Econazole depleted calcium release-activated calcium (CRAC) current through blockade of voltage-dependent Ca^{2+} channels

Econazol menghambat *calcium release-activated calcium (CRAC) current* melalui penghambatan kanal Ca^{2+} tergantung voltase

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Abstract

Econazole is an azole antifungal agent which can block the calcium release-activated calcium (CRAC) current in human leukaemic T cell line. The phenomenon is also possible to occur in mast cell such as RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells. In the study, we investigated effect of econazole on $^{45}Ca^{2+}$ uptake into the cells in response to thapsigargin, an ATP-dependent Ca^{2+} (SERCA) inhibitor, by direct measurement of radiolabelled Ca^{2+} uptake in cells. The mechanism underlying this effect of econazole was studied using molecular modelling. In present study, econazole inhibited $^{45}Ca^{2+}$ influx into mast cells in absence of mast cells inducer, thapsigargin. Moreover, econazole potently suppressed the $^{45}Ca^{2+}$ influx induced by thapsigargin. It was supported that econazole also inhibited Ca^{2+}-induced tracheal contraction. The increase of Ca^{2+} was stimulated by the opening of voltage-dependent Ca^{2+} channels activated by KCl-induced membrane depolarization. Based on molecular docking study, score of interaction (equal to energy of interaction) of 3FGO, a main protein target on Ca^{2+}-ATPase, with native ligan, thapsigargin and econazole were -76.941, -117.205, and -92.277, respectively. The interaction of thapsigargin and Ca^{2+}-ATPase was more stable than this of econazole and Ca^{2+}-ATPase. It suggests that it would be difficult for econazole to block the interaction of thapsigargin with Ca^{2+}-ATPase to increase intracellular Ca^{2+}. In conclusion, econazole inhibited the increase of intracellular Ca^{2+} involving the blokade of voltage-dependent Ca^{2+} channels, but not involving the Ca^{2+}-ATPase pathway.
Key words : econazole, Ca^{2+}-ATPase, CRAC current, thapsigargin

Abstrak

Econazol merupakan obat antifungi yang dapat menghambat *calcium release-activated calcium (CRAC) current* pada *human leukaemic T cell line*. Fenomena tersebut juga dapat terjadi pada *RBL-2H3* (*rat basophilic leukemia*) *cells*. Penelitian ini mempelajari pengaruh econazol terhadap pengambilan $^{45}Ca^{2+}$ (*$^{45}Ca^{2+}$ uptake*) menuju ke dalam sel yang diinduksi thapsigargin, suatu *ATP-dependent Ca^{2+}* (*SERCA*) *inhibitor*. Mekanisme aksi yang dipelajari adalah efek econazol terhadap kanal Ca^{2+} tergantung voltase dan pompa Ca^{2+}-ATPase. Pada penelitian ini, econazol menghambat influks $^{45}Ca^{2+}$ menuju ke dalam sel mast baik tanpa dan dengan induksi thapsigargin. Econazol juga menghambat kontraksi otot polos yang diinduksi Ca^{2+} ekstraseluler. Pada percobaan tersebut, peningkatan Ca^{2+} dirangsang oleh terbukanya kanal Ca^{2+} tergantung voltase, yang diaktivasi oleh depolarisasi membran akibat pemberian KCl. Pada kajian *molecular docking*, skor interaksi (sebanding dengan energi interaksi) antara

3FGO (protein targe utama pada Ca^{2+}-ATPase) dengan ligan asli, thapsigargin dan econazol berturut-turut adalah 76,941, -117,205, dan -92,277. Interaksi antara thapsigargin dengan Ca^{2+}-ATPase lebih stabil dibandingkan interaksi antara econazol dengan dengan Ca^{2+}-ATPase. Hal tersebut menunjukkan bahwa econazol tidak dapat menghambat interaksi antara thapsigargin dengan Ca^{2+}-ATPase untuk meningkatkan Ca^{2+} intraseluler. Sebagai kesimpulan, econazol menghambat peningkatan Ca^{2+} intraseluler dengan menghambat kanal Ca^{2+} tergantung voltase, namun tidak mempengaruhi jalur Ca^{2+}-ATPase.
Kata kunci : econazol, Ca^{2+}-ATPase, CRAC current, thapsigargin

