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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 nonexsicitable 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.

Intracellular Ca\(^{2+}\) has a critical role in signal transduction in various cell types. In electrically nonexcitable cells, the stimulation of cell surface receptors produces a transient intracellular Ca\(^{2+}\) increase via Ca\(^{2+}\) influx from inositol triphosphate (IP\(_3\))-sensitive Ca\(^{2+}\) stores, and a subsequent sustained Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) (SOC) channels, noted as Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels (1, 2). Depletion of the stores stimulates a Ca\(^{2+}\) current called I\(_{\text{CRAC}}\). A sustained Ca\(^{2+}\) influx is critical for Ca\(^{2+}\)-sensitive transcription of NF-AT and the expression of genes such as the IL-2 gene (3, 4). The nuclear transport of NF-AT requires a sustained increase in the intracellular Ca\(^{2+}\) concentration, which is regulated by SOC channels (4). Cyclosporin A and FK506 inhibit the calcineurin-dependent NF-AT dephosphorylation, which is required for nuclear transport and function of NF-AT in the expression of IL-2 and other cytokine genes (5, 6). Therefore, a sustained Ca\(^{2+}\) influx through SOC channels is a key event for the activation of T lymphocytes and production of cytokines. Furthermore, the Ca\(^{2+}\) influx through SOC channels participates in various immune and inflammatory responses, because I\(_{\text{CRAC}}\) was reported in other inflammatory leukocytes including basophils, macrophages (7, 8), dendritic cells (9), and CTLs (10). Dendritic cells and CTLs are noted as critical immune regulatory cells. Recently, it was reported that the expression of SOC channels was up-regulated in activated T lymphocytes, but low numbers of SOC channels were sufficient to maintain a resting state (11). This suggests that SOC channels play an important role in immune responses and inflammatory events.

In the present study, we investigated the effects of YM-58483, a pyrazole derivative, on Ca\(^{2+}\) influx and IL-2 production in Jurkat cells in vitro, and on T lymphocyte-mediated immune responses in vivo using a mouse model of delayed-type hypersensitivity (12).

Materials and Methods

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 Yamashita Pharmaceutical Company (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). Eczonazole, iomycin, 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 Nacali 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

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with HRP and protein G-Sepharose gel (4 Fast Flow) were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Anti-NF-ATc2 (4G6-G51.1) 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 obtained from JRH Biosciences (Lenexa, KS). Each chemical compound was dissolved in DMSO for experiments in vitro. TNBC 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&F-96365 (14) were used as reference compounds for the blocker of SOC channels, and nifedipine was used as a reference compound for the blocker of voltage-operated Ca<sup>2+</sup> (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 in a 37°C, humidified 5% CO<sub>2</sub> incubator (CPD-170W; Hirasaki 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 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% CO<sub>2</sub> incubator.

**Measurement of intracellular Ca<sup>2+</sup>** by spectrophotofluorometer

Jurkat cells (2 × 10<sup>6</sup> cells/ml) were suspended in HBSS of the following composition (mM): NaCl, 137; KCl, 5.8; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 2.5; glucose, 5; and HEPES, 10, pH 7.4, and were loaded with 1 μM fura 2-AM at room temperature for 45 min. Following centrifugation (200 × g, 24°C for 3 min, himac CF 7D2; Hitachi, Tokyo, Japan), cells were successively washed 3 times with ionic-uniformed dye and resuspended in Ca<sup>2+</sup>-free HBSS. The fluorescence of the cell suspension was monitored by spectrophotofluorometer (CAF-110; JASCO, Tokyo, Japan) at an emission wavelength of 500 nm and excitation wavelengths of 340 and 380 nm, respectively. The concentration of intracellular Ca<sup>2+</sup> was calculated from the 340/380 nm fluorescence ratio (R) with the standard equation (18). The Rmax value was obtained from the 25 μM ionomycin-induced fluorescence ratio, and the Rmin value from the fluorescence ratio on additional treatment with 50 mM EGTA after ionomycin. A dissociation constant (Kd) of 224 nM was used for the calculation of intracellular Ca<sup>2+</sup>.

**Anti-CD3 mAb-induced increase in intracellular Ca<sup>2+</sup>**

TCR-cross talk-linked intracellular Ca<sup>2+</sup> responses were induced by 10 μg/ml human anti-CD3 mAb (UCHT1). Each concentration of compound was added 1 min before TCR stimulation in the absence or presence of extracellular Ca<sup>2+</sup> (2 mM CaCl<sub>2</sub>).

