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3-(13-Hydroxytridecyl)-1-[13-(3-Pyridyl)Tridecyl] Pyridinium Chloride (YM-53792), a Novel Inhibitor of NF-AT Activation

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ABSTRACT. A compound, YM-53792, was identified as an inhibitor of interleukin-2 (IL-2) gene promoter activity, using a Jurkat cell-based reporter system in which the luciferase gene is regulated by the IL-2 gene promoter. Production of IL-2, interleukin-4 (IL-4) and interleukin-5 (IL-5) from human peripheral blood mononuclear cells was suppressed by YM-53792 in a dose-dependent fashion. Since expression of these cytokine genes is known to be regulated by NF-AT, we examined whether the promoter activity created by multimerization of NF-AT elements was inhibited with YM-53792. YM-53792 inhibited this promoter activity, but not AP-1- and NF-κB-driven promoter activities nor SV40 enhancer/promoter activity. In addition, electrophoretic mobility shift assays did not detect NF-AT/DNA complexes when nuclear extract prepared from YM-53792-treated, PMA/A23187-stimulated Jurkat cells was used, whereas AP-1/DNA complexes were observed. These results suggest that YM-53792 specifically inhibits the activation of NF-AT. BIOCHEM PHARMACOL 54;9:999–1005, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. IL-2 promoter activity; cytokine production; inhibition of NF-AT activation; Jurkat cells; PMA/A23187 stimulation; YM-53792

Several transcription factors that bind to the enhancer/promoter region of the interleukin-2 (IL-2)§ gene have been identified. These factors include NF-AT, Oct, CD28RE, NF-κB and AP-1. Many studies have demonstrated that NF-AT plays a major role in the regulation of IL-2 gene expression [see 1, 2 for reviews].

At present, four NF-AT family proteins, NF-ATp [3], NF-ATc [4], NF-ATx [or NF-AT4] [5, 6] and NF-AT3 [6], have been reported. These proteins are highly related within a 290-amino-acid domain (rel-like domain), which is distantly related to the rel domain of NF-κB family proteins [5, 7]. Since all the NF-AT genes were cloned from T cell cDNA libraries, they may play roles in transcription of the IL-2 gene. NF-ATx mRNA is expressed at a higher level in thymus than in periphery in immune tissues [5] and NF-ATp is expressed at high levels in resting T cells [5], whereas NF-ATc is induced in response to stimulation with PMA plus ionomycin in the T cell line [4]. NF-AT3 mRNA, however, is expressed predominantly outside the immune system [6]. When T cells were activated with T cell receptor (TCR) plus CD28 or PMA plus ionomycin, an NF-AT family protein present in the cytoplasm is dephosphorylated by a calcium/calmodulin-dependent phosphatase calcineurin, activated with cytoplasmic Ca^{2+} ions increased by TCR activation. The dephosphorylated NF-AT then translocates into the nucleus [8, 9] and subsequently associates with AP-1 induced by protein kinase C (PKC) or Ras signaling pathways. Dephosphorylated NF-AT/AP-1 complex then binds to the NF-AT recognition DNA sequence leading to induction of IL-2 gene expression [9, 10]. Furthermore, it has been reported that the rel-like domain of NF-AT proteins is sufficient for cooperative interactions with AP-1 and DNA binding [6, 7]. It seems, however, that each NF-AT protein would have a specific function, but physiological roles of NF-AT family proteins have not been fully elucidated.

In this report, a compound, YM-53792, was identified as an inhibitor of IL-2 gene promoter activity by using a Jurkat cell-based reporter system in which the luciferase gene is regulated by the IL-2 gene promoter. YM-53792 inhibited production of IL-2, IL-4 and IL-5 from peripheral blood mononuclear cells (PBMCs) and the promoter activity created by a multimerization of NF-AT elements. Furthermore, a shifted band derived from NF-AT/DNA complexes

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§ *Abbreviations:* CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PKC, protein kinase C; RLU, relative light unit; TCR, T cell receptor.

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was not detected in a electrophoretic mobility shift assay (EMSA) using nuclear extract prepared from YM-53792-treated, PMA/A23187-stimulated Jurkat cells. These results suggest that YM-53792 specifically suppressed the activation of NF-AT.

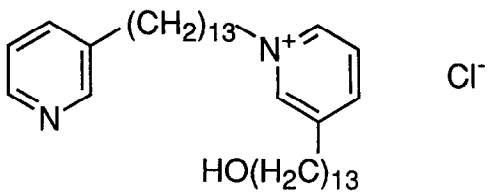

FIG. 1. Chemical structure of YM-53792.