Introduction

Econazole is an azole antifungal agent with a broad spectrum activity, that penetrates the stratum corneum and achieves effective concentrations down to the mid-dermis. It is poorly absorbed into the blood (less than 1 %). Clinically, econazole is used only for tropical application (Rang, *et al.,* 2003; Brunton, *et al.,* 2008). The chemical structure of econazole is shown at Fig.1.

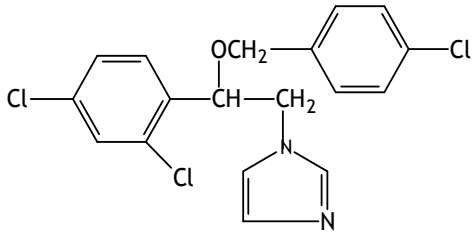

Figure 1. Chemical structure of Econazole.

Mast cells have long been considered to be involved in many different acute and chronic inflammatory processes including acting in delayed and immediate hypersensitivity reactions (Bienenstock, *et al.,* 1987; Galli, 1993). Type I hypersensitivity reaction or allergy can be developed in present of allergen such as grass pollen, product from dead house dust mites, foodstuffs or some drug by evoking the production of antibodies of the IgE type that bind to mast cell and eoshinophils. Subsequently, the mast cells release some allergy mediators such as histamine, eicosanoids and cytokines. Among them, histamine has most important role in the inflammatory reactions and immune responses. Therefore, these mediators induce location-dependent effects such as hay fever in the nose, the initial phase of asthma in the bronchial tree, urticaria in the skin or gastrointestinal tractus irritations (Rang, *et al.,* 2003). Allergy reaction in our body can be triggered by allergens, such as grass pollen, dust mite, certain foodstuffs or some drugs, that evoke the production of IgE type then attach to mast cell via the high-affinity FcεRI receptors on the cell surface (Rang, *et al.,* 2003). The cross-linkage of certain allergen into IgE antibody molecules on FcεRI receptors can generate a series of intracellular signalling such as the activation of protein tyrosine kinases and an increase of intracellular Ca^{2+} levels. Finally, these subsequent signalling events trigger the granule exocytosis releasing histamine from mast cells (Metcalfe *et al.,* 1997).

There are only few studies about the effect of econaloze, known as an antifungal agent, on rat mast cells. Previously, econazole blocked calcium release-activated calcium (CRAC) current in human leukaemic T cell line. Since this phenomenon also occurs in rat mast cells, it will be interesting to investigate the effect of econazole in rat mast cell such as RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells. In this article we studied the inhibitory effect of econazole on Ca^{2+} uptake into mast cells in response to thapsigargin, an ATP-dependent Ca^{2+} (SERCA) inhibitor, by direct measurement of radiolabelled Ca^{2+} uptake in cells. Furthermore, the mechanism underlying this effect of econazole was studied using molecular modelling.

Methodology

Materials

Econazole and thapsigargin were purchased from Sigma-Aldrich (St. Louis USA). The chemical structure of the compound is shown in Fig.1. Eagle's minimum essential medium (MEM) and antibiotics (combination of penicillin G sodium and streptomycin sulfate) were purchased from Gibco (Grand Island, NY, USA). Fetal calf serum was obtained from JRH Biosciences (Kansas, USA). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Dojindo (Kumamoto, Japan). Radiolabelled-calsium from Dupont-NEN (Boston,

USA). The Krebs solution with this following composition (mM) : NaCl 118.0; KCl 4.4; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; $MgSO_4$ 1.1; KH_2PO_4 1.2; glucose 11.0 (Sayah *et al.,* 1997), were purchased from Merck & Co., Inc. (New Jersey, USA). The solution must be made freshly, and continuously bubbled with 95% O_2 and 5% CO_2. Composition of Ca^{2+}-free Krebs solution was the same as that of previous Krebs except $CaCl_2$ was replaced by editic acid 0.1 mmol/L (Pang, *et al.,* 2003).

