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A Pyrazole Derivative, YM-58483, Potently Inhibits Store-Operated Sustained Ca\(^{2+}\) Influx and IL-2 Production in T Lymphocytes

Jun Ishikawa,\(^1\)* Keiko Ohga,* Taiji Yoshino,* Ryuichi Takezawa,* Atsushi Ichikawa,† Hirokazu Kubota,‡ and Toshimitsu Yamada*  

In nonexcitable cells, Ca\(^{2+}\) entry is mediated predominantly through the store depletion-dependent Ca\(^{2+}\) channels called store-operated Ca\(^{2+}\) (SOC) or Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels. YM-58483, a pyrazole derivative, inhibited an anti-CD3 mAb-induced sustained Ca\(^{2+}\) influx in acute T cell leukemia, Jurkat cells. But it did not affect an anti-CD3 mAb-induced transient intracellular Ca\(^{2+}\) increase in Ca\(^{2+}\)-free medium, nor anti-CD3 mAb-induced phosphorylation of phospholipase C\(\gamma\)1. It was suggested that YM-58483 inhibited Ca\(^{2+}\) influx through SOC channels without affecting the TCR signal transduction cascade. Furthermore, YM-58483 inhibited thapsigargin-induced sustained Ca\(^{2+}\) influx with an IC\(_{50}\) value of 100 nM without affecting membrane potential. YM-58483 inhibited by 30-fold the Ca\(^{2+}\) influx through SOC channels compared with voltage-operated Ca\(^{2+}\) channels, while econazole inhibited both SOC channels and voltage-operated Ca\(^{2+}\) channels with an equivalent range of IC\(_{50}\) values. YM-58483 potently inhibited IL-2 production and NF-AT-driven promoter activity, but not AP-1-driven promoter activity in Jurkat cells. Moreover, this compound inhibited delayed-type hypersensitivity in mice with an ED\(_{50}\) of 1.1 mg/kg. Therefore, we concluded that YM-58483 was a novel store-operated Ca\(^{2+}\) entry blocker and a potent immunomodulator, and could be useful for the treatment of autoimmune diseases and chronic inflammation. Furthermore, YM-58483 would be a candidate for the study of capacitative Ca\(^{2+}\) entry mechanisms through SOC/CRAC channels and for identification of putative Ca\(^{2+}\) channel genes. *Inflammation Research, Pharmacology Laboratories, 1Molecular Medicine Research, Molecular Medicine Laboratories, and †Medicinal Chemistry Research II, Pharmaceutical Co., Ltd., Tsukuba-shi, Ibaraki, Japan. E-mail address: ishikaj@yamanouchi.co.jp

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\(^{3}\)Abbreviations used in this paper: IP\(_3\), inositol triphosphate; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\); FLIPR, fluorometric imaging plate reader; MC, methylcellulose; PLC\(\gamma\)1, phospholipase C\(\gamma\)1; SOC, store-operated Ca\(^{2+}\); TNCB, 2,4,6-trinitrochlorobenzene; VOC, voltage-operated Ca\(^{2+}\).

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Materials and Methods  

**Materials**

YM-58483, 4-methyl-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide, and SK&F 96365 were synthesized by Yamaneuchi Pharmaceutical (Tokyo, Japan). YM-58483 was identified from the screen of a chemical library in a high throughput screening to find novel agents of inhibiting thapsigargin-induced sustained Ca\(^{2+}\) influx in Jurkat cells. Cyclosporin A was purchased from Sandoz (Basel, Switzerland). Econazole, ionomycin, nifedipine, thapsigargin, PMA, and penicillin-streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO). Fura 2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Prednisolone, 2,4,6-trinitrochlorobenzene (TNCB), EGTA, NaCl, KCl, MgCl\(_2\), 6H\(_2\)O, CaCl\(_2\), 2H\(_2\)O, D(+)-glucose, and HEPES were obtained from Nacalai Tesque (Kyoto, Japan). DMSO, acetone, and ethanol were purchased from Kanto Chemical (Tokyo, Japan). Methylcellulose (MC) was obtained from Shin-Etsu Chemical (Tokyo, Japan). Human anti-CD3 mAb, UCHT1, and C105 were purchased from R&D Systems (Minneapolis, MN) and Leinco Technologies (St. Louis, MO), respectively. Anti-phospholipase C\(\gamma\)1 (PLC\(\gamma\)1) mAb and anti-tyrosine phosphorylation mAb (4G10) conjugated with HRP were bought from Upstate Biology (Charlottesville, VA). Protease inhibitor mixture was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Sheep anti-mouse Ig Ab conjugated.
with HRP and protein G-Sepharose gel (4 Fast Flow) were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Anti-NF-ATc2 (4G6-G5.1) mAb was purchased from BD Pharmingen (San Diego, CA). RPMI 1640 medium, DMEM/F-12 medium, and heat-inactivated horse serum were purchased from Life Technologies (Grand Island, NY). FBS was purchased from JRH Biosciences (Lenexa, KS). Each chemical compound was dissolved in DMSO for experiments in vitro. TNCB was dissolved in 7% (w/v) and 0.25% (w/v) with a solvent containing 10% acetone and 90% ethanol. YM-58483 and prednisolone were suspended in 0.5% MC for oral administration.