**Thapsigargin-induced increase in intracellular Ca<sup>2+</sup>**

To investigate the inhibitory effects of compounds on Ca<sup>2+</sup> influx through SOC channels, Ca<sup>2+</sup> influx was evoked by 1 μM thapsigargin in Jurkat cells. 1) For the pretreatment experiment, Jurkat cells were stimulated by thapsigargin in Ca<sup>2+</sup>-free HBSS. Each concentration of compound was added 10 min after thapsigargin, and Ca<sup>2+</sup> influx was evoked by addition of exogenous 2 mM CaCl<sub>2</sub>. 2) For the posttreatment study, each concentration of compound was added after a sustained intracellular Ca<sup>2+</sup> influx had been evoked in the presence of extracellular Ca<sup>2+</sup> 10 min after thapsigargin stimulation.

**Measurement of intracellular Ca<sup>2+</sup> in 96-well microplates**

A 96-well microplate assay system was used in the comparative study on channel inhibition. Fluorescence of fura 2 was measured in a fluorescence microplate reader (Fluostar; SLT Labinstruments, Salzburg, Austria) with an excitation wavelength of 340 and 380 nm at an emission wavelength of 500 nm. The concentration of intracellular Ca<sup>2+</sup> in each well was calculated from the fluorescence ratio with the standard equation. The Rmax value was obtained from 25 μM ionomycin-treated wells. The Rmin value was obtained from 25 μM ionomycin- and 50 mM EGTA-treated wells.

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

Inhibition of SOC channels was evaluated in Jurkat cells (2 × 10<sup>6</sup> cells/ml). In 96-well microplates (MS-8496K; Sumitomo Bakelite, Tokyo, Japan), 200 μl of fura 2-loaded Jurkat cells was stimulated with 1 μM thapsigargin for 30 min, and the intracellular Ca<sup>2+</sup> 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 VOC 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 × 10<sup>6</sup> 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 mM KCl for 20 min, and the intracellular Ca<sup>2+</sup> concentration was measured at the endpoint of 20 min.

**Measurement of membrane potential**

Membrane potential was measured using the fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Jurkat cells (1 × 10<sup>6</sup> cells/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 cells 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 pipettor allowed simultaneous addition of compound concentrations (20 μl) delivered at a rate of 10 μl/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 × 10<sup>6</sup> cells/ml) were treated with varying concentration of compounds for 10 min at room temperature. The cells were stimulated with 20 μg/ml CD3 mAb (C105) for 2 min to induce PLCγ1 activity. The cell lysates were centrifuged at 15,000 × g for 20 min. Clarified lysate was incubated for 1 h at 4°C with 1 μg of anti-PLCγ1 mAb and 40 μl of 50% slurry of protein G-Sepharose. After washing, immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech AB). Membranes were reconstituted with BlockAce (Dainippon Pharmaceutical, Tokyo, Japan), followed by immunoblotting with HRP-conjugated anti-phosphotyrosine mAb for 1 h at room temperature. Subsequently, the membranes were reblotted with anti-PLCγ1 mAb and HRP-conjugated anti-mouse Ig Ab. Membranes were developed using ECL (Amersham Pharmacia Biotech AB). Data were analyzed using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

**IL-2 production assay**

Jurkat cells (5 × 10<sup>6</sup> cells/ml) were placed in a 96-well microplate and incubated with 20 μg/ml PHA (Sigma-Aldrich) in the CO<sub>2</sub> 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 using the human IL-2 ELISA system (Amersham 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 reporter gene expression assay**

IL-2 gene expression was evaluated in a stable IL-2 reporter cell line, IL-2/Jurkat cells (16), established from Jurkat cells with a reporter plasmid (GVI4L). The promoter region of the IL-2 gene. IL-2/Jurkat cells (2 × 10<sup>6</sup> cells/ml) were placed in a 96-well microplate (Nunclon Surface; Nalge Nunc International, Rochester, NY), and were stimulated with 20 μg/ml PHA in the CO<sub>2</sub> incubator for 20 h. 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 using 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).
NF-AT and AP-1 reporter gene assays