Culture of RBL-2H3 cells

RBL-2H3 cells (Department of Pharmacology, School of Medicine, Ehime University Japan) were cultured in MEM containing 15% fetal calf serum and antibiotics (penicillin and streptomycin) in a flask in a humidified atmosphere (5% CO_2) at 37 °C as described by Barsumian *et al.* (1981).The flask of 25 cm^2 containing approximately 1 x 10^5 cells/mL were used in the experiments. All addition into the medium was sterilized by filtration in a 0.22-μm millipore filter.

Assay of $^{45}Ca^{2+}$ Uptake

This experiment was conducted using RBL-2H3 cells in a 24 well-plate (Ali, *et al.,* 1993). After overnight incubation at 37 °C, the cells were washed twice with 500 μL of PIPES buffer and then preincubated for 10 min at 37 °C in 180 μL PIPES buffer either without (as a negative control) or with the drug. After preincubation, 20 μL of PIPES buffer containing 5μCi/mL of $^{45}Ca^{2+}$ and 5μM of thapsigargin as Ca^{2+} uptake stimulant was added into each well, and the plate was incubated at 37° C for 15 min. After this time, the reaction was stopped by washing with ice-cold Ca^{2+}-free buffer containing 100 μM La^{3+}. The cells were lysed with 0.3 mL of 0.1% Triton X, and 100 μL of the solution was combined with 10 mL of scintillation cocktail for radioactivity counting. The values were expressed as the percentage of maximum uptake in the absence of inhibitor compounds.

The contraction of trachea induced by extracelluler Ca^{2+}

Male guinea pigs weighing 300-450 g were sacrificed, and the trachea was gently removed and cut transversely into six rings, and the rings were cut longitudinally into strips. The strips of isolated trachea were then mounted in organ bath containing 20 mL Krebs solution (pH 7.4) aerated with 95% O_2 and 5% CO_2; and maintained at 37° C. The tissue strips were equilibrated for at least 60 min under a resting tension of 1 g, and the Krebs solution was replaced with the fresh solution every 15 min. Isotonic contractions were recorded by a level transducer (type 368 HSE, Germany) connected to a bridge amplifier (type 336 HSS, Germany) and a recorder (Kipp & Zonen BBD 41, The Netherlands). The tissue strips were 60-min equilibrated in Ca^{2+}-free Krebs solution. KCl solution (80 mmol/L) was administered in the strips for 5 min to depolarize the membrane of smooth muscle cells in order to activate voltage-dependent Ca^{2+} channels. Subsequently, $CaCl_2$ solution (10 mM) was added to contract the strips. Then, the tissue strips were washed and equilibrated. After administration of KCl for 5 min, the tissue strips were incubated with econazole (50 μM) for 10 min, and then followed by $CaCl_2$ to contract the strips.

Molecular docking study

The action mechanism of econazole through inhibition on Ca^{2+} ATPse of sarcoplasmic reticulum was investigated by using molecular docking study. The structure of econazole was docked onto the main protein target of Ca^{2+} ATPse, namely 3FGO.PDB. The docking was performed using PLANTS (Protein Ligand ANT System).

Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. *P*-values less than 0.05 were considered significant.