Econazole (13) and SK/EF-96365 (14) were used as reference compounds because of their blocking of SOC channels, and nifedipine was used as a reference compound for the blocker of voltage-operated Ca2+ (VOC) channels (15).

**Cell culture**

Human T cell leukemia, Jurkat cells (E6-1) were purchased from American Type Culture Collection (Manassas, VA), and were cultured in RPMI 1640 medium with 10% FBS and a 100 U/ml penicillin-streptomycin solution at 37°C, humidified 5% CO2 incubator (CPD-170W; Hirasawa Works, Tokyo, Japan). A stable IL-2 reporter cell line, IL-2/Jurkat cells (16), and a murine neuroendocrine cell line, PC12-h cells (17), were kindly donated by Drs. K. Kuromitsu and S. Hashimoto, respectively (Yamanouchi Pharmaceutical). PC12-h cells were cultured in rat type I collagen-coated flasks (Biocoat; BD Biosciences, Bedford, MA) in DMEM/F-12 medium with 10% horse serum, 7% FBS, and 100 U/ml penicillin and streptomycin in a 37°C, humidified 5% CO2 incubator. Several chemicals were used for the present study: econazole (13), SK/EF-96365 (14), nifedipine (15), and nocodazole (16). SK/EF-96365 was synthesized in K. Kuromitsu’s laboratory. Nocodazole was purchased from Wako Pure Chemical (Osaka, Japan). A stable IL-2 reporter cell line, IL-2/Jurkat cells (20) was employed for the present study.

**Membrane preparation**

Membrane potential was measured using the fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Jurkat cells (1 × 10⁶/ml) were preincubated with 50 mM KCl for 20 min, and the intracellular Ca²⁺ concentration was measured at the endpoint of 20 min.

**Measurement of membrane potential**

Membrane potential was measured using the fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Jurkat cells (1 × 10⁶/ml) were suspended in HBSS buffer, and mixed with an equal amount of the membrane potential dye (FLIPR membrane potential assay kit; Molecular Devices). They were treated with 90 µl of dye and were added to each well of 96-well black/clear-bottom microplate (BD Biosciences), and incubated at 37°C for 30 min before experiment. Laser intensity of the FLIPR was set to provide a basal fluorescence signal of 2000 relative fluorescence, and measurements of fluorescence were taken at 6-s interval for the first 3 min, then at 20-s intervals for the remaining 8 min. An on-board 96-well pipetter allowed simultaneous addition of compound concentrations (20 µl) delivered at a rate of 10 µ/s, 10 s after the start of readings. Fluorescence changes were captured by a cooled charge-coupled device camera and integrated to an on-line personal computer. The depolarization level was presented as percentage of 50 mM KCl-induced response using values of 10 min after induction.