MATERIALS AND METHODS

Construction of Reporter Plasmids

To construct the reporter plasmid pGVIL-2, the promoter region of the IL-2 gene (−373 to +47) [11] was amplified by polymerase chain reaction (PCR) using human placental genomic DNA (Clontech Laboratories, Inc.) as a template and synthetic oligonucleotides 5′-GGGCTCGAGCA CAATATGCTATTCACATGT-3′ and 5′-CCCAAGCT TGTGGCAGGAGTTGAGGTT-3′ as primers, and ligated upstream of the luciferase gene in the SV40 promoter-deleted plasmid pGV-P (Toyo Ink MFG, Co., Ltd.). For construction of reporter plasmids, pGL(AP-1)$_4$ and pGL(NF-AT)$_4$, consisting of four tandem repeats of the AP-1 binding site in the human metallothionein IIa promoter and the human IL-2 distal NF-AT binding site, respectively, four oligonucleotides, AP1-1 (5′-GATCCG CAAGTGACTCAGCGCGA-3′, AP1-2 (5′-GATCTC GCGCTGAGTCACTTGCG-3′), NFAT-1 (5′-GATCCAGGAGGAAAAACTGTTTCATACA GAAGGC GA-3′) and NFAT-2 (5′-GATCTCGCCTT CTGTATGAAACAGTTTTTCCTCCTG-3′), were chemically synthesized. Sets of oligonucleotides AP1-1/AP1-2 and NFAT-1/NFAT-2 were annealed and then ligated successively four times in *Bgl*II site of plasmids pGL2-Promoter (Promega Co.) and pGL2.TATA*, generating pGL(AP-1)$_4$ and pGL(NF-AT)$_4$, respectively. A reporter plasmid pMT101 containing the luciferase gene driven by four tandem repeats of κB motif in the immunogloblin κ light chain promoter was kindly provided by Dr. M. Takeuchi (Yamanouchi Pharmaceutical Co., Ltd.)

Cells

Human Jurkat cells obtained from ATCC, stable reporter cell lines established from Jurkat cells, and mouse EL-4 cells were cultured in RPMI 1640 (Life Technologies Co.) containing 10% (v/v) fetal calf serum (Life Technologies Co.), 100 U/mL penicillin G and 100 μg/mL streptomycin (Life Technologies Co.). PBMCs were prepared by centrifugation over Ficoll–Hypaque (Pharmacia Biotech KK.) from peripheral blood of healthy human volunteers.

Establishment of Stable Reporter Cell Lines

Jurkat cells (7–10 times 10^6 cells/0.4 mL of RPMI 1640) were transfected with 20 μg of pGVIL-2 plus 2 μg of pSV$_2$-bsr (Funakoshi Co.) or 20 μg of pGL(AP-1)$_4$ plus 2 μg of pcDNAI/neo (Invitrogen Co., San Diego, CA) by means of electroporation under conditions of 280V and 960 μF in 0.4 cm cuvettes (Nippon Bio-Rad Laboratories KK). Plasmids pSV$_2$-bsr and pcDNAI/neo carrying the blasticidin- and neomycin-resistant genes, respectively, were used as selection markers. After addition of 10 mL of RPMI 1640 supplemented with 10% (v/v) fetal calf serum, cells were cultured for 40 hr and subsequently incubated in the presence of 6 μg/mL of blasticidin (Funakoshi Co.) or 2 mg/mL of G418 (Life Technologies Co.). An IL-2/Jurkat reporter cell line, in which highest induction (*ca.* 17-fold) of the luciferase gene driven by the IL-2 promoter was observed in response to 10 ng/mL of PMA (Sigma Chemical Co.) plus 1 μM of calcium ionophore A23187 (Calbiochem-Novabiochem Co.) was selected from among approximately 30 blasticidin-resistant clones. An AP-1/Jurkat reporter cell line, in which luciferase gene induction was highest in response to 10 ng/mL of PMA, was chosen from among *ca.* 20 G418-resistant clones. To isolate an SV40/Jurkat reporter cell line as a negative control in which the luciferase gene is constitutively expressed, a reporter plasmid pGV-C (Toyo Ink MFG. Co., Ltd.) and pSV$_2$-bsr were simultaneously electroporated into Jurkat cells and the SV40/Jurkat cell line subsequently obtained by the method described above.