Result and Discussion

The effect on $^{45}Ca^{2+}$ influx

The effect of econazole on intracellular Ca^{2+} signaling was investigated by direct measurement of radiolabelled Ca^{2+} uptake in RBL-2H3 cells. In absence of thapsigargin, the $^{45}Ca^{2+}$ influx into mast cells occurred normally, however, in present of thapsigargin the influx of $^{45}Ca^{2+}$ increased 3-4 time fold. Thapsigargin causes Ca^{2+} uptake by stimulating opening of cell membrane Ca^{2+} channels. In absence of thapsigargin, econazole inhibited the normal $^{45}Ca^{2+}$ influx into mast cells (Fig. 2).

In presence of thapsigargin, econazole markedly inhibited intracellular $^{45}Ca^{2+}$ influx stimulated by thapsigargin incorporation dose-dependently (Fig. 3). Intracellular accumulation of $^{45}Ca^{2+}$ was almost completely inhibited at 100 μM, the highest concentration tested. Econazole at the concentration of 3, 10 and 100 μM significantly decreased the $^{45}Ca^{2+}$ influx by 11.64±3.37%, 52.62±5.21%, and

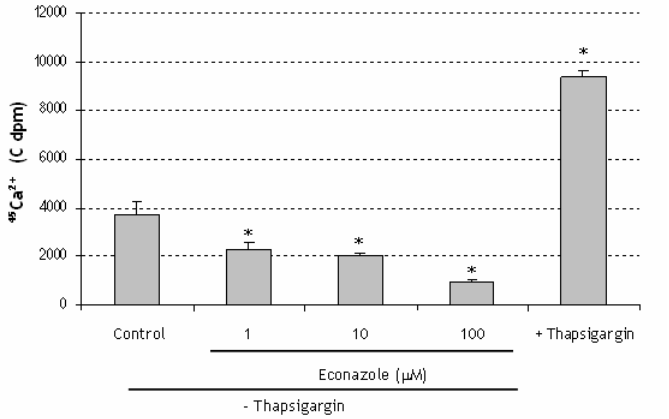

Figure 2. Effects of econazole (1, 10, 100 μM) or thapsigargin (0.5μM) on intracelluler $^{45}Ca^{2+}$ in RBL-2H3 cells. The data were representative of 3-5 independent experiments. *Significant difference ($P<0.05$) compared to the control value.

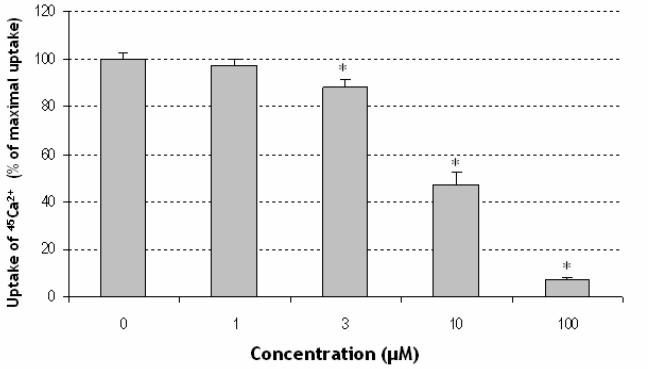

Figure 3. Inhibition of $^{45}Ca^{2+}$ uptake by econazole in thapsigargin (0.5μM)-stimulated RBL-2H3 cells. The data were representative of 3-5 independent experiments. *Significant difference ($P<0.05$) compared to the control value.

92.42±0.64%, respectively. IC_{50} value of inhibitory effect of econazole on $^{45}Ca^{2+}$ influx is 9.55 μM.

Effect on the contraction of trachea induced by extracelluler Ca^{2+}

The administration of KCl (80 mmol/L) functions to depolarize the cells membrane of tracheal smooth muscle. In the study, the administration of $CaCl_2$ (10 mM) obviously stimulated contraction of tracheal smooth muscle in Ca^{2+}-free Krebs solution. Pre-treatment with econazole (50 μM) inhibited the contraction effect of $CaCl_2$. The incubation of marmin at 10 min prior to $CaCl_2$ markedly decreased the observation time-response curve of $CaCl_2$ (Fig 4).

