



HOKKAIDO UNIVERSITY

Title	Roles of Muscarinic Receptors m1 and m2 on Interleukin 2 Production in Human Peripheral Blood Lymphocytes and Human Leukemic T Cell Line Jurkat Cells
Author(s)	Fujino, Hiromichi
Degree Grantor	北海道大学
Degree Name	博士(薬学)
Dissertation Number	甲第4231号
Issue Date	1997-09-30
DOI	https://doi.org/10.11501/3130586
Doc URL	https://hdl.handle.net/2115/51445
Type	doctoral thesis
File Information	000000315776.pdf



DOCTORAL THESIS

**Roles of Muscarinic Receptors m1 and m2 on
Interleukin 2 Production in Human Peripheral
Blood Lymphocytes and Human Leukemic T Cell
Line Jurkat Cells**

ヒト末梢血リンパ球およびヒト株化T細胞 Jurkat のインターロイキン2産生における m1、m2 ムスカリン性受容体の役割

Hiromichi Fujino

*Faculty of Pharmaceutical Sciences
Hokkaido University*

Under the Supervision of
Professor Dr. Yasuyuki Nomura

Sapporo 1997

①

DOCTORAL THESIS

**Roles of Muscarinic Receptors m1 and m2 on
Interleukin 2 Production in Human Peripheral
Blood Lymphocytes and Human Leukemic T Cell
Line Jurkat Cells**

ヒト末梢血リンパ球およびヒト株化T細胞 Jurkat のインターロイキン2産生における m1、m2 ムスカリン性受容体の役割

Hiromichi Fujino

*Faculty of Pharmaceutical Sciences
Hokkaido University*

Under the Supervision of
Professor Dr. Yasuyuki Nomura

Sapporo 1997

Table of Contents

	page
Table of Contents	1
Acknowledgments	3
Abbreviations	4
Summary	6
General Introduction	8
Materials	9
Experimental Procedures	11
Section 1: Roles of muscarinic receptors on T cell antigen receptor mediated interleukin-2 production in human peripheral blood lymphocytes	
1-1. Introduction	20
1-2. Results	21
1-3. Discussion	24
1-4. Conclusion of Section 1	26
Section 2: Roles of muscarinic receptors on interleukin-2 production in Jurkat cells	
Part 1: Roles of m1 receptors	
2-1. Introduction	36
2-2. Results	37
2-3. Discussion	43
2-4. Conclusion of Section 2, Part 1	47
Part 2: Roles of m2 receptors	
2-5. Introduction	63

2-6.	Results	64
2-7.	Discussion	68
2-8.	Conclusion of Section 2, Part 2	70
Section 3: Interleukin-2 production enhanced by cyclic AMP pretreatment		
3-1.	Introduction	84
3-2.	Results	86
3-3.	Discussion	89
3-4.	Conclusion of Section 3	91
General conclusions		101
References		103

Acknowledgments

I gratefully acknowledge Professor Yasuyuki Nomura for supervision of the work.

I thank Dr. Toshihiko Murayama, and Dr. Takashi Uehara (Department of Pharmacology), Professor Hiroyoshi Ariga, Dr. Takahiro Taira, Dr. Hirotake Kitaura, and Dr. Yasuhiro Imamura (Department of Molecular biology), Professor Sanae M.M. Iguchi-Ariga (College of Medical Technology), Professor Yukiko Tokumitsu (Department of Physiological Chemistry), for critical review of the manuscripts and valuable discussions.

I also appreciate critical reading of the manuscript by Dr. Yasunobu Okuma (Department of Pharmacology) and Mr. Bishonga Christopher (Faculty of Veterinary Medicine) and their valuable comments.

I express my sincere thank to Professor Morio Kanno (Faculty of Medicine) and Shoshisha Foundation for consideration to my research support.

I am also grateful to Mr. Masatoshi Sakai (Department of Hygienic Chemistry) for preparation of hPBL.

Finally, I want to express my deep gratitude to my parents, my sister and my friends for encouraging me throughout the works.

Abbreviations

ACh:	acetylcholine
AIDS:	acquired immunodeficiency syndrome
ATP:	adenosine triphosphate
bp:	base pair
BSA:	bovine serum albumin
$[Ca^{2+}]_i$:	intracellular Ca^{2+} concentration
CaM:	calmodulin
cAMP:	adenosine 3', 5'-cyclic monophosphate
CNS:	central nervous system
CsA:	cyclosporin A
DAG:	diacylglycerol
DBcAMP:	N_6, O_2 -dibutryl cAMP
DTT:	dithiothreitol
EIA:	enzyme immunoassay
ELISA:	enzyme-linked immunosorbent assay
ERK:	extracellular signal regulated protein kinase
FACS:	fluorescence-activated cell sorter
FCS:	fetal calf serum
FKBP:	FK506 binding protein
G-protein:	GTP-binding protein
HEPES:	4-(2-hydroxyethyl)-1-piperazineethane sulfate
hPBL:	human peripheral blood lymphocytes
IL-2:	interleukin-2
IMDM:	Iscov's modified Dulbecco's medium
Ins (1, 4, 5) P_3 :	inositol 1, 4, 5-trisphosphate

JNK:	c-Jun N-terminal kinase
kb:	kilobase
mAb:	monoclonal antibody
MAPK:	mitogen-activated protein kinase
MAPKK:	MAP kinase kinase
MEK:	MAP or ERK kinase
Oxo-M:	oxotremorine-M
PBS:	phosphate buffered saline
PHA:	phytohemagglutinin
PI:	phosphatidylinositol
PI (4, 5) P ₂ :	phosphatidylinositol 4, 5-bisphosphate
PKA:	protein kinase A
PKC:	protein kinase C
PLC:	phospholipase C
PMA:	phorbol 12-myristate 13-acetate
PMSF:	phenylmethylsulfonyl fluoride
PYK2:	proline-rich tyrosine kinase 2
RT-PCR:	reverse transcription-polymerase chain reaction
SCID:	severe combined immunodeficiency
SDS:	sodium dodecylsulfate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
TBST:	tris buffered saline
TCR:	T cell antigen receptor

Summary

In this study, I found the existence of muscarinic acetylcholine (mACh) receptors in human peripheral blood lymphocyte (hPBL) as well as in human leukemic T cell line Jurkat cells, and examined the roles of the mACh receptors in interleukin-2 (IL-2) production in the cells.

In the first section, I examined the effects of a nonselective muscarinic receptor agonist, oxtemorine-M (Oxo-M), on IL-2 production in hPBL. The mACh receptor subtype genes have been characterized as m1 to m5 (1). I found that m1 and m2 subtype receptor mRNAs are expressed in hPBL and the pretreatment of the receptors with Oxo-M enhanced IL-2 production (2). I demonstrated that Jurkat cells also expressed m1 and m2 receptor mRNAs.

In the second section, I examined the physiological roles of the m1 and m2 receptors in Jurkat cells. The results showed that the m1 subtype stimulation enhanced IL-2 production, while the m2 subtype stimulation decreased it through the regulation of mitogen-activated protein kinase (MAPK)- and c-Jun N-terminal kinase (JNK)-mediated pathways.

In the third section, I examined the effects of cyclic AMP (cAMP) on IL-2 production. Pretreatment with dibutyryl cAMP (DBcAMP) for 24 hr enhanced IL-2 production in Jurkat cells. Fluorescence-activated cell sorter (FACS) analysis demonstrated that Jurkat cells were arrested in G1 phase by DBcAMP treatment. The enhancement of IL-2 production seemed to be correlated to the cell cycle progression. Although the m2 receptors are known to couple with Gi protein which inhibits cAMP accumulation. There were no effects of the m2 receptor stimulation on the cell cycle in Jurkat cells.

In the sections 2 and 3, stimulation of m2 receptors for 18 hr did not affect the amount of cAMP, after PMA/A23187 stimulation for 30 min caused decreases of AP-1.

Concomitant stimulation with m2 receptors and PMA/A23187 for 30 min decreased the amount of cAMP and caused decreases of AP-1 and NF- κ B. The relationships of these events must be clarified in the future.

These findings may be important steps to further clarify the functional roles and the intracellular mechanism of the neuro-immune interaction.

General Introduction

In Alzheimer's disease, cholinergic activity is low in the central nervous system (CNS). Muscarinic receptor expression in hPBL of patients is markedly decreased, although the receptor-ACh-affinity is not changed (3). The functions of T cells have been shown to decrease with increasing age in several immuno-dysfunction diseases (4), *e.g.*, severe combined immunodeficiency (SCID) or acquired immunodeficiency syndrome (AIDS). These findings suggest that a selective muscarinic receptor agonist may be therapeutically useful for the treatment of several diseases which result from dysfunction of TCR/CD3 complexes in T cells. It is important to further clarify the functional roles and the intracellular mechanism of the neuro-immune interaction.

Several bi-directional interactions are known to exist between nervous and immune systems *via* neurotransmitters and cytokines. Lymphoid organs such as spleen, lymph nodes and thymus contain vagus nerve terminals, which may contact with lymphocytes (5). In addition, receptors for many neurotransmitters, including catecholamines and acetylcholine (ACh), have been detected on the surface of lymphocytes (6, 7). ACh is a neurotransmitter which exists in both central and peripheral nervous systems. Cholinergic system may hence take part in the immuno-neuroendocrine system. Despite of several reports on the existence of ACh receptors in lymphocytes, biochemical mechanisms of the roles of ACh system in the immune system have not been elucidated in detail. In this study, I examined the roles of muscarinic acetylcholine receptors expressed in T lymphocytes on interleukin-2 (IL-2) production.

Materials

[γ - ³² P] ATP:	NEN Research Products
4-DAMP:	Research Biochemical Inc.
A23187:	Sigma
AFDX-116:	Nippon Boehringer Ingelhei
anti-human rabbit polyclonal IgG c-Fos (sc-52):	Santa Cruz Biotechnology Inc.
anti-human rabbit polyclonal IgG c-Jun (sc-44):	Santa Cruz Biotechnology Inc.
anti-human mouse monoclonal IgG CD2 (XIX. 8):	Cymbus Bioscience
anti-human mouse monoclonal IgG CD3 (NU-T3):	Nichirei
anti-human CD3 coated T-25 flask:	AIS inc.
anti-human mouse monoclonal IgG CD4 (NU-TH/I):	Nichirei
anti-human mouse monoclonal IgG CD8 (NU-TS/C):	Nichirei
anti-human mouse monoclonal IgG IL-2 receptor:	CHEMICON
anti-human rabbit polyclonal IgG JNK-1 (sc-474):	Santa Cruz Biotechnology Inc.
AP-1 oligonucleotide set:	Oncogene Science Inc.
aprotinin:	Sigma
cAMP enzyme immunoassay kit:	Amersham Life Science
CsA:	Sandoz Ltd.
DBcAMP:	Sigma
DTT:	Sigma
ECL Western detection kit:	Amersham Life Scinece
FCS:	GIBCO/BRL
Ficoll-Paque:	Pharmacia/LKB
FK-506:	Fujisawa Pharmaceutical Co. Ltd.
forskolin:	Sigma
Fura-2/AM:	Dojin

H-89:	Seikagaku kogyo
herbimycin A:	Wako Pure Chemical Ind. Ltd.
human c-Jun (1-79) substrate (sc-4113):	Santa Cruz Biotechnology Inc.
human fetal brain MATCHMAKER cDNA:	Clontech Laboratories Inc.
human γ -globulin:	Wako Pure Chemical Ind. Ltd.
IL-2 ELISA kit:	NEN/Dupont, IWAKI GLASS
IMDM:	GIBCO/BRL
leupeptin:	Sigma
luciferase lysis buffer:	PicaGene™ TOYO INK
luciferase substrate:	PicaGene™ TOYO INK
NF- κ B oligonucleotide set:	Oncogene Science Inc.
Oct-1 oligonucleotide set:	Oncogene Science Inc.
orthophosphoric acid:	Wako Pure Chemical Ind. Ltd.
sodium orthovanadate:	Sigma
Oxo-M:	Research Biochemical Inc.
p42/p44 MAP kinase enzyme assay kit:	Amersham Life Science
PD98059:	NEN Research Products
pGV-B:	PicaGene™ TOYO INK
PHA:	Sigma
pirenzepine:	Nippon Boehringer Ingelheim
PMA:	Sigma
PMSF:	Wako Pure Chemical Ind. Ltd.
propidium iodide:	Wako Pure Chemical Ind. Ltd.
RPMI 1640:	GIBCO/BRL
SB203580:	Smithkline Beecham
thymidine:	Wako Pure Chemical Ind. Ltd.

Experimental procedures

Preparation of human peripheral blood lymphocytes (hPBL): hPBL were extracted from the venous blood of healthy volunteers by dextran sedimentation followed by Ficoll-Paque gradient centrifugation, and the cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FCS in a humidified atmosphere of 10% CO₂ and 90% air at 2×10^6 cells/ml. Trypan blue dye staining revealed that the viability of hPBL was more than 98%.

Positive panning separation of CD3-positive T cells from hPBL: CD3-positive T cells were positively selected from hPBL by a panning technique (8). Briefly, the antibody-coated flasks were washed three times with a PBS-Ca²⁺-/Mg²⁺-free medium (PBS/CMF) prior to panning. Approximately 5×10^7 fresh hPBL were incubated at room temperature with 4 ml of PBS/CMF containing 0.5% human γ -globulin and then introduced into the anti-CD3 mAb coated T-25 flasks. After incubation for 1 hr at room temperature, non-attached cells were removed by pipetting and 10 ml of the "release medium" (10% FCS-containing RPMI-1640) was added. After 72 hr culture in a humidified atmosphere of 5% CO₂ at 37 °C, adherent cells were removed from the flasks by pipetting. The cells were then replated at $0.5 - 1 \times 10^6$ cells/ml in a fresh RPMI-1640 medium.

Assay of IL-2 production: The amount of IL-2 produced by cultured T cells was