Induction of Luciferase Gene Expression and Measurement of Luciferase Activity

In the case of reporter cell lines, IL-2/Jurkat and AP-1/Jurkat reporter cells (5 times 10^4 cells/0.1 mL of RPMI 1640 supplemented with 10% (v/v) fetal calf serum) cultured in flat-bottom 96-well plates (Microlite 1, Dynatech Laboratories, Inc.) were stimulated with PMA (10 ng/mL) plus A23187 (1 μM) and PMA (10 ng/mL) alone, respectively. YM-53792 (Fig. 1) or cyclosporine A (CsA, Sandoz Laboratories) was added 30 min before stimulation. The reaction was stopped by addition of 50 μL of solubilization buffer (10 mM Tris-HCl/pH 7.8, 0.5 mM $MgCl_2$, 10 mM dithiothreitol and 0.1% (v/v) Triton X-100). Luciferase activities were measured with a ML3000 luminometer (Dynatech Laboratories, Inc.) after addition of 50 μL of substrate solution (5 mM luciferin, 2 mM coenzyme A, 2 mM ATP, 0.5 mM $MgCl_2$, and 2 mM $Mg(OH)_2$ in 10 mM Tris-HCl/pH 7.8 solution). In the case of transient expression assays, Jurkat cells (7 times 10^6 cells) transfected with 20 μg of pGVIL-2, pGL(NF-AT)$_4$, pGL(AP-1)$_4$ or pMT101 by electroporation were cultured for 40 hr and then plated into half of a 96-well plate. Cells were stimulated for 6 hr and

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luciferase activities were determined by ML3000 luminometer. Relative activities were calculated as below:

$$\text{Relative activities (\%)} = \frac{[\text{RLU/sample in stimulation} - \text{RLU in unstimulation}]}{[\text{RLU in stimulation} - \text{RLU in unstimulation}]} \times 100$$

RLU: relative light unit.

Measurement of Cytokine Production

Human PBMCs (2 times 10^5/0.1 mL RPMI 1640 containing 10% (v/v) fetal calf serum) were stimulated with 10 ng/mL of PMA plus 1 μM of A23187 for 24 hr. Test compounds were added 30 min before stimulation. The amounts of IL-2 and IL-4 produced from PBMCs were quantitated using ELISA kits purchased from Amersham International plc and Sceti Co., Ltd., respectively. Production of IL-5 was determined by means of an ELISA in which rat antimouse IL-5 antibody, TRFK5 (PharMingen cross-reacting antibody to human IL-5), was used as the capture antibody. The polyclonal rabbit antihuman IL-5 antibody (Genzyme) followed by a donkey anti-rabbit immunoglobulin $F(Ab')_2$ fragment conjugated with horseradish peroxidase (Amersham) was used as the detecting antibody system. Mouse EL-4 cells (5×10^4/0.1 mL of RPMI 1640 containing 10% (v/v) fetal calf serum) were stimulated with PMA and A23187 for 24 hr. Test compounds were added 30 min before stimulation. Amounts of IL-2 were quantitated using an ELISA kit purchased from Amersham.

Electrophoretic Mobility Shift Assay (EMSA)

NF-AT oligonucleotides (30-bp double-stranded oligonucleotides corresponding to nucleotide positions −285 to −254 of the human IL-2 gene promoter, 5′-GGAGGA AAAACTGTTTCATACAGAAGGCGT-3′) [12], mutated NF-AT oligonucleotides (5′-CGTCCTAAAACT GTTTCATACAGAAGGCGTT-3′, mutated nucleotides are shown underlined) [13], and AP-1 oligonucleotides (19 bp double-stranded oligonucleotides containing the AP-1 site of the human metallothionein IIa promoter) [14] were ^{32}P-end labeled with T4 polynucleotide kinase (Takara Shuzo) in the presence of [γ-^{32}P]ATP (Amersham). IL-2/Jurkat reporter cells were treated with YM-53792 for CsA for 1 hr and then stimulated with PMA plus A23187 for 3 hr in the presence of YM-53792 or CsA. After incubation, nuclear extracts were prepared by the method of Mattila et al. [15]. Two micrograms of nuclear extracts were incubated with 10 fmol (*ca.* 4×10^4 cmp) of the ^{32}P-labeled oligonucleotides at room temperature for 20 min in 10 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol and 1 μg poly(dIdC/dIdC) (Pharmacia Biotech KK) in a final volume of 20 μL. DNA-protein complexes were resolved by electrophoresis on 4% (w/v) polyacrylamide gels at room temperature in TAE buffer (6.7 mM Tris-HCl/pH 7.9, 3.3 mM sodium acetate and 1 mM EDTA). After drying, DNA-protein complexes were analyzed by FUJIX BAS2000 bio-image analyzer (Fuji Film Co. Ltd.).