Activity on Ca^{2+} ATPase : molecular docking study

In the study, we also investigated the action mechanism of econazole whether through inhibition on Ca^{2+} ATPse of sarcoplasmic reticulum by using molecular docking study. The structure of econazole was docked onto the main protein target of Ca^{2+} ATPse, namely 3FGO. PDB. The result shown that the score of interaction of econazole with 3FGO. PDB was -92.277, and this of thapsigargin with 3FGO.PDB was -117.205.

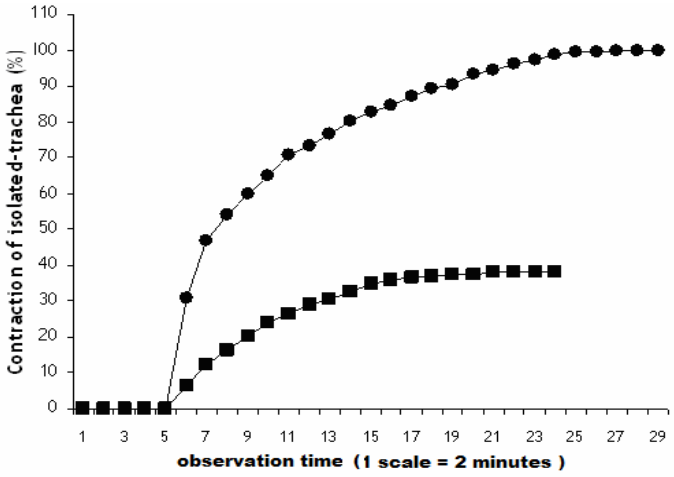

Figure 4. Time observation-response curves to $CaCl_2$ in the absence (●) or presence of econazole at concentration of 50 μM (■) in Ca^{2+}-free Krebs solution (Data represent mean±SEM, n=3).

Whereas, the energy of interaction of native ligan with 3FGO.PDB was -76.941. The score of interaction is equal to energy of interaction. The molecular interaction of econazol with 3FGO. PDB, a main protein target of Ca^{2+} ATPse represented at fig. 5-6.

Allergy reaction can be triggered by allergens that evoke the production of IgE type then attach to mast cell via the high-affinity FcεRI receptors on the cell surface. The cross-linkage of certain allergen into IgE antibody molecules on FcεRI receptors stimulate an increase of intracellular Ca^{2+} levels that trigger histamine-containing granule exocytosis from mast cells (Rang, *et al.,* 2003; Metcalfe, *et al.,* 1997). Thapsigargin, a sesquiterpene lactone isolated from the plant *Thapsia garginica*, is a stimulant for histamine release from mast cells. Its target is the ATP-dependent Ca^{2+} pump in the endoplasmic reticulum, and it can increase the concentration of cytosolic free calcium ion (Patkar, *et al.,* 1979; Brayden, *et al.,* 1989). Ca^{2+} release from intracellular store plays a major role in the opening of cell membrane Ca^{2+} channels to cause Ca^{2+} influx in mast cells (Metcalfe, *et al.,* 1997).

In phenomenon of calcium release activated calcium (CRAC) current in mast cells, endoplasmic reticulum has an important role as an intracellular pool in activation of calcium influx by plasma membrane channels, named as store-operated calcium channels (SOC channels). In turn, this activation induced CRAC current that selective for calcium ion (Zweifach and Lewis , 1993; Parekh, *et al.,* 1997; Alonso, *et al.,* 2008). There are two components have main role in homeostasis of intracellular calcium i.e. STIM1 (store Ca^{2+} sensor), and pore-forming CRAC channels subunit CRACM1 (or Orai1). STIM1 is a transmembrane protein of the sarcoplasmic/ endoplasmic reticulum that contribute as a crucial calcium sensor coupling the process of store depletion with calcium influx through the CRAC channels. CRACM1 is a calcium-release-activated calcium channel in the plasma membrane, and an important molecular component for SOC channels (Alonso, *et al.,* 2008; Roos, *et al.,* 2005, Vig and Kinet, 2007).