**Immunoprecipitation assay and Western blotting**

Jurkat cells (4 × 10⁷ cells/ml) were treated with varying concentrations of compounds for 10 min at room temperature. The cells were stimulated with 1 µM for anti-CDC2 mAb (C) or 1 µM for anti-PLCγ mAb for 20 min after stimulation. Jurkat cells (2 × 10⁶ cells/ml) were placed in a 96-well microplate (Nunclon Sterile Surface; Nunc International, Rochester, NY), and were stimulated with 20 µl of PHA (Sigma-Aldrich) in the CO2 incubator for 20 h, and the supernatant was collected from these cells after centrifugation (200 × g, 24°C for 3 min). The concentration of IL-2 in each supernatant was measured by the human IL-2 ELISA system (human IL-2 ELISA Kit DuoSet; Genzyme, Cambridge, MA). OD values at 450 nm were measured by microplate reader (Spectra Max 190; Molecular Devices).

**IL-2 production assay**

Jurkat cells (5 × 10⁶ cells/ml) were treated with 200 µl of anti-CD3 mAb (200 µg/ml) for 30 min, and the intracellular Ca²⁺ concentration was measured at an endpoint of 30 min. Each compound was added at the same time during thapsigargin stimulation.

**Comparative study on the inhibition of SOC channels**

It was noted that L-type VOC channels were expressed in PC12 cells (19). The inhibition of VOC channels was assessed in PC12-h cells (1 × 10⁶ cells/ml) after detachment from the flask using the protein digestive enzyme, Actinase E (Kaken Pharmaceutical, Tokyo, Japan), and fura 2 loading. In 96-well microplates, 200 µl of fura 2-loaded PC12-h cells was stimulated with 50 nM KCl for 20 min, and the intracellular Ca²⁺ concentration was measured at the endpoint of 20 min.

**Measurement of membrane potential**

Membrane potential was measured using the fluorescent imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Jurkat cells (1 × 10⁶/ml) were suspended in HBSS buffer, and mixed with an equal amount of the membrane potential dye (FLIPR membrane potential assay kit; Molecular Devices). Then 90 µl of dye and 90 µl of dye were added to each well of 96-well black/clear-bottom microplate (BD Biosciences), and incubated at 37°C for 30 min before experiment. Laser intensity of the FLIPR was set to provide a basal fluorescence signal of 2000 relative fluorescence, and measurements of fluorescence were taken at 6-s interval for the first 3 min, then at 20-s intervals for the remaining 8 min. An on-board 96-well pipetter allowed simultaneous addition of compound concentrations (20 µl) delivered at a rate of 10 µ/s, 10 s after the start of readings. Fluorescence changes were captured by a cooled charge-coupled device camera and integrated to an on-line personal computer. The depolarization level was presented as percentage of 50 mM KCl-induced response using values of 10 min after induction.
NF-AT and AP-1 reporter gene assays
Jurkat cells (1 × 10^7 cells/ml) were transfected with 40 μg of pGL-
TATA, pGL(AP-1) or pGL(NF-AT), by means of electroporation under the condition of 280 V and 960 μF in 0.4-cm cuvettes (Gene Pulser Cuvette; Bio-Rad Laboratories, Hercules, CA). After 24 h transfection, pGL-
TATA-transfected cells were stimulated with 10 ng/ml PMA and 1 μM ionomycin, or 10 ng/ml PMA, pGL(AP-1) or pGL(NF-AT)-transfected cells were stimulated with PMA and ionomycin for 16 h. Compounds were added at the same time during stimulation at 0.1% DMSO. Luciferase activities were measured the same as IL-2 reporter gene assay. Each reporter plasmid was kindly provided by S. Kuromitsu (16).

NF-AT dephosphorylation assay
Jurkat cells (1 × 10^7 cells/ml) were tested with varying concentration of compounds for 30 min at 37°C. The cells were stimulated with 1 μM ionomycin for 30 min at 37°C. After stimulation, the cells were centrifuged at 200 × g for 2 min, and were solubilized in 100 μl of Triton X-100 lysis buffer. The cell lysate was centrifuged at 15,000 × g for 20 min; the clarified lysate was subjected to SDS-PAGE; and NF-ATc2 was detected by Western blotting with anti-NF-ATc2 mAb.