RESULTS

Identification of YM-53792 as an Inhibitor of IL-2 Promoter Activity

To identify novel inhibitors of IL-2 promoter activity, compounds were screened by using IL-2/Jurkat reporter cells in which luciferase activity was induced *ca.* 17-fold in response to PMA/A23187 (Fig. 2A). A compound YM-53792, 3-(13-hydroxytridecyl)-1-[13-(3-pyridyl)tridecyl]pyridinium chloride (Fig. 1), was identified to be an inhibitor of IL-2 promoter activity. YM-53792 is an intermediate product during total synthesis of cyclostelletamine C, a bispyridinium macrocyclic alkaloid which has muscarinic acetylcholine receptor antagonistic activity [16]. YM-53792 inhibited induction of the luciferase gene expression driven by the IL-2 promoter in a dose-dependent manner (Fig. 2B). The IC_{50} was approximately 50 nM. Inhibitory activity of YM-53792 was *ca.* 10-fold lower than that of CsA (Fig. 2B). In our assay system, the basal IL-2 promoter activity was too low to allow reliable assessment of the inhibition of basal activity by YM-53792. Cytotoxicity of YM-53792 in Jurkat cells was not observed for 24 hr up to 1 μM by the method using alarmar blue (Iwaki Glass Co., Ltd.) (data not shown), suggesting that the inhibitory activity of YM-53792 was not due to cytotoxicity. Additionally, other intermediates and cyclostelletamine C did not have inhibitory activities on the IL-2 gene promoter up to 1 μg/mL (data not shown).

To examine whether or not YM-53792 inhibits other promoter activities, we established two reporter cell lines, AP-1/Jurkat and SV40/Jurkat, in which the luciferase gene is regulated with tandem repeats of AP-1 binding site and SV40 enhancer/promoter, respectively. YM-53792 did not inhibit these promoter activities up to 1 μM (Fig. 2C), suggesting that the inhibitory activity of YM-53792 is specific.

Blocking of Cytokine Production in Human PBMCs with YM-53792

Since expression of the IL-2 gene was inhibited with YM-53792 in Jurkat cells (Fig. 2B), we examined whether YM-53792 inhibits production of IL-2 in mouse EL-4 cells and human PBMCs. Mouse EL-4 cells were used because IL-2 production can't be detected in our Jurkat subline. Endogenous IL-2 production in EL-4 cells stimulated with PMA/A23187 was suppressed by *ca.* 60% at 1 μM of YM-53792 (Fig. 3A). The IC_{50} was approximately 20-fold higher in EL-4 cells (Fig. 3A) compared to Jurkat cells (Fig. 2B). Since we also observed a lower inhibitory activity of CsA in EL-4 cells (compare Fig. 3A to Fig. 2B), the lower

