Extracellular Signal Regulated Protein Kinase and c-Jun N-Terminal Kinase are Involved in m1 Muscarinic Receptor-Enhanced Interleukin-2 Production Pathway in Jurkat Cells

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We have previously shown that m1 and m2 muscarinic receptors were expressed on human peripheral blood lymphocytes (HPBL) and that pre-stimulation of these receptors enhanced phytomenadione (PHB)-induced interleukin-2 (IL-2) production. Possible intracellular signal pathways of muscarinic receptors to regulate IL-2 production were examined in human T cell line Jurkat cells. Pretreatment of the cells with muscarinic receptor agonist, oxotremorine M (Oxo-M), enhanced IL-2 production induced by phorbol 12-myristate 13-acetate (PMA)/A23187, while Oxo-M by itself did not affect IL-2 production. The enhancement of IL-2 production by Oxo-M was inhibited by 4-diphenylacetoxoy-N-methylpiperidinemethiodide (4-DAMP) an m1/m3 receptor antagonist. When the cells were pretreated with AF-DX116, an m2 antagonist, the IL-2 production enhanced by Oxo-M was further stimulated. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that m1 and m2 muscarinic receptors exist on Jurkat cells.

The stimulation of m1 receptors enhanced the PMA/A23187-induced binding activity to AP-1 consensus sequences in IL-2 promoter and production of c-Fos and c-Jun protein. The stimulation of m1 receptors did not modify the DNA binding of NF-kB, NF-AT or Oct-1. When m1 receptors were stimulated, activities of mitogen-activated protein kinase (MAPK) extracellular signal regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) were increased, while p38 MAPK was not affected. Incubation with Oxo-M induced a transient increase in [Ca++]i, which was abolished by pretreatment with 4-DAMP. Treatment with cyclosporin A markedly decreased the PMA/A23187-induced IL-2 promoter activity. This treatment, however, did not affect the enhancement of the promoter activity induced by m1 receptor stimulation.

The results suggest that transcription factor AP-1 is involved in the m1 receptor-mediated enhancement of IL-2 transcript in Jurkat cells, and that pathways via MAPK/ERK and JNK, but not via p38 MAPK, are involved in the m1 receptor-mediated enhancement of IL-2 promoter activity.

Key words: muscarinic receptor; interleukin-2; neuroimmune interaction; Jurkat cell

Several bidirectional interactions are known to exist between nervous and immune systems via neurotransmitter and cytokine release. Receptors for many neurotransmitters including catecholamines and acetylcholine (ACH) have been detected on the cell surface of lymphocytes. Fujii et al. indicated that a human T cell line synthesized ACh and suggested that ACh can be an autacoid modulating T cell-dependent immune responses. However, the biochemical mechanisms of the roles of the ACh system in the immune system have not been elucidated in detail. Interleukin-2 (IL-2) has a pivotal role in regulating the proliferation and differentiation of hematopoietic cells.

We have shown that muscarinic ACh receptors m1 and m2 are expressed on human peripheral blood lymphocytes (HPBL) and that pretreatment of these receptors with oxotremorine-M (Oxo-M) enhanced IL-2 production. The IL-2 promoter activity is regulated by the 300 bp region adjacent to the transcription initiation site. This region contains specific binding sites for various transcription factors including AP-1, NF-xB, NF-AT and Oct-1, and cooperation of these factors is required for maximal activation of the IL-2 promoter. In this study, we examined the binding activity of each transcription factor of the IL-2 promoter that was affected by the muscarinic receptor-mediated signals on IL-2 production.

Recently, work in a number of laboratories has greatly elucidated the mitogen-activated protein kinase (MAPK) cascades that play critical roles in mediating several intracellular actions. The MAPK superfamily, c-Jun N-terminal kinase (JNK) and p38 MAPK, have been identified. Muscarinic receptors are typical G protein-coupled receptors. MAPK activation by /beta/-subunits of G protein is initiated by tyrosine phosphorylation events in COS-7 cells. Furthermore, the signals from muscarinic receptors affect the MAPKs in various cells. However, the effect of muscarinic receptor stimulation on the MAPKs cascade in lymphocytes has not been established.