The effect of econazole on intracellular Ca^{2+} has long been studied but is still interesting for further investigation. The previous study, econazole and miconazole inhibited the depolarization-induced and receptor-operated contraction of guinea-pig isolated trachea (Li, *et al.,* 1997). Related to our study, econazole was also able to block Ca^{2+} release activated Ca^{2+} current (ICRAC) in T lymphocytes at the extracellular site. In order to inhibite ICRAC, the compound interacts with extracellular domain of the channel protein directly, rather than affects intracellular pathways (Christian, *et al.,* 1996). Huang, *et al.*

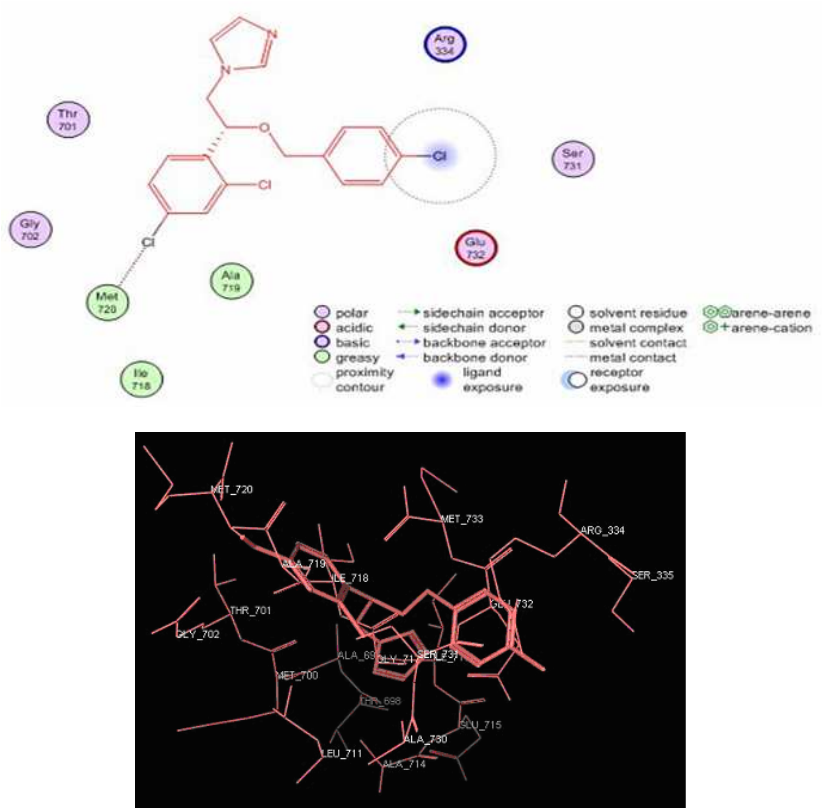

Figure 5. Molecular interaction of econazol with 3FGO. PDB, a main protein target of Ca^{2+} ATPse using PLANTS (Protein Ligand ANT System).

(2005) reported that econazole depleted the intracellular Ca^{2+} stores, and totally prevented thapsigargin from releasing more Ca^{2+} in human PC3 prostate cancer cells.