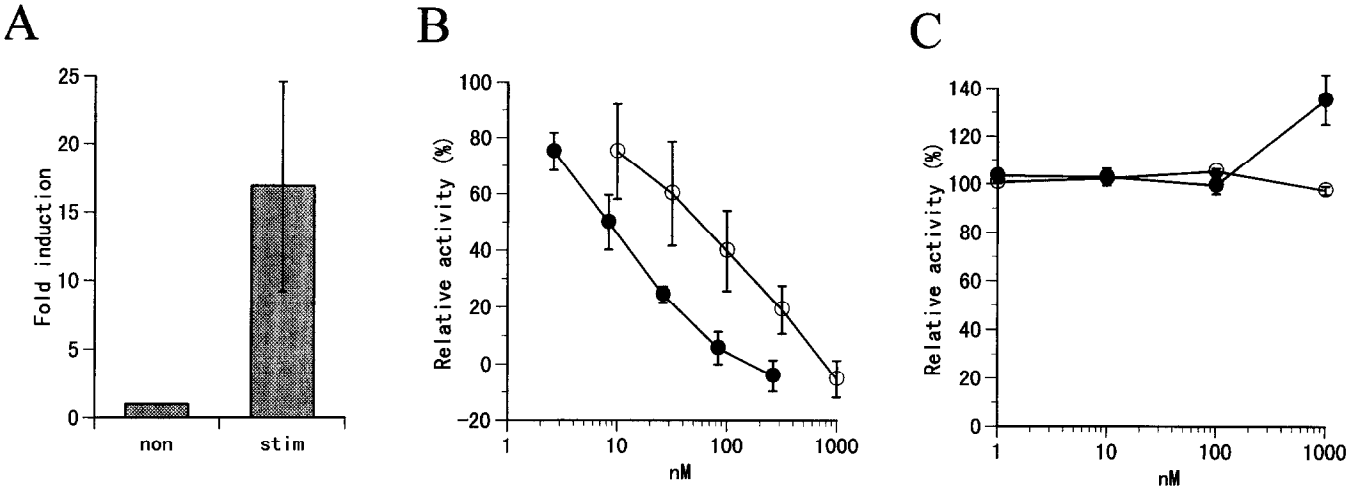

FIG. 2. (A) Induction of luciferase gene expression. IL-2/Jurkat reporter cells were incubated with or without PMA plus A23187 for 24 hr and luciferase activities were then measured. Basal and stimulated RLUs were 17 ± 2 and 290 ± 157, respectively. (B) Effects of YM-53792 (○) and CsA (●) on IL-2 promoter activity. IL-2/Jurkat reporter cells were treated with YM-53792 or CsA for 30 min and then stimulated with PMA plus A23187. After 24 hr incubation, luciferase activities were determined. The values shown represent the means of duplicate determinations from three separate experiments. (C) Effect of YM-53792 on AP-1-dependent promoter and SV40 enhancer/promoter activities. AP-1/Jurkat reporter cells (●) were treated with YM-53792 for 30 min and then stimulated with PMA for 6 hr. SV40/Jurkat reported cells (○) were incubated with YM-53792 for 6.5 hr. The values shown represent the means of duplicate determinations from four separate experiments. Basal and stimulated RLUs of AP-1/Jurkat were 207 ± 39 and 1590 ± 204, respectively. The RLU of SV40/Jurkat was 14561 ± 851.

inhibitory activity of YM-53792 obtained in EL-4 cells may be cell-dependent. YM-53792 also blocked IL-2 production in PBMCs stimulated with PMA and A23187 (Fig. 3B). CsA repressed IL-2 production in EL-4 cells and PBMCs at *ca.* 20- to 50-fold lower concentrations than was observed with YM-53792 (Figs. 3A, B). These results suggest that the inhibition of IL-2 gene expression by YM-53792 was not unique to Jurkat cells. In addition, production of IL-4 and IL-5 was suppressed with YM-53792 in a dose-dependent manner (Figs. 3C, D). The sensitivity of cytokine production to inhibitors seemed to differ between PBMCs prepared from different volunteers. Since PBMCs used in Figs. 3B to 3D were prepared from different volunteers, the inhibitory potencies of YM-53792 in regard to these cytokine productions cannot be compared.

Inhibition of NF-AT-Dependent Transcriptional Activation with YM-53792

NF-AT is known to be involved in expression of the IL-2, IL-4, and IL-5 genes [17–19]. Indeed, CsA, which is known as an NF-AT inhibitor, strongly blocked production of IL-2, IL-4 and IL-5 (Fig. 3). To examine the possibility that YM-53792 suppressed NF-AT activity specifically, its inhibitory activity was analyzed in transient expression assays in Jurkat cells using reporter plasmids by which the promoter activities were created by the IL-2 gene and the multimerization of NF-AT, AP-1 and NF-κB binding sites. The inhibitory patterns of IL-2- and NF-AT-promoter-dependent gene expression in Jurkat cells were almost the same, with IC_{50} values of 150 nM (Fig. 4A). These IC_{50} values were approximately 3-fold higher than those observed in stably transfected cell assays (compare Fig. 4A to Fig. 2B). It is likely that the different inhibitory activities of YM-53792 obtained in the reporter cell assay and the transient expression assay were due to different incubation times (24 and 6 hr). We next analyzed its effect on AP-1- and NF-κB-dependent promoter activities using reporter plasmids pGL(AP-1)$_4$ and pMT101. YM-53792 did not affect AP-1- and NF-κB-dependent promoter activity up to 0.1 μM (Fig. 4B). At higher concentrations of 0.3 to 1 μM, the NF-κB and AP-1 promoters [pMT101 and pGL(AP-1)$_4$] were inhibited 20% and enhanced 40%, respectively (Fig. 4B). These results suggest that YM-53792 specifically inhibits NF-AT-dependent promoter activity.