In this study, we found that activation of muscarinic receptors enhanced IL-2 production; we evaluated the effect of muscarinic receptors on regulatory elements of the IL-2 promoter and identified the pathways mediated by MAPKs in Jurkat cells.

MATERIALS AND METHODS

Materials: Cyclosporin A (CsA) and AF-DX116 (11-[(2-[diethylamino)methyl]-1-piperidinyl][acyetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) were kind gifts from Sandoz (Basel, Switzerland) and Nippon Boehringer Ingelheim (Hyogo, Japan), respectively. SB203580 was a gift from Smithkline Beecham (King of Prussia, PA, U.S.A.). Iscove's modified Dulbecco's medium (IMDM) and FCS were from Gibco/BRL (Gaithersburg, MD, U.S.A.). Oxo-M and 4-DAMP (4-diphenylacetoxoy-N-methylpiperidinemethiodide) were purchased from Research Biochemicals.© 2000 Pharmaceutical Society of Japan
cal (Natick, MA, U.S.A.). Fura-2/AM was obtained from Dojin Laboratories (Kumamoto, Japan). A23187, aprotinin, DTT, leupeptin, sodium orthovanadate and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical (St. Louis, MO, U.S.A.). Anti-human rabbit polyclonal IgG c-Fos (sc-52), c-Jun (sc-44), JNK-1 (sc-474), p38 (sc-535), human c-Jun (1-79) substrate (sc-4113) and human ATF-2 (1-96) substrate (sc-4114) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Oligonucleotide sets of AP-1, NF-kB and Oct-1 were from Oncogene Science (Cambridge, U.K.). Human fetal brain MATCHMAKER cDNA was from Clontech Laboratories (Palo Alto, CA, U.S.A.). Human γ-globulin, orthophosphoric acid and PMSF were from Wako Pure Chemical (Osaka, Japan). IL-2 enzyme-linked immunosorbent assay (ELISA) kit was from Iwaki Glass (Chiba, Japan). [γ-32P] ATP and PD98059 were from DuPont-New England Nuclear (Boston, MA, U.S.A.). Luciferase lysis buffer, substrate and pGEM-B were from Toyo Inc. (Tokyo, Japan). Enhanced chemiluminescence (ECL) Western detection kit and p42/p44 MAP kinase enzyme assay kit were purchased from Amersham Life Science (Buckinghamshire, U.K.).

**Assay of IL-2 Production** The amount of IL-2 was determined using an ELISA kit for human IL-2. Briefly, cell-free culture supernatants were obtained by centrifugation at 500×g for 5 min, and were assayed according to the manufacturer's instructions and analyzed on an automated ELISA plate reader (MTP-120, Corona Electric).

**RNA Isolation and Reverse Transcription-PCR (RT-PCR) Analysis** Total cellular RNA was extracted from Jurkat cells with 4 M guanidinium isothiocyanate as described by Chomczynski and Sacchi.17 The RNA samples were spectrophotometrically quantified by monitoring absorbance at 260 and 280 nm. PCR primers (21 nucleotides) were designed for human muscarinic receptors m1—m5.18 The sequences corresponding to base numbers relative to the coding initiation site, the sizes of expected PCR products and the registered sequence numbers (GenBank) were reported previously.53 Computer analyses revealed that all the primers were 100% homologous to their target sequences but had no significant homologies with any other genes registered in the GenBank data base. Aliquots of 1 μg of total RNA were incubated at 37°C for 60 min with a mixture of 100 units of RT, 1× first strand buffer, 10 mM DTT, 0.5 mm of each dNTP, and 50 units RNase inhibitor in a final volume of 20 μl. The reaction mixture was further incubated for 10 min at 70°C to inactive RT. As a control template, a human fetal brain MATCHMAKER cDNA library was digested with EcoRI and extracted.53 Aliquots of 2 μl of the RT products or the control template were mixed with 1 μl of TUB DNA polymerase, 200 μM each of sense and antisense primers in a buffer containing 1× TUB buffer and 0.2 mm of each dNTP in a final volume of 20 μl. The mixture was overlain with 30 μl of liquid paraffin to prevent evaporation and then amplified by 40 cycles of PCR. The PCR products were separated by electrophoresis in 1.5% agarose gels in 1× Tris-borate/EDTA (TBE) buffer. The gels were stained with ethidium bromide and photographed.

