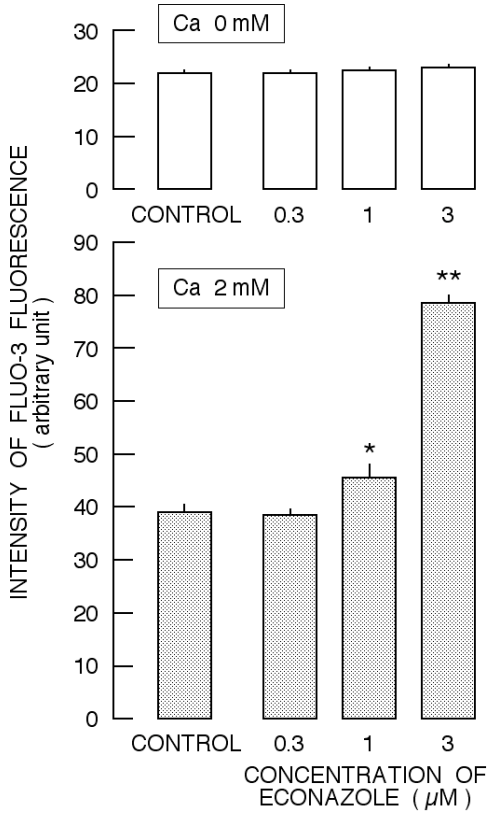

Fig.1. *Effect of econazole on mean intensity of Fluo-3 fluorescence in the absence and presence of external Ca^{2+}. Column and bar indicate average and SD of 4 experiments. Asterisks (* and **) show significant increase ($P < 0.05$ and $P < 0.01$, respectively) in the intensity, comparing with control.*

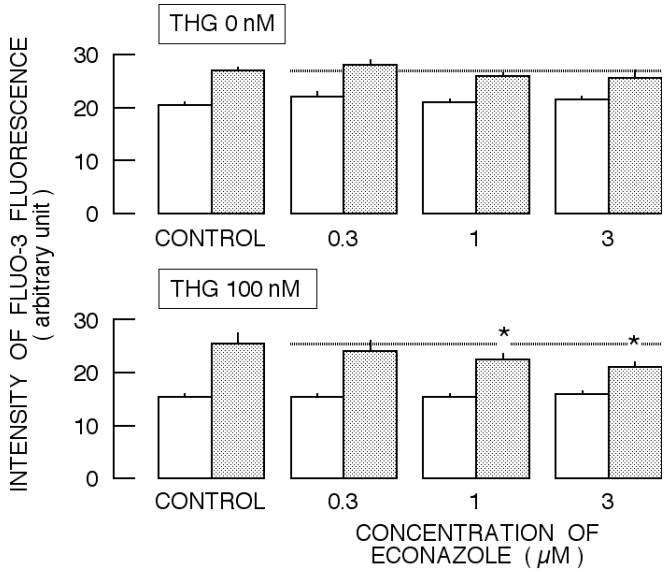

Fig. 2. *Effect of econazole on mean intensity of Fluo-3 fluorescence augmented by reintroduction of external Ca^{2+} in absence and presence of thapsigargin. Asterisk (*) shows significant attenuation ($P < 0.05$ and $P < 0.01$, respectively) of the intensity, comparing with control augmentation. The bar indicates the control level after the reintroduction.*

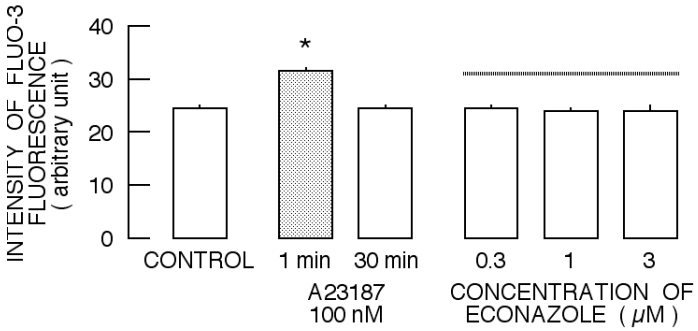

Fig. 3. *Effect of econazole on mean intensity of Fluo-3 fluorescence at 30 min after the start of application of A23187. The increase at 1 min after the start of A23187 application was significant ($P < 0.01$), comparing with control. The bar indicates the A23187-induced level at 1 min in the presence of econazole.*