Inhibition of NF-AT Activation with YM-53792

Since the specific inhibition of YM-53792 on NF-AT-dependent activity is implied by the results described, we examined whether YM-53792 inhibits the activation of NF-AT in EMSA using nuclear extract prepared from YM-53792-treated, PMA/A23187-stimulated IL-2/Jurkat reporter cells. Complexes of NF-AT/DNA (Fig. 5, indicated by solid arrow) were observed when cells were stimulated with PMA and A23187 (lane 2), but not when they were unstimulated (lane 1). The presence of a 100-fold molar excess of NF-AT oligonucleotides competed out the complexes, but mutated NF-AT oligonucleotides had no effect on complex formation (data not shown), indicating that the complexes detected in EMSA were NF-AT-specific. Both YM-53792 and CsA strongly inhibited formation of NF-AT/DNA complexes at 1 μM (Fig. 5, lanes 4

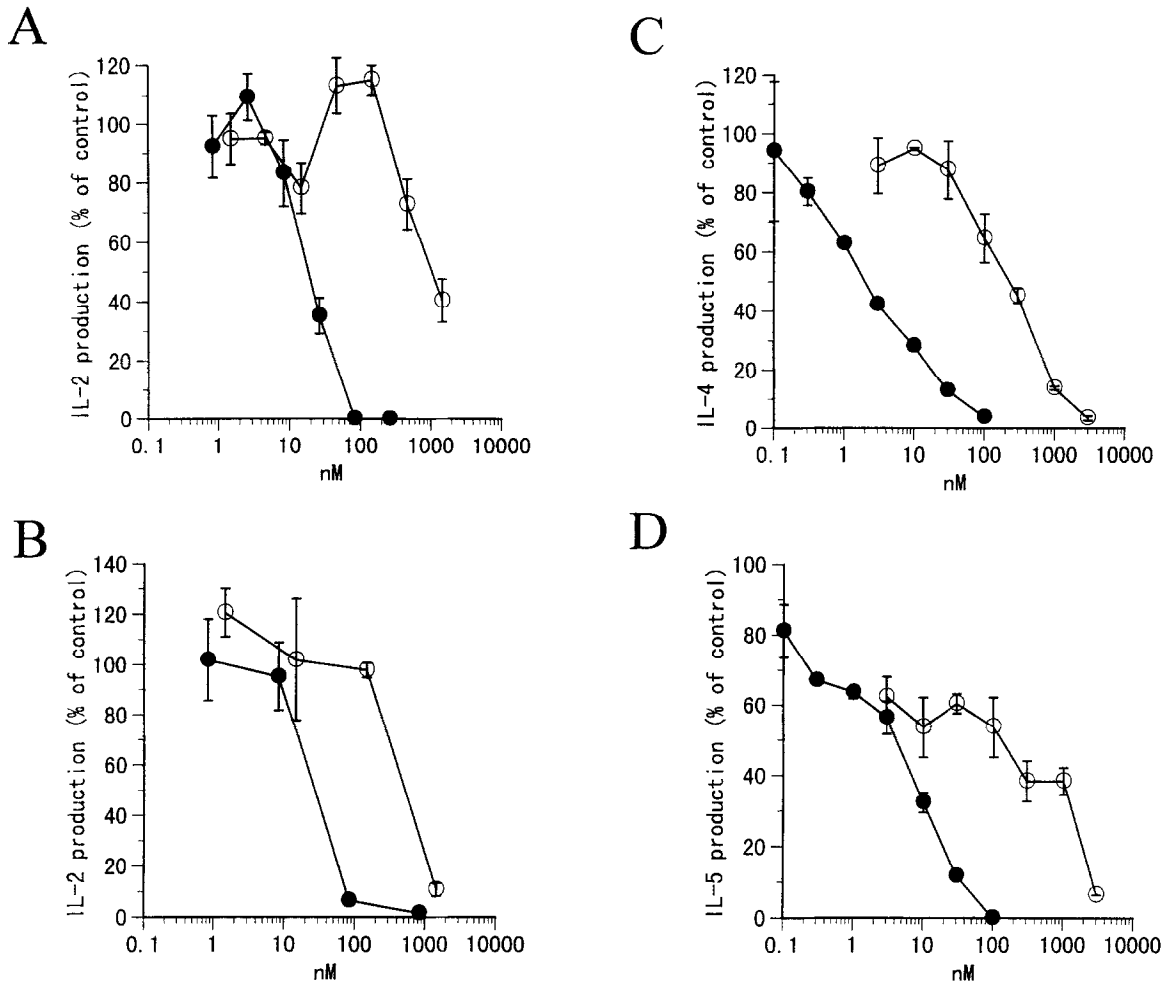

FIG. 3. Effect of YM-53792 and CsA on cytokine production in mouse EIL-4 cells and human PBMCs. Mouse EL-4 cells and human PBMCs were treated with YM-53792 (○) or CsA (●) for 30 min and then stimulated with PMA and A23187 for 24 hr. The amounts of IL-2 (A) produced in EL-4 cells and those of IL-2 (B), IL-4 (C) and IL-5 (D) in PBMCs were determined by ELISA. The values shown represent the means of duplicate determinations from one or two separate experiments. Cytokines synthesized in cultures of stimulated cells: IL-2 from EL-4 cells, 123 ± 9.2 ng/mL; IL-2, IL-4 and IL-5 from PBMCs, 2.21 ± 0.67 ng/mL, 1.39 ± 0.16 ng/mL and 77 ± 4.4 pg/mL, respectively.