**Cell Culture and Transient Transfection** Jurkat cells were cultured in IMDM supplemented with 10% FCS. Aliquots of 10 μg of the pIL-2LUC plasmid carrying the human IL-2 promoter of 587 bp linked to the luciferase cDNA were transfected into the cells (2×10^5 or 4×10^5 cells/ml) by the DEAE-dextran method and the cells were incubated for 48—72 h before harvesting.

**Luciferase Assays** The transfected cells were harvested, washed with PBS, lysed using 200 μl of a detergent solution (lysis buffer), and centrifuged at 12000 rpm for 10 min. The supernatants were standardized and subjected to luciferase reaction in a total of 20 μl of lysis buffer with 100 μl of luciferase substrate in the vials. Immediately after mixing, the light intensities emitted by the samples were measured on a luminometer (luminometer ATP-300, Advantec Toyo, Ltd.). The extract from the cells transfected with the promoterless plasmid pGEM-B was assayed in parallel as a control.

**Band Shift Assay** Nuclear extracts of Jurkat cells were prepared as described by Dignam et al.,21 with minor modifications.23 The extract was resuspended in, and dialyzed against a buffer containing 20 mM HEPES-KOH (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM MgCl2, 0.2 mM EDTA and 0.5 mM DTT. As probes, 1 μg aliquots of annealed oligonucleotides were end-labeled using [γ-32P] and T4 polynucleotide kinase, purified by gel filtration through Sephadex G50, separated in and eluted from 10% polyacrylamide gels. Binding reactions were carried out by incubating 0.2—1 pmol of end-labeled DNA (10000 cpm) with 2.5 μg of nuclear extract proteins and 2 μg of poly(dI-dC) at room temperature for 15 min in a buffer containing 50 mM KCl, 1 mM EDTA, 4% Ficoll 400, 1 mM DTT and 4 mM MgI2 in a final volume of 15 μl. After incubation, the reaction mixtures were loaded onto 4% polyacrylamide gels in 0.25× TBE buffer and electrophoresed at 10 V/cm. For competition experiments, non-labeled double-stranded oligonucleotides (5- or 50-fold excess amount of the labeled probe) were added to the reaction mixture prior to addition of nuclear extracts. The sequences of oligonucleotides used were as follows:

- **AP-1**: GATCCTATGTCAGCTACGATCTCGTGACTCAGGGG
- **NF-kB**: GATTCGGGGAGGATTTCCGCTGGGACTTCGCCAGGG
- **NF-AT**: ACGCCCCAAAGAGGAATTTGTTTTCATCAGAC
- **Oct-1**: GATCATTGGCATGATCGATCGCATTGGCAGATGATC

**Western Blotting Analysis** Cells were harvested, washed with 3 ml of ice-cold PBS, and lysed in 500 μl of a lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysates were centrifuged at 120000 rpm for 20 min at 4°C and the supernatants were boiled for 5 min with SDS sample buffer as previously described by Chen and Blenis24 and stored at -70°C until use. The samples were subjected to 7.5% SDS-PAGE, then transferred to nitrocellulose filters at 100 V for 1.5 h at 4°C. The filters were then blocked with Tris-buffered saline/Tween (TBST) (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 1% BSA for 1 h and incubated with the primary antibody in TBST for 1 h at room temperature. After washing three times with TBST, the filters were incubated with the secondary antibody (anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate) for 30 min. After washing with TBST three times, antibody-reactive
bands were visualized with a chemiluminescence detection kit (ECL, Western detection kit).