TNCB-induced contact hypersensitivity in mice
Five-week-old male CD-1 mice were obtained from Japan SLC (Shizuoka, Japan). The animals were treated with 100 μl of a 7% TNCB solution in ethanol on the abdominal region after cutting their abdominal hair under anesthesia at day 0. Seven days after the TNCB sensitization, the thickness of both ears from extracellular medium in Ca^2+ store in Ca^2+ medium. The peak Ca^2+ influx from Ca^2+ store and sustained Ca^2+ flux in the absence of extracellular Ca^2+ (Fig. 1). Neither YM-58483 nor econazole inhibited the transient increase in intracellular Ca^2+ in the absence of extracellular Ca^2+ (Fig. 1B). Moreover, YM-58483 (3 μM) did not affect the baseline intracellular Ca^2+ in Jurkat cells (Fig. 2).

Effects on anti-CD3 mAb-induced tyrosine phosphorylation of PLCγ1
YM-58483 (0.3–3 μM) and econazole (0.3–3 μM) did not affect the anti-CD3 mAb-induced tyrosine phosphorylation of PLCγ1 in Jurkat cells (Fig. 3, A and C). The amount of PLCγ1 protein did not change on stimulation with anti-CD3 or any compound (Fig. 3B).
Effects on the sustained Ca\(^{2+}\)/H\(^{100}\) influx via SOC channels

Thapsigargin evoked a transient increase in intracellular Ca\(^{2+}\)/H\(^{100}\) in the absence of extracellular Ca\(^{2+}\)/H\(^{100}\), and the subsequent addition of 2 mM CaCl\(_2\) induced a sustained Ca\(^{2+}\)/H\(^{100}\) influx (Fig. 4). Pretreatment with YM-58483 (0.01–10 \(\mu\)M) and econazole (0.1–10 \(\mu\)M) 1 min before addition of 2 mM CaCl\(_2\) inhibited the sustained Ca\(^{2+}\)/H\(^{100}\) influx in a concentration-dependent manner with IC\(_{50}\) values of 100 and 680 nM, respectively (Figs. 4 and 5; Table I). Furthermore, YM-58483 and econazole reduced concentration dependently the already evoked Ca\(^{2+}\)/H\(^{100}\) influx with the equivalent IC\(_{50}\) values compared with pretreatment (Figs. 5 and 6; Table I).

Comparison of inhibitory effects on SOC and VOC channels

YM-58483 inhibited thapsigargin-induced Ca\(^{2+}\)/H\(^{100}\) influx through SOC channels in Jurkat cells with an IC\(_{50}\) value of 150 ± 11 nM, which was equivalent to the value for the spectrofluorometer system (Fig. 7A; Table II). The VOC channel blocker, nifedipine, potentely inhibited the high concentration KCl-induced Ca\(^{2+}\)/H\(^{100}\) influx through VOC channels in PC12-h cells with an IC\(_{50}\) of 2.7 ± 0.41 nM, but not that through SOC channels (2.0% at 10 \(\mu\)M). The IC\(_{50}\) value of YM-58483 for VOC channels was 4700 ± 550 nM, which was 30-fold less than that for SOC channels (Fig. 7B; Table II).

Econazole and SK&F-96365 showed no selectivity for SOC channels and VOC channels (Table II).

Effects on membrane potential

The high concentration KCl (50 mM) caused remarkable depolarization in Jurkat cells (Fig. 8). YM-58483 (3 and 10 \(\mu\)M) did not affect membrane potential (Fig. 8, A and C). In contrast, econazole (3 and 10 \(\mu\)M) caused slight, but significant depolarization in a concentration-dependent manner (Fig. 8, B and C).

Effects on PHA-induced IL-2 production

The supernatant from Jurkat cells stimulated with PHA for 20 h contained a significant amount of IL-2 compared with that from nonstimulated cells (PHA (−), 15 pg/ml; PHA (+), 2126 pg/ml; \(p < 0.01\) by Student’s \(t\) test, \(n = 10\)). YM-58483 and cyclosporin A inhibited this IL-2 production in a concentration-dependent manner.
manner with IC\textsubscript{50} values of 17 ± 3.2 and 3.2 ± 0.45 nM, respectively (Fig. 9A; Table III). Econazole and SK&F-96365 also inhibited IL-2 production in a concentration-dependent manner, but these inhibitory effects were weaker than that of YM-58483 (Fig. 9B; Table III). The VOC channel blocker, nifedipine, did not inhibit IL-2 production from Jurkat cells.