In present study, econazole inhibited $^{45}Ca^{2+}$ influx into mast cells in absence of thapsigargin. In this condition (without thapsigargin), ATP-dependent Ca^{2+} pump work normally in the sarcoplasmic reticulum, and can control the level of cytosolic free calcium ions. In presence of thapsigargin, ATP-dependent Ca^{2+} pump is inhibited causing increasing of cytosolic free calcium ions, and then activate the voltage-dependent Ca^{2+} channels, so that stimulate Ca^{2+} influx. Moreover, Ca^{2+} influx and protein kinase C sinergically trigger the mediator release from mast cells. Econazole potently suppressed the $^{45}Ca^{2+}$ influx induced by thapsigargin. It indicates that econazole alters some intracellular signalling events in mast cells, especially by blocking Ca^{2+} uptake into mast cells. In the study, econazole also inhibited Ca^{2+}-induced tracheal contraction. The tracheal smooth muscle was previously evoked by KCl to cause membrane depolarization, then activates the voltage-dependent Ca^{2+} channels. This condition allowed Ca^{2+} influx from extracellular side through these channels. The increase of intracellular Ca^{2+} from Ca^{2+} influx caused the formation of complex of intracellular Ca^{2+}-calmodulin. The MLCK is activated by binding to the complex. The contraction of smooth muscle is triggered when myosin light chain (MLC) is phosphorylated by myosin-light-chain kinase (MLCK) (Rang *et al*, 2003). The last finding supports the previous result that econazole depleted the intracellular Ca^{2+} through blockade of Ca^{2+} influx.

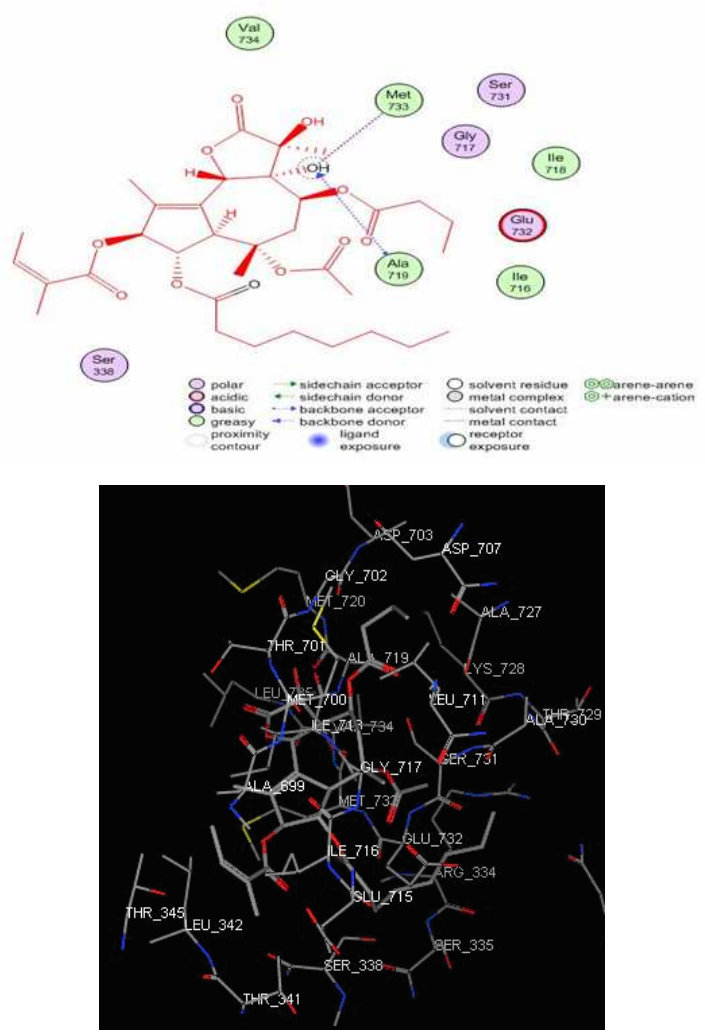

Figure 6. Molecular interaction of thapsigargin with 3FGO.PDB, a main protein target of Ca^{2+} ATPse using PLANTS (Protein Ligand ANT System).