**p42/p44 MAPK Assay** Cell lysates were prepared and the supernatants were stored as described above for Western blotting analysis. The activity of p42/p44 MAPK/ERK (extracellular signal regulated protein kinase) was assayed using the BIOTRAK™ MAPK assay system. Aliquots of 15 μl of each sample were added to 10 μl of a buffer containing HEPES (pH 7.4), sodium orthovanadate, 0.05% sodium azide and the peptide corresponding to EGF (epidermal growth factor) receptor carrying the Thr669 phosphorylation site as a substrate. The reaction was triggered by adding 5 μl of magnesium-[32P]ATP buffer (optimized solution of ATP in buffer containing HEPES (pH 7.4) and magnesium chloride), incubated for 30 min at 30°C and terminated by the addition of 10 μl of stop reagent (solution of orthophosphoric acid containing carnosine red). Thirty microliters of each terminated reaction mixture was applied to the center of a paper disk, and each disk was washed twice with 250 ml of 75 mM orthophosphoric acid for 2 min and then three times with 250 ml distilled water for 2 min. The paper disks were dried, put in 20 ml scintillation vials to which 10 ml of liquid scintillation cocktail was added, and counted in a scintillation counter for 32P.

**Protein Kinase Assay** Cell lysates were prepared as described above for Western blotting analysis. The lysates were centrifuged at 12000 rpm for 20 min at 4°C and the supernatants were applied to 50 μl of protein G-Sepharose beads (50% slurry) for 1 h at 4°C to remove nonspecific immune complexes. Fifteen micrograms of anti-JNK-1 mAb or anti-p38 mAb was added to the flow-through fraction, and the mixture was incubated for 4 h at 4°C. Specific immune complexes were precipitated with 30 μl of protein G-Sepharose beads (50% slurry) and washed five times with 1 ml of the ice-cold lysis buffer. Immune-complex kinase assays were performed as previously described by Chen and Blenis (24) using c-Jun (1-79) or ATF-2 (1-96) as a substrate. The reactions were carried out in 30 μl at 30°C for 30 min, stopped by the addition of 30 μl of 2× SDS sample buffer, and analyzed on SDS-polyacrylamide gels. The gels were dried and autoradiographed.

**Measurement of Intracellular Ca2+ Concentration ([Ca2+]i)** Jurkat cells at 4×106 cells/ml in IMDM medium containing 0.3% BSA were incubated with 5 μM Fura-2/AM at 37°C for 20 min. To remove free Fura-2/AM, the cell suspension was diluted 10-fold in IMDM medium, incubated at 37°C for 30 min, and centrifuged at 3000rpm for 5 min. The cells were resuspended in Tyrode-HEPES buffer at 2×106 cells/ml and monitored for intracellular Fura-2 fluorescence with a fluorescence spectrophotometer (Hitachi F-2000) in a cuvette controlled at 37°C with stirring. The fluorescence intensity in 1×106 cells was monitored at 510 nm after excitation at 340/380 nm. The equation [Ca2+]i=Kd(R−Rmin)/(Rmax−R)×Fmax(340 nm)/Fmin(380 nm) was used, where R is the ratio in the fluorescence intensity of Fura-2-loaded cells at 340/380 nm, Fmin is the fluorescence intensity of the Ca2+-free dye at 340 nm, determined in a 10 mM EGTA solution of Triton X-100-lysed cells, and Fmax is the fluorescence intensity of the Ca2+-saturated dye at 380 nm, determined in Triton X-100-lysed cell suspension containing 1 mM CaCl2. The limiting ratios, Rmin (Ca2+-free dye) and Rmax (Ca2+-saturated dye), were determined in Triton X-100-lysed cell suspensions using EGTA or Ca2+ buffers, respectively. The change in the ratio upon stimulation (R) was then used to calculate the change in [Ca2+]i. The published Kd value for the Fura-2/Ca2+ interaction, 224 nm, was used for the calculations (25).