**Effects on IL-2, NF-AT, and AP-1 reporter gene expression**

YM-58483, econazole, and cyclosporin A inhibited IL-2 reporter gene expression in a concentration-dependent manner with IC\textsubscript{50} values of 1.31, 1700 ± 350, and 4.8 ± 1.8 nM, respectively (Fig. 10; Table IV). SK&F-96365 also inhibited IL-2 reporter gene expression in a concentration-dependent manner (51% at 10 \textmu M).

YM-58483 and cyclosporin A inhibited PMA/ionomycin-induced NF-AT reporter gene activity in a concentration-dependent manner in pGL(NF-AT)\textsubscript{3} -transfected Jurkat cells (Fig. 11). In contrast, these compounds did not affect PMA-induced AP-1 reporter gene activity in pGL(AP-1)\textsubscript{4} -transfected cells. Then pGL-TATA reporter gene activity was not affected by the stimulation of PMA/ionomycin or PMA.

**Effects on NF-AT dephosphorylation**

YM-58483 (0.03–3 \textmu M) and cyclosporin A (0.01–1 \textmu M) inhibited PMA/ionomycin-induced dephosphorylation of NF-AT in a concentration-dependent manner in Jurkat cells (Fig. 12).

**FIGURE 5.** Typical pattern on posttreatment with YM-58483 (0.01–10 \textmu M) and econazole (0.1–10 \textmu M) of the sustained Ca\textsuperscript{2+} influx in Jurkat cells. Cells (2 × 10\textsuperscript{6}/ml) were stimulated with 1 \textmu M thapsigargin for 10 min, and each concentration of compound was added 10 min after the stimulation with thapsigargin.

**FIGURE 6.** Effects on Ca\textsuperscript{2+} influx in Jurkat cells. A, Effect of pretreatment with YM-58483 (■, 0.01–10 \textmu M) and econazole (●, 0.03–10 \textmu M) on exogenous 2 mM Ca\textsuperscript{2+} induced sustained Ca\textsuperscript{2+} influx in thapsigargin-stimulated cells (see Fig. 3). B, Effect of posttreatment with YM-58483 (■, 0.003–10 \textmu M) and econazole (●, 0.01–10 \textmu M) on the thapsigargin-induced sustained Ca\textsuperscript{2+} influx (see Fig. 4). Each point is calculated from the values of 10 min (see Figs. 3 and 4) and represents the mean ± SE of three to five experiments.

**FIGURE 7.** Effects on SOC and VOC channels. A, Effects of YM-58483 (●, 0.003–10 \textmu M) and econazole (▲, 0.003–10 \textmu M), SK&F-96365 (■, 0.003–10 \textmu M), and nifedipine (●, 0.003–1 \textmu M) on Ca\textsuperscript{2+} influx through SOC channels induced by 1 \textmu M thapsigargin in Jurkat cells. B, Effects of YM-58483 (●, 0.003–10 \textmu M), econazole (▲, 0.003–10 \textmu M), SK&F-96365 (■, 0.003–10 \textmu M), and nifedipine (●, 0.0003–1 \textmu M) on Ca\textsuperscript{2+} influx through VOC channels induced by 50 mM KCl in PC12-h cells. Each point represents the mean ± SE of 8–10 experiments.

<table>
<thead>
<tr>
<th>IC\textsubscript{50} Value (nM)</th>
<th>Pretreatment\textsuperscript{a}</th>
<th>Posttreatment\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM-58483</td>
<td>100 (68–140)</td>
<td>140 (110–180)</td>
</tr>
<tr>
<td>Econazole</td>
<td>680 (100–6100)</td>
<td>690 (460–1100)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each concentration of compound was added 10 min after thapsigargin stimulation in the absence of extracellular Ca\textsuperscript{2+}; and Ca\textsuperscript{2+} influx was evoked by addition of exogenous 2 mM CaCl\textsubscript{2}.

\textsuperscript{b}Each concentration of compound was added 10 min after the stimulation of thapsigargin.

**Table 1. Effects of YM-58483 and econazole on thapsigargin-induced sustained Ca\textsuperscript{2+} influx in Jurkat cells**
Membrane potential.