To investigate whether action mechanism of econazole through the pathway of Ca^{2+} ATPse of sarcoplasmic reticulum, we used the molecular docking study. The structure of econazole was docked onto 3FGO.PDB, a main protein target on Ca^{2+} ATPase using PLANTS (Protein Ligand ANT System). The score of interaction of FGO with native ligan, thapsigargin and econazole were -76.941, -117.205, and -92.277, respectively. The score of interaction is equal to energy of interaction. It suggests that both compounds could interact with Ca^{2+} ATPase, and the affinity of thapsigargin to the pump is more potent than this of econazole. Inhibition on the Ca^{2+} ATPse of sarcoplasmic reticulum could decrease the Ca^{2+} movement to sarcoplasmic reticulum. In turn, it could increase intracellular Ca^{2+}, and stimulated the Ca^{2+} influx. Fig. 2, thapsigargin increased the intracellular $^{45}Ca^{2+}$ potently. Based on the molecular docking study, econazole could inhibit Ca^{2+}-ATPase, and then increase the intracellular Ca^{2+}. In fact, econazole decreased intracellular $^{45}Ca^{2+}$ in RBL-2H3 cells (fig. 2). This fact indicates that econazole decrease the intracellular Ca^{2+} not involving the Ca^{2+}-ATPase pathways.

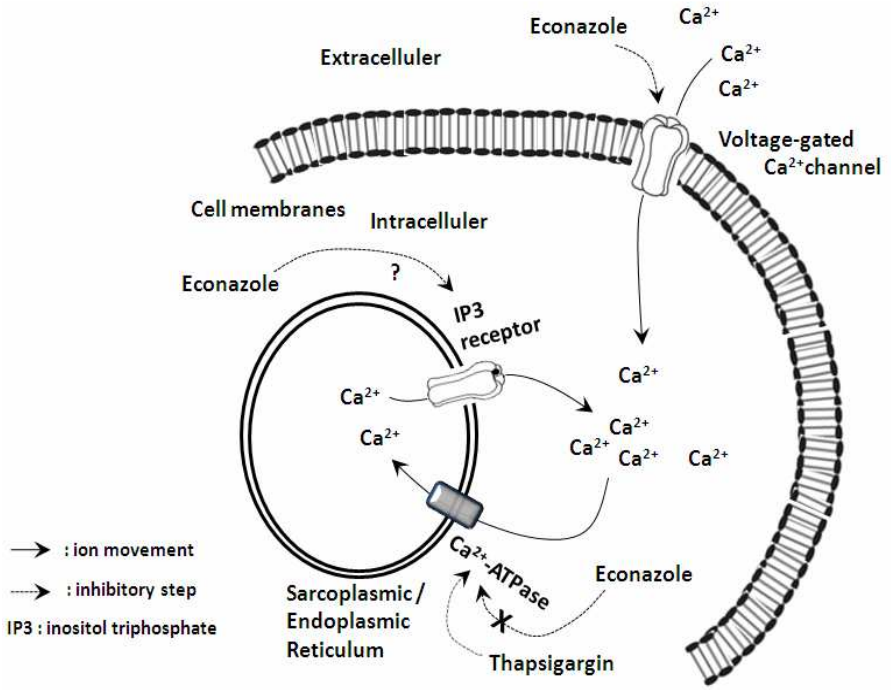

Figure 7. Proposed mechanism of actions of econazole.

Based on the docking study, the affinity of thapsigargin to Ca^{2+}-ATPase was higher than this of econazole. It would be difficult for econazole to block the interaction of thapsigargin with Ca^{2+}-ATPase to increase intracellular Ca^{2+}. In fact, econazole potently inhibited the intracellular $^{45}Ca^{2+}$ in RBL-2H3 cells induced by thapsigargin (fig. 3). These findings indicate that econazole decreased the intracellular Ca^{2+} not involving the Ca^{2+}-ATPase pathways. Econazole might blockade the Ca^{2+} uptake at cell membranes through blockade of voltage-dependent Ca^{2+} channels (Fig. 7).

Conclusion

In conclusion, econazole inhibited the increase of intracellular Ca^{2+} induced by thapsigargin not involving the blockade of the Ca^{2+}-ATPase. Econazole might blockade the Ca^{2+} uptake at cell membranes.

Acknowledgements

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