**Statistics** Results were expressed as the mean ± S.E. Statistical analysis was performed with Dunnett's test for multiple comparison after one-way analysis of variance. Student's t-test was also used for comparison between the two. Significant values were those with p<0.05 and p<0.01.

**RESULTS**

**Effect of Muscarinic Agonist on IL-2 Production in Jurkat Cells** Incubation of Jurkat cells with 100 μM Oxo-M, a muscarinic receptor agonist alone did not induce IL-2 production (data not shown). However, pretreatment of the cells with Oxo-M dose dependently (0.1—100 μM) enhanced IL-2 production induced by 10 μM PMA together with 500 nM A23187 (Fig. 1A).

One hundred μM Oxo-M enhanced the PMA/A23187-induced IL-2 production by approximately 175%. Pretreatment with an m1/m3 antagonist, 4-DAMP, or a non-selective antagonist, atropine inhibited the 100 μM Oxo-M-induced enhancement of IL-2 production (Fig. 1B). At a dose of 1 μM of both 4-DAMP and atropine, the Oxo-M-induced enhancement was completely inhibited.

**Expression of Muscarinic Receptor mRNA in Jurkat Cells** To investigate which subtypes of muscarinic receptor mRNA are expressed in Jurkat cells, RT-PCR analyses were carried out on total RNA extracted from Jurkat cells as we have previously shown for hPBL. As shown in Fig. 2, expressions of subtypes m1 and m2, but not of m3—m5, were detected in Jurkat cells, similar to those in hPBL. The specificity of the primers was evaluated using human fetal brain.
Fig. 2. Expression of Muscarinic Receptor mRNA in Jurkat Cells

RT-PCR was used to amplify signals from mRNA isolated from Jurkat cells. The size (bp) of the Hind 1 pluc19 fragments in the last lane is indicated. For each set of subtype-specific primers, mRNA was treated with (+) or without (−) reverse transcriptase. The blank lanes are the absence of reverse transcriptase (absence of contamination from cellular DNA or amplified products).

Fig. 3. Effects of AF-DX116 on Oxo-M Enhanced PMA/A23187-Induced IL-2 Production in Jurkat Cells

AF-DX116 (0.1–10 μM) was added 30 min before Oxo-M treatment. Cells were pretreated with 100 μM Oxo-M for 24 h prior to 10 nM PMA plus 500 nM A23187 treatment for 24 h. Each point is the mean ± S.E. of three independent determinations. The value of Oxo-M-enhanced IL-2 production is shown as 100%.

cDNA as control.9)  

Roles of m2 Receptors in IL-2 Production Since not only m1 but also m2 receptors were expressed in Jurkat cells, the possible role of m2 receptor in IL-2 production in Jurkat cells was examined. When the cells were pretreated with 1 mM AF-DX116, an m2 antagonist, the Oxo-M-induced enhancement of IL-2 production was further stimulated by approximately 150% (Fig. 3).

Effect of Muscarinic Agonist on IL-2 Promoter Activity in Jurkat Cells To measure the IL-2 promoter activity, IL-2-LUC, a plasmid carrying the human IL-2 promoter of 587 bp linked to the luciferase cDNA,10) was transfected into Jurkat cells. The IL-2 promoter activity was maximally increased by combined treatment with 1 nM PMA and 500 nM A23187 (Fig. 4A). The PMA/A23187-induced IL-2 promoter activity was enhanced by pretreatment with 100 μM Oxo-M. This enhancement by Oxo-M was abolished when the cells were treated with an m1/m3 antagonist, 1 μM 4-DAMP (Fig. 4B).