B 50 mM KCl (•) was used. F Potential assay kit for 10 min. As a positive control of depolarization, 50 mM KCl-induced membrane potential was measured by FLIPR system using FLIPR membrane

Effects on the membrane potential in Jurkat cells. Membrane potential was measured by FLIPR system using FLIPR membrane

FIGURE 8. Effects on the membrane potential in Jurkat cells. Membrane potential was measured by FLIPR system using FLIPR membrane potential assay kit for 10 min. A, Effect of YM-58483 (3 μM, ○; 10 μM, △) on the membrane potential. B, Effect of econazole (3 μM, △; 10 μM, ▲) on the membrane potential. C, The net depolarization level was presented as percentage of the 50 mM KCl-induced fluorescence change. Each compound was compared with 0.1% DMSO at the values of 10 min. Each point and column represents the mean ± SE of seven experiments. *, p < 0.05; **, p < 0.01 vs 0.1% DMSO (Dunnett’s multiple range test).

TNCC-induced contact hypersensitivity in mice

TNCC-induced ear swelling was significantly increased in control animals compared with the vehicle-challenged negative control

Discussion

The stimulation of TCRs with anti-CD3 mAb results in the initiation of a signal transduction cascade leading to T cell activation. The earliest biochemical event in this cascade is tyrosine phosphorylation of PLCγ1 (20), which leads to the formation of IP3 and is followed by the stimulation of IP3-sensitive Ca2+ stores and an increase in intracellular Ca2+. In the presence of extracellular Ca2+, anti-CD3 mAb evoked a transient peak, followed by a sustained increase in intracellular Ca2+. In contrast, anti-CD3 mAb evoked only a transient increase in intracellular Ca2+ in the absence of extracellular Ca2+, in which the maximum Ca2+ concentration was evidently diminished by the levels of econazole-induced inhibition observed in the presence of extracellular Ca2+. It is noted that econazole is a blocker of store-operated Ca2+ entry through SOC channels (21), and inhibits ICRCRAC interacting with the outside of the membrane in lymphocytes (22). These results suggest that the transient peak of intracellular Ca2+ observed in the presence of extracellular Ca2+ consists of two components, one derived from Ca2+ stores and one from the extracellular environment. In the absence of extracellular Ca2+, YM-58483 and econazole partially, but significantly inhibited the anti-CD3 mAb-induced transient increase in intracellular Ca2+, and YM-58483 completely inhibited the sustained Ca2+ influx. YM-58483 and econazole did not inhibit the transient increase in intracellular Ca2+ in the absence of extracellular

FIGURE 9. Effects of YM-58483 (C, 0.0003–1 μM), cyclosporin A (●, 0.0003–1 μM), econazole (▲, 0.003–10 μM), SK&F-96365 (■, 0.3–10 μM), and nifedipine (♦, 0.3–10 μM) on PHA-induced IL-2 production in Jurkat cells. Jurkat cells (5 × 10⁶/ml) were incubated with PHA (20 μg/ml) for 24 h in the 96-well microplates. IL-2 content in supernatant was measured by ELISA. Each point represents the mean ± SE of four to five experiments.
Table III. Inhibitory effects of YM-58483, other Ca\(^{2+}\) entry blockers, and cyclosporine A on PHA-induced IL-2 production from Jurkat cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) Value (nM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM-58483</td>
<td>17 ± 3.2</td>
<td>89% at 1 (\mu)M</td>
</tr>
<tr>
<td>Econazole</td>
<td>&gt;10,000</td>
<td>63% at 10 (\mu)M</td>
</tr>
<tr>
<td>SK&amp;F-96365</td>
<td>&gt;10,000</td>
<td>40% at 10 (\mu)M</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>&gt;10,000</td>
<td>12% at 10 (\mu)M</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>3.2 ± 0.45</td>
<td>92% at 1 (\mu)M</td>
</tr>
</tbody>
</table>

Moreover, these compounds did not affect the phosphorylation of PLC-\(\gamma\) on TCR stimulation. Therefore, it is suggested that YM-58483 as well as econazole inhibit the sustained Ca\(^{2+}\) influx without affecting the TCR signal transduction cascade leading to the stimulation of IP\(_3\)-sensitive Ca\(^{2+}\) stores.