and 6). However, only CsA was effective at 0.1 μM (lane 5). Up to ten micromolar of YM-53792 did not affect NF-AT/DNA complex formation in the *in vitro* binding assay when nuclear extract prepared from cells stimulated with PMA plus A23187 was incubated with NF-AT oligonucleotides (data not shown), indicating that the target site of YM-53792 was not NF-AT/DNA binding. Another band which migrated faster than the NF-AT/DNA complexes was nonspecific, since it did not disappear in the presence of NF-AT and AP-1 oligonucleotides (data not shown). Additionally, neither YM-53792 nor CsA repressed AP-1-dependent complex formation (Fig. 5, lanes 9–12). These results strongly suggest that YM-53792 specifically inhibited NF-AT activation.

DISCUSSION

In this paper, we show that YM-53792 specifically inhibits NF-AT activation involved in expression of cytokine genes. In fact, several NF-AT sites have been reported in the IL-2, IL-4 and IL-5 gene promoters [17–19]. The NF-AT site, designated P element, in the IL-4 gene promoter was reported to be involved in the responsiveness to the stimulation of PMA/A23187 [20]. Furthermore, five NF-AT binding sites, P0 to P4, were reported in the promoter region of the murine IL-4 gene, and each was shown to interact with NF-AT [21]. These NF-AT sites cooperatively confer CsA-sensitive and ionomycin-inducible promoter activity. In the case of the mouse IL-5 gene, the NF-AT site, designated the IL-5P, could play an important role in its expression [19]. Our results are consistent with these reports.

Baine et al. [22] reported that WIN53078 and WIN61058, structurally different from CsA and YM-53792, acted as inhibitors of IL-2 transcription. NF-AT/DNA complexes were retarded in mobility shift assays when using nuclear extracts prepared from cells incubated with these compounds, suggesting either that a suppressor(s) induced