Effects of m1 Receptor Stimulation on Activation of Nuclear Transcription Factors in Jurkat Cells Induction of the IL-2 promoter activity depends on the region 300 bp upstream from the transcription initiation site.13) This region contains the specific binding sites for AP-1, NF-κB, NF-AT and Oct-1, and cooperation of these transcription factors is required for the maximal activation of the IL-2 promoter.8)

To investigate effects of m1 receptor stimulation on activation of nuclear transcription factors in Jurkat cells, we examined the binding activity of the extracts to the target elements for AP-1, NF-κB, NF-AT and Oct-1 by band shift assays. For the specific stimulation of m1 receptor, the cells were treated with Oxo-M in the presence of m2 antagonist AF-DX116.

The band shift analysis by excess amounts of unlabeled AP-1 oligonucleotides confirmed that the bands at the positions indicated by arrowheads were due to the specific AP-1 nucleoprotein complex (the right-end panel of Fig. 5A).

DNA binding of AP-1 was increased in response to PMA/A23187, and this increase was enhanced by the pretreatment with AF-DX116/Oxo-M at 2–2.5 h of incubation period (Fig. 5A and B). In Western blot analysis, similar increases in c-Fos and c-Jun proteins in response to PMA/A23187 were observed, and this increase was also enhanced by the pretreatment with AF-DX116/Oxo-M (Fig. 5C). DNA binding of NF-κB increased in response to PMA/A23187; this increase was, however, not affected by the pretreatment with AF-DX116/Oxo-M (Fig. 6A). DNA binding of NF-AT and Oct-1 were not modified by PMA/A23187, and were not affected by the stimulation of AF-DX116/Oxo-M (Fig. 6B and C).

Roles of MAP Kinase Family in m1 Receptor-Mediated Enhancement of IL-2 Promoter Activities in Jurkat Cells Possible involvement of MAPK/ERK and MAPK/ERK kinase (MEK) pathways in the m1 receptor-mediated enhancement of IL-2 promoter activity was examined. PMA/A23187-induced IL-2 promoter activity was enhanced by 100 μM Oxo-M in the presence of AF-DX116. Pretreatment of cells with 3 μM PD98059, a specific inhibitor of the MEK20 significantly inhibited the m1 receptor-mediated enhancement of IL-2 promoter activity (Fig. 7A), while pretreatment of cells with 1 μM SB203580, a specific inhibitor of p38 MAPK21 did not affect the m1 receptor-mediated enhancement of IL-2 promoter activity (Fig. 7B). The results
Fig. 5. Effects of m1 Receptor Stimulation on PMA/A23187-Induced AP-1 Consensus Sequence Binding Proteins in Jurkat Cells

The cells were treated with or without 1 μM AF-DX116 for 15 min and 100 μM Oxo-M for 1 h prior to treatment with 1 nM PMA plus 500 nM A23187 for 0 to 3 h. Then, band shift assays were performed using each nuclear extract. A, Typical results of band shift assays are shown. The bands indicated by arrowheads were shown to be specifically decreased by competition analysis, using homologous unlabeled oligonucleotides (×5 or ×50 of the labeled probe). B. The quantities of each band are shown in the bar chart. C, Typical data of c-Fos and c-Jun are shown. The amounts of c-Fos and c-Jun were measured by Western blotting analysis.

suggested that pathways via MAPK/ERK and MEK, but not via p38 MAPK, are involved in the m1 receptor-mediated enhancement of IL-2 promoter activity.

The next series of experiments examined the effects of m1 receptor stimulation on activities of the MAPK superfamily were examined. The cells were stimulated with 100 μM Oxo-M in the presence of m2 receptor blocker, AF-DX116. As shown in Fig. 8A, the stimulation of m1 receptor by Oxo-M for 10 min enhanced MAPK/ERK activity to about 130% of control. This enhancement was abolished by pretreatment with 1 μM 4-DAMP for 20 min (data not shown). PMA by itself also increased the MAPK activity. The phosphorylation of c-Jun, a substrate for JNK was increased at 10 to 30 min after the stimulation by Oxo-M (Fig. 8B), while Oxo-M did not affect in the rate of phosphorylation of ATF-2, a substrate for p38 MAPK (Fig. 8C).

Effects of Oxo-M on [Ca^{2+}], and CsA on m1 Receptor-Mediated IL-2 Promoter Activity in Jurkat Cells

Incubation with 100 μM Oxo-M induced a transient [Ca^{2+}], rise, and this rise in [Ca^{2+}], was abolished by pretreatment with 1 μM 4-DAMP (Fig. 9A). Whether or not the Oxo-M-induced elevation of [Ca^{2+}], is involved in the Oxo-M-induced enhancement of IL-2 promoter activity, we examined the effect of CsA, an inhibitor of calcineurin, on this activity. Treatment with 1 μM CsA remarkably decreased the PMA/A23187- induced IL-2 promoter activity, although this treatment did not affect the enhancement of the promoter activity induced by AF-DX116/Oxo-M (Fig. 9B).

These results suggest that the stimulation of muscarinic receptor induces increase in [Ca^{2+}], in Jurkat cells; however, the calcineurin pathway may not be involved in the Oxo-M-induced enhancement of IL-2 production.

DISCUSSION

Muscarinic Receptors in Jurkat Cells

Muscarinic re-
Receptors were expressed in hPBL that consists of enriched T cells and a small population of B cells and monocytes, and stimulated of the receptors resulted in enhancement of PHA-induced IL-2 production. To confirm the involvement of muscarinic receptors in IL-2 production, we used human T cell line Jurkat cells instead of hPBL. The presence of muscarinic receptors on human lymphocytes has been demonstrated in several studies using radioligand binding assays. RT-PCR techniques have revealed the existence of at least five distinct genes (m1—m5) in T cells. As we showed in this study, there are m1 and m2 muscarinic receptor mRNA subtypes in Jurkat cells as well as in hPBL. On the other hand, Ewa and Nordberg showed that m3, m4 and m5 muscarinic receptor mRNAs were expressed in purified human T cells, while m1 and m2 subtypes were not detected. They further reported that both m3 and m5 subtypes were expressed in Peer cells, a human leukemic T cell line. Although Kawashima et al. showed the expression of m4 and m5 subtype mRNAs in the CEM, MOLT-3 and Jurkat cells, the m3 subtype mRNA expression was detected in both CEM and MOLT-3, but not in Jurkat cells. These differences are probably due to the condition of the T cells in the laboratory.

We detected m1 and m2 receptors in Jurkat cells and stimulation of these receptors enhanced both IL-2 production and the promoter activity induced by PMA/A23187. This enhancement was inhibited by 4-DAMP (m1 and m3 receptor antagonist) and atropine (non-selective muscarinic receptor antagonist), while AF-DX116 (m2 receptor antagonist) further enhanced IL-2 production. Therefore, stimulation of m1 receptors alone enhanced IL-2 production and stimulation of m2 receptors alone might inhibit it, although stimulation of both types of m1 and m2 receptors together appeared to enhance it. The cell surface molecules such as CD3, CD2 and IL-2 receptor A subunit were measured by flow cytometric analysis using monoclonal antibodies, but expression of these molecules was not affected by muscarinic receptor stimulation (data not shown). These results suggest that m1 receptor stimulation does not regulate the expression of cell surface molecules involved in the TCR response in Jurkat cells.