Jurkat cells (1 × 10^7 cells/0.4 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)-transfected cells were stimulated with PMA, and 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. Kuroimitsu (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 the abdominal region after cutting their abdominal hair under anesthesia at day 0. Seven days after the TNCB sensitization, the thickness of both ears outside of the ear pinnas. Only a solvent was applied for negative control outside the ear pinnas. The animals were treated with 100 μl of a 7% TNCB solution in the abdominal region after cutting their abdominal hair under anesthesia at day 0. Seven days after the TNCB sensitization, the thickness of both ears was measured, and 10 μl of 0.25% TNCB was applied both inside and outside of the ear pinnas. Only a solvent was applied for negative control mice. Ear thickness was measured using a dial thickness gauge (Peacock; Ozaki MFG, Tokyo, Japan) 24 h after the TNCB challenge, and changes in swelling were calculated from the value pre-exposure. YM-58483 (1–30 mg/kg) and prednisolone (0.3–3 mg/kg) were administered orally 1 h before exposure to TNCB, and 0.5% MC was administered orally in negative control and control animals.

Statistical analysis

Data are represented by the mean ± SE. Statistical analyses were performed by SAS (Cary, NC). Percent inhibition of Ca2+ influx, IL-2 production, and IL-2 gene expression was calculated for each concentration of compound as follows: (solvent control – compound group)/(solvent control) × 100. The IC50 was calculated from the percent inhibition value for nonstimulation and control stimulation, and the ED50 was calculated from the value for the negative control and control. Statistical analysis of the effect was performed by Student’s t test or Dunnett’s multiple range test.

Results

Effects on anti-CD3 mAb-induced increase in intracellular Ca2+

Anti-CD3 mAb (10 μg/ml) induced a transient and then sustained increase in intracellular Ca2+ in the presence of extracellular Ca2+. YM-58483 (3 μM) and econazole (3 μM) partially inhibited the transient increase, and completely inhibited the sustained increase in Jurkat cells. It shows the typical pattern of 0.1% DMSO (Fig. 1A) and 3 μM econazole inhibited the transient increase in intracellular Ca2+ in the absence of extracellular Ca2+, but maximum Ca2+ concentration was evidently diminished by the compound-induced inhibition in the presence of extracellular Ca2+ (Fig. 1B). Neither YM-58483 nor econazole inhibited the transient increase in intracellular Ca2+ in the absence of extracellular Ca2+ (Fig. 1B). Moreover, YM-58483 (3 μM) did not affect the baseline intracellular Ca2+ 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).

![FIGURE 1](http://www.jimmunol.org/Download) Effect of YM-58483 (3 μM) and econazole (3 μM) on anti-CD3 mAb-induced increase in intracellular Ca2+ in Jurkat cells. A. Anti-CD3 mAb-induced transient Ca2+ influx and sustained Ca2+ influx in the presence of extracellular Ca2+. TCR stimulation by anti-CD3 mAb triggered initial transient Ca2+ influx from Ca2+ store and sustained Ca2+ influx from extracellular medium in Ca2+ medium. The peak Ca2+ concentration of transient phase (filled column) and the Ca2+ concentration of sustained phase at 10 min after stimulation (hatched column) were shown in the figure. B. Anti-CD3 mAb-induced transient Ca2+ influx in the absence of extracellular Ca2+. TCR stimulation by anti-CD3 mAb evoked only transient Ca2+ influx from Ca2+ store in Ca2+-free medium. The peak Ca2+ concentration of transient phase (filled column) was shown in the figure. Each column represents the mean ± SE of five experiments.

![FIGURE 2](http://www.jimmunol.org/Download) Effect of YM-58483 on the baseline intracellular Ca2+ in Jurkat cells. It shows the typical pattern of 0.1% DMSO (upper) and 3 μM YM-58483 (lower) on the baseline Ca2+ caused by 2 mM CaCl2 after Ca2+-free condition.
Effects on the sustained Ca\(^{2+}\)/flux via SOC channels

Thapsigargin evoked a transient increase in intracellular Ca\(^{2+}\) in the absence of extracellular Ca\(^{2+}\), and the subsequent addition of 2 mM CaCl\(_2\) induced a sustained Ca\(^{2+}\) 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\(^{2+}\) influx in a concentration-dependent manner with IC\(_{50}\) values of 100 and 680 nM, respectively (Figs. 4 and 5; Table I). Further-

more, YM-58483 and econazole reduced concentration dependently the already evoked Ca\(^{2+}\) 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\(^{2+}\) 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, potently inhibited the high concentration KCl-induced Ca\(^{2+}\) 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

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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 10 ± 3.1, 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).