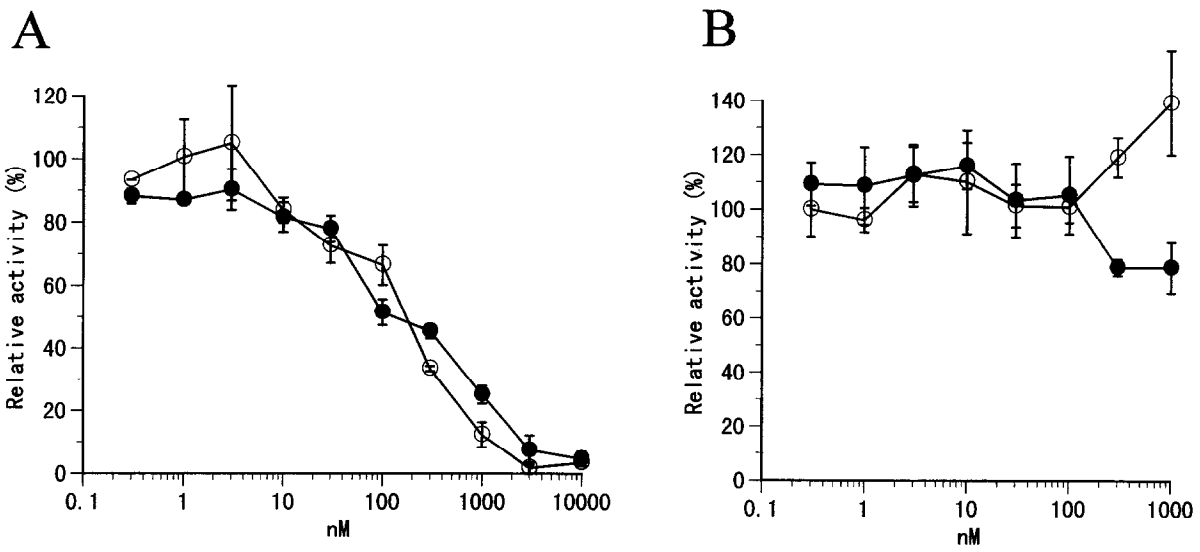

FIG. 4. Inhibition of NF-AT activity with YM-53792 in Jurkat cells. (A) Reporter plasmids pGL(NF-AT)$_4$ (●) and pGVIL-2 (○) were transiently transfected into Jurkat cells. After 40 hr transfection, cells were treated with YM-53792 for 30 min and then stimulated with PMA and A23187 for 6 hr. The values shown represent the means of duplicate determinations. These results are from one of three separate experiments with similar results. Basal and stimulated RLUs: IL-2, 3 ± 1 and 74 ± 20, NF-AT, 3 ± 1 and 106 ± 30. (B) Reporter plasmids pGL(AP-1)$_4$ (○) and pMT101 (●), were transiently transfected into Jurkat cells. After 40 hr transfection, cells were treated with YM-53792 for 30 min and then stimulated with PMA for 6 hr. The values shown represent the means of duplicate determinations from three separate experiments. Basal and stimulated RLUs: AP-1, 116 ± 53 and 402 ± 159, NF-κB, 32 ± 2 and 164 ± 24.

with WIN compounds binds NF-AT or that WIN compounds directly interact with the NF-AT/DNA complex, resulting in a conformational change that alters the mobility of the complex. In the case of YM-53792, however, the shifted band derived from the NF-AT/DNA complex disappeared in EMSA (Fig. 5, lane 4), indicating that inhibitory mechanisms of YM-53792 and WIN compounds could be different, although the inhibitory mechanism of YM-53792 is unknown.

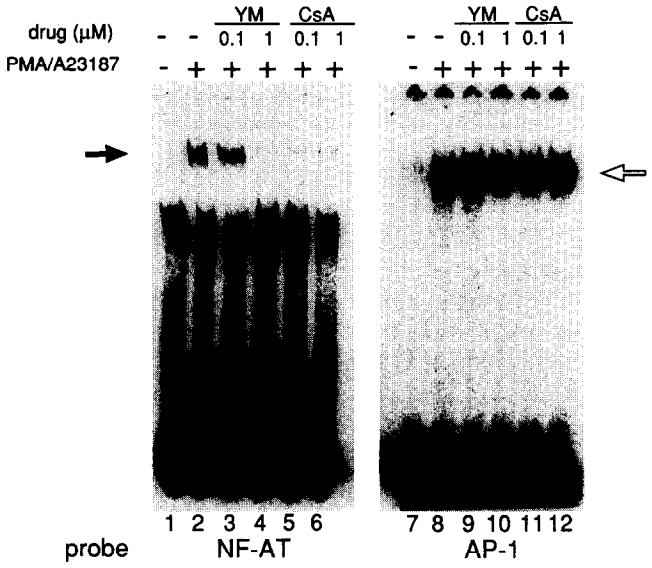

FIG. 5. Effect of YM-53792 or CsA on EMSA using NF-AT or AP-1 probe. IL-2/Jurkat reporter cells were treated with YM-53792 or CsA for 1 hr and then stimulated with PMA plus A23187 for 3 hr in the presence of YM-53792 or CsA. After incubation, nuclear extracts were prepared. EMSA was carried out using 2 μg of nuclear extract and ^{32}P-labeled NF-AT oligonucleotides (NF-AT) or AP-1 oligonucleotides (AP-1) as probes. Solid and open arrows indicate the NF-AT/DNA and the AP-1/DNA complexes, respectively.

Although CsA and FK506 are clinically used as immunosuppressive agents, they have severe adverse effects, such as neurotoxicity and nephrotoxicity, which limit their long-term use [23, 24]. CsA binding to cyclophilin or FK506 binding to FK binding protein inhibits calcineurin activity effectively [8, 25]. Inactivation of these immunophilins directly or indirectly blocks the translocation of NF-AT from the cytoplasm to the nucleus, thus blocking the transcription of the IL-2 gene mediated with NF-AT [8, 15]. It seems that these side-effects are related to the inhibition of the activation of calcineurin, a ubiquitous enzyme with presumably multiple functions [26]. Since side-effects of CsA and FK506 are based on inhibition of calcineurin activity, improved immunosuppressive agents would be expected to act at sites downstream of a specific calcineurin-regulated signaling pathway. In preliminary experiments that overexpress a constitutive active form of calcineurin, YM-53792 did not inhibit activation of NF-AT, but CsA did*. This result prompted us to examine the inhibitory mechanism of YM-53792 on NF-AT activation. Further studies of its function are in progress. It is hoped that the analysis of the mechanism of YM-53792 action in particular may allow the regulation of a new target molecule(s) for novel immunosuppressive agents.

*Kuromitsu S, et al., unpublished data.

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