Ca²⁺- and MAPK-Pathways in IL-2 Promoter Stimulation of muscarinic receptors increases phospholipase C activity and thus [Ca²⁺], from intracellular stores and protein kinase C activity. The Ca²⁺-pathway via calcineurin may be involved in IL-2 production by stimulating the translocation of transcription factors such as NF-AT from cytoplasm into nucleus. In this study, pretreatment of Jurkat cells with CsA, which blocks translocation of NF-AT to nucleus by in-
Fig. 9. Oxo-M-Induced [Ca\textsuperscript{2+}]\textsubscript{i} Rise and Effects of CsA on m1 Receptor-Mediated IL-2 Promoter Activity in Jurkat Cells

A. The time course of the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was examined after addition of 100 \mu M Oxo-M (upper) or 1 \mu M 4-DAMP for 20 min prior to treatment with 100 \mu M Oxo-M (lower). B. Cells were pretreated with 1 \mu M CsA for 2 h prior to treatment with 1 \mu M AF-DX116 and 100 \mu M Oxo-M or 24 h, and then treated with 1 \mu M PMMA and 300 \mu M A23187 for 24 h. Cells were transfected and the average of data normalized to the value of PMMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity as 100\% (± S.E.). **, p < 0.01 versus the value in the absence of AF-DX116/Oxo-M. Statistical analysis was performed using the Student’s t-test.

ubrinating the activity of calcineurin,\textsuperscript{34,35} remarkably diminished the IL-2 promoter activity. In addition, the stimulation of muscarinic receptor evoked a transient [Ca\textsuperscript{2+}]\textsubscript{i} increase in Jurkat cells. Band shift assay, however, showed little difference of the NF-AT-DNA complex during the m1 stimulation. These results suggest that m1 receptor-mediated enhancement of IL-2 promoter activity may not be due to activation of the calcineurin pathway. In our experiment, NF-AT was constantly detected within 3 h after the m1 receptor stimulation. One explanation for the existence of NF-AT, or Oct-1, in the Jurkat cells even before the m1 receptor stimulation may be the fact that the Jurkat cells are cell lines. Since cell lines constantly undergo mitosis, unlike normal T cells, it may be necessary for cells to induce nucleofactors through the cell cycle.

As mentioned in Introduction, m1 receptor stimulation may activate MAPKs through protein kinase C or \beta y subunits of G proteins. However, there were no remarkable differences in the NF-\kappaB complex between the cells pretreated with and without m1 receptor stimulation in our system. Therefore, it is likely that m1 receptor-mediated pathways do not activate NF-\kappaB.

Involvement of MAPKs in Muscarinic Receptor Stimulation The results of the present study indicate that ERK and JNK, but not p38 kinase, are involved in the m1 receptor-mediated enhancement of IL-2 production by stimulating AP-1 activity. There are some reports that MAPK signal transduction pathways are involved in IL-2 gene transcription in T cells: Ras, the JNK cascade, and one or more of AP-1\textsuperscript{36} raf-1, MEK1, and ERK 1/2.\textsuperscript{37} Therefore, ERK and JNK may activate AP-1 proteins via the activation of c-Fos and c-Jun, respectively. Since stimulation of the m1 receptor itself did not induce IL-2 production or promoter activity, c-Fos and c-Jun by themselves did not seem to induce the IL-2 production sufficiently. Another possibility is that the enhancement of AP-1 binding by AF-DX116 and Oxo-M is due to m2 receptor inhibition. The inhibition of m2 receptors may lead to activation of adenylate cyclase because m2 receptors negatively regulate cyclic AMP production.\textsuperscript{38} These events may cause activation of protein kinase A which may possibly modulate AP-1 activity. The possibility of m2 receptor activation under resting conditions should be elucidated in the future. In conclusion, our results identified the functional roles and the intracellular pathways through the m1 muscarinic receptor to IL-2 promoter in Jurkat cells showing that there may be relationships between ACh and T cells. These results show a model of neuroimmune interaction through neurotransmitter receptor signaling in immunocompetent cells.

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