3.3. Effect of econazole on Fluo-3 fluorescence augmented by A23187 under external Ca^{2+}-free condition

Under external Ca^{2+}-free condition, A23187 at 100 nM increased mean intensity of Fluo-3 fluorescence and the intensity gradually returned to control level within 30 min in continued presence of A23187 (Fig. 3, Oyama *et al.*, 1994). This change in the fluorescence intensity is due to the release of Ca^{2+} from intracellular Ca^{2+} stores by A23187 and the extrusion of Ca^{2+} by membrane Ca^{2+} pump (Oyama *et al.*, 1994). The intensity of Fluo-3 fluorescence at 30 min after the start of application of A23187 in the presence of 0.3-3 μM econazole was similar to that in the absence of econazole (Fig. 3). Thus, it is suggested that econazole exerts no inhibitory action on membrane Ca^{2+} pump.

4. DISCUSSION

Econazole, an imidazole antifungal, is proposed to possess a therapeutic potential against tuberculosis (Ahmad *et al.*, 2005, 2006. 2007, 2008). However, this agent inhibits capacitative Ca^{2+} influx (Mason *et al.*, 1993; Hornestein *et al.*, 1996; Christian *et al.*, 1996). Furthermore, econazole exerts multiple effects on cellular Ca^{2+} signal (Jan *et al.*, 1999). Therefore, one may argue the possibility that econazole at therapeutic concentrations exerts cytotoxic effect that is related to Ca^{2+} because

intracellular Ca^{2+} is known to play physiological, pathological, and toxicological roles.

In present study, econazole at micromolar concentrations (1-3 μM) increased membrane Ca^{2+} permeability, resulting in the increase in intracellular Ca^{2+} concentration under normal Ca^{2+} condition (Fig. 1), and attenuated the thapsigargin-related increase in intracellular Ca^{2+} concentration *via* reintroducing external Ca^{2+} by inhibiting capacitative Ca^{2+} influx (Fig. 2). Of two reciprocal effects described above, econazole-induced increase in membrane Ca^{2+} permeability is predominant in affecting intracellular Ca^{2+} concentration because of larger augmentation of Fluo-3 fluorescence by econazole under normal Ca^{2+} condition (Fig. 1). On the contrary, econazole at 1-3 μM did not induce Ca^{2+} release from intracellular Ca^{2+} site (Fig. 1) and did not affect membrane Ca^{2+} pump (Fig. 3). Thus, econazole at micromolar concentrations may disturb intracellular Ca^{2+} homeostasis, resulting in some cytotoxic effects.

Possible therapeutic concentration of econazole against tuberculosis is 0.1-0.3 μM (Ahmad *et al.*, 2005). As shown in Figs. 1-3, 0.3 μM econazole did not change Fluo-3 fluorescence in rat thymocytes. Thus, econazole at submicromolar concentrations, presumably therapeutic, exerts no action on intracellular Ca^{2+} homeostasis. Therefore, it is concluded that econazole at submicromolar concentrations exerts no cytotoxic effect that is related to Ca^{2+}. However, our recent study by using FluoZin-3 reveals that submicromolar econazole greatly increases mean intensity of FluoZin-3 fluorescence in the presence of physiological concentrations of Zn^{2+} (Kinazaki *et al.*, In submitted). Therefore, it may be suggested that the side effect of submicromolar econazole is related to Zn^{2+}, but not Ca^{2+}. In this aspect, further study will be necessary.

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