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

<table>
<thead>
<tr>
<th>IC\textsubscript{50} Value (nM) (95% confidence limit)</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.

**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 × 10⁶/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 (\textbullet, 0.01–10 \textmu M) and econazole (\textcircled{e}, 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), econazole (△, 0.003–10 \textmu M), SK&F-96365 (■, 0.003–10 \textmu M), and nifedipine (●, 0.003–10 \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.
TNB-induced contact hypersensitivity in mice

TNB-induced ear swelling was significantly increased in control animals compared with the vehicle-challenged negative control (Fig. 13). YM-58483 and prednisolone inhibited dose dependently TNB-induced ear swelling in sensitized mice with ED50 values of 1.1 and 3.7 mg/kg, respectively (Fig. 13).

**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 ICRAC 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 presence 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 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. As a positive control of depolarization, 50 mM KCl (●) was used. 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).

**FIGURE 9.** Effects of YM-58483 (○, 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.
Ca\textsuperscript{2+}. Moreover, these compounds did not affect the phosphorylation of PLC\_y1 on TCR stimulation. Therefore, it is suggested that YM-58483 as well as econazole inhibit the sustained Ca\textsuperscript{2+} influx

\begin{tabular}{|c|c|c|}
\hline
\textbf{IL-2 Production} & & \\
\hline
\textbf{IC\textsubscript{50} Value (nM)} & \% Inhibition & \\
\hline
YM-58483 & 17 ± 3.2 & 89\% at 1 \mu M \\
Econazole & >10,000 & 63\% at 10 \mu M \\
SK&F-96365 & >10,000 & 40\% at 10 \mu M \\
Nifedipine & >10,000 & 12\% at 10 \mu M \\
Cyclosporine A & 3.2 ± 0.45 & 92\% at 1 \mu M \\
\hline
\end{tabular}

\textbf{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 × 10\textsuperscript{5}/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 activity (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).

\begin{tabular}{|c|c|c|}
\hline
\textbf{IL-2 Luciferase Gene Expression} & & \\
\hline
\textbf{IC\textsubscript{50} Value (nM)} & \% Inhibition & \\
\hline
YM-58483 & 10 ± 3.1 & 99\% at 1 \mu M \\
Econazole & 1,700 ± 350 & 86\% at 10 \mu M \\
SK&F-96365 & >10,000 & 51\% at 10 \mu M \\
Cyclosporine A & 4.8 ± 1.8 & 112\% at 1 \mu M \\
\hline
\end{tabular}

\textbf{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 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)\textsubscript{4}-transfected cells (right). Lower, Effects of YM-58483 (0.0003–3 \mu M) and cyclosporin A (0.0001–1 \mu M) on PMA/ionomycin (PI)-induced NF-AT reporter gene activity in pGL(NF-AT)\textsubscript{3}-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 PI (Dunnett’s multiple range test).
manner. These results indicated that YM-58483 inhibited Ca\(^{2+}\) influx without affecting membrane potential, and econazole inhibited Ca\(^{2+}\) influx partly through membrane depolarization. Therefore, it is suggested that YM-58483 inhibited store-operated Ca\(^{2+}\) entry by directly interacting with SOC channels or channel-regulatory cofactors at the membrane surface without affecting the depletion of Ca\(^{2+}\) stores.

It has been reported that econazole and SK&F-96365 inhibited store-operated Ca\(^{2+}\) influx through SOC channels (13, 14, 22, 23). Indeed, econazole and SK&F-96365 inhibited Ca\(^{2+}\) influx through SOC channels in lymphocytes, but these compounds also inhibited Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) entry blocker, which selectively inhibits Ca\(^{2+}\) through SOC channels, not VOC channels.

Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) and calmodulin (5, 6). In our preliminary studies, cyclosporin A, unlike YM-58483, did not inhibit the store-operated Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\)-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\(\gamma\), which activates both PI3 and Ras/Raf pathways (26, 27). YM-58483 did not inhibit PMA/ionomycin-induced phosphorylation of PLC\(\gamma\) and TCR-stimulated transient Ca\(^{2+}\) efflux from Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) signaling is enhanced in activated T lymphocytes, and inhibition of Ca\(^{2+}\) influx is important for the regulation of T lymphocytes. In the present study, YM-58483 did not affect baseline intracellular Ca\(^{2+}\) 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\(^{2+}\) entry mechanisms through SOC/CRAC channels and putative Ca\(^{2+}\) entry channel genes.

Acknowledgments

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References