YM-58483 and econazole inhibited a sustained Ca\(^{2+}\) influx following pretreatment, and reduced the already evoked sustained Ca\(^{2+}\) influx after posttreatment. When YM-58483 interacts with some unidentified cytosolic molecules, which regulate Ca\(^{2+}\) influx from SOC channels, the inhibitory effects of these compounds on posttreatment must be weaker than that pretreatment and must indicate a slow reducing pattern. But, in the present study, YM-58483 and econazole inhibited a sustained Ca\(^{2+}\) influx both pre- and posttreatment to the same degree. Moreover, YM-58483 and econazole rapidly reduced the sustained Ca\(^{2+}\) influx posttreatment. It is noted that membrane depolarization reduces Ca\(^{2+}\) influx in lymphocytes (7). Indeed, it was reported that KCl-induced membrane depolarization inhibited Ca\(^{2+}\) influx in the CD3-activated Jurkat cells (18). In the present study, YM-58483 did not change membrane potential in Jurkat cells. In contrast, econazole caused slight, but significant depolarization in a concentration-dependent manner.

Table IV. Inhibitory effects of YM-58483, other Ca\(^{2+}\) entry blockers, and cyclosporine A on PHA-induced IL-2 reporter gene expression in Jurkat cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) Value (nM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM-58483</td>
<td>10 ± 3.1</td>
<td>99% at 1 (\mu)M</td>
</tr>
<tr>
<td>Econazole</td>
<td>1,700 ± 350</td>
<td>86% at 10 (\mu)M</td>
</tr>
<tr>
<td>SK&amp;F-96365</td>
<td>&gt;10,000</td>
<td>51% at 10 (\mu)M</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>4.8 ± 1.8</td>
<td>112% at 1 (\mu)M</td>
</tr>
</tbody>
</table>

FIGURE 10. Effects on PHA-induced IL-2 gene expression in IL-2/Jurkat cells. A, Effects of YM-58483 (0.0003–1 \(\mu\)M), cyclosporin A (0.0003–1 \(\mu\)M), econazole (0.003–10 \(\mu\)M), and SK&F-96365 (0.003–10 \(\mu\)M) on IL-2 reporter gene expression. Stable IL-2 reporter clone IL-2/Jurkat cells (2 \(\times\) 10\(^3\)/ml) were incubated with PHA (20 \(\mu\)g/ml) for 24 h in 96-well microplates. IL-2 gene expression was detected as luciferase activity and presented as fold induction from the nonstimulated luciferase (vehicle). B, Percentage of inhibition of YM-58483 (●), cyclosporin A (○), econazole (▲), and SK&F-96365 (■) on IL-2 reporter gene expression. Each point represents the mean ± SE of four experiments. **, \(p < 0.01\) vs PHA (Dunnett’s multiple range test).

FIGURE 11. Effects on NF-AT and AP-1 transcriptional activity in Jurkat cells. Upper, Effects of the stimulation of 10 ng/ml PMA and 1 \(\mu\)M ionomycin or 10 ng/ml PMA on luciferase activity in pGL-TATA-transfected cells (left). Effects of YM-58483 (0.3 and 3 \(\mu\)M) and cyclosporin A (0.1 and 1 \(\mu\)M) on PMA-induced AP-1 reporter gene activity in pGL(AP-1)-transfected cells (right). Lower, Effects of YM-58483 (0.0003–3 \(\mu\)M) and cyclosporin A (0.0001–1 \(\mu\)M) on PMA/ionomycin (P/I)-induced NF-AT reporter gene activity in pGL(NF-AT)-transfected cells. Reporter gene activity was detected as luciferase activity and presented as fold induction from the nonstimulated luciferase activity (vehicle). Each column represents the mean ± SE of four to six experiments. **, \(p < 0.01\) vs P/I (Dunnett’s multiple range test).
manner. These results indicated that YM-58483 inhibited Ca\textsuperscript{2+} influx without affecting membrane potential, and econazole inhibited Ca\textsuperscript{2+} influx partly through membrane depolarization. Therefore, it is suggested that YM-58483 inhibited store-operated Ca\textsuperscript{2+} entry by directly interacting with SOC channels or channel-regulatory cofactors at the membrane surface without affecting the depletion of Ca\textsuperscript{2+} stores.

It has been reported that econazole and SK&F-96365 inhibited store-operated Ca\textsuperscript{2+} influx through SOC channels (13, 14, 22, 23). Indeed, econazole and SK&F-96365 inhibited Ca\textsuperscript{2+} influx through SOC channels in lymphocytes, but these compounds also inhibited Ca\textsuperscript{2+} influx through VOC channels in PC12-h cells over the same range of concentrations. In contrast, the effect of YM-58483 on SOC channels was 30 times more selective against Ca\textsuperscript{2+} influx than that through VOC channels, indicating that YM-58483 is a selective SOC channel blocker. These results suggest that YM-58483 is a novel Ca\textsuperscript{2+} entry blocker, which selectively inhibits Ca\textsuperscript{2+} through SOC channels, not VOC channels.

Ca\textsuperscript{2+} influx through SOC channels is a key event for immune responses, including IL-2 production in lymphocytes. The IL-2 gene is up-regulated by NF-AT, which needs a sustained increase of intracellular Ca\textsuperscript{2+} via the activation of SOC channels (4). YM-58483 inhibited IL-2 production from PHA-stimulated Jurkat cells as potently as cyclosporin A. It is noted that cyclosporin A inhibits the interaction of calcineurin with NF-AT, and calcineurin is activated by Ca\textsuperscript{2+} and calmodulin (5, 6). In our preliminary studies, cyclosporin A, unlike YM-58483, did not inhibit the store-operated Ca\textsuperscript{2+} influx in Jurkat cells (data not shown). Furthermore, YM-58483 as well as cyclosporin A inhibited IL-2 reporter gene expression in PHA-stimulated stable IL-2/Jurkat cells. Econazole and SK&F-96365 also inhibited IL-2 production and IL-2 reporter gene expression. Therefore, it is suggested that YM-58483 inhibits the transcription of NF-AT and production of IL-2 by inhibiting Ca\textsuperscript{2+} influx. Indeed, YM-58483 inhibited NF-AT dephosphorylation and NF-AT reporter gene activity without affecting AP-1 reporter gene activity as well as cyclosporin A in the present study. It is noted that AP-1 activation is regulated by Ca\textsuperscript{2+}-independent Ras/Raf pathway (24, 25), which supports that YM-58483 did not affect PMA-induced AP-1 reporter gene activity in the present study. TCR-mediated signaling is coupled to the downstream phosphorylation of PLC\textgamma, which activates both PI\textgamma and Ras/Raf pathways (26, 27). YM-58483 did not inhibit PMA/ionomycin-induced phosphorylation of PLC\textgamma and TCR-stimulated transient Ca\textsuperscript{2+} efflux from Ca\textsuperscript{2+} store, indicating that YM-58483 regulates the activation of NF-AT without affecting TCR signaling. Therefore, it is suggested that the inhibition of sustained Ca\textsuperscript{2+} influx is main mechanism of the immune modulatory action of this compound. Furthermore, YM-58483 as well as prednisolone orally inhibited TNCB-induced contact hypersensitivity in mice. The contact hypersensitivity is regarded as a prototype of T lymphocyte-mediated delayed-type hypersensitivity reactions (12). This suggested that YM-58483 could be a useful immune modulator in autoimmune diseases or chronic inflammation. Therefore, it is concluded that YM-58483 is an in vivo effective novel Ca\textsuperscript{2+} entry blocker, and is a new-type immune modulatory agent via the different mechanism from well-known immunosuppressants such as cyclosporin A or FK506.

It was recently reported that PHA stimulates the increased number of SOC channels in T lymphocytes (11), indicating that intracellular Ca\textsuperscript{2+} signaling is enhanced in activated T lymphocytes, and inhibition of Ca\textsuperscript{2+} influx is important for the regulation of T lymphocytes. In the present study, YM-58483 did not affect baseline intracellular Ca\textsuperscript{2+} levels, as shown in Fig. 2, suggesting that YM-58483 would not influence resting lymphocytes. Therefore, YM-58483 could be a potent regulator for the activation of T lymphocytes, and could be useful for the treatment of autoimmune diseases and chronic inflammation. Furthermore, YM-58483 could be a useful tool for the study of capacitative Ca\textsuperscript{2+} entry mechanisms through SOC/CRAC channels and putative Ca\textsuperscript{2+} entry channel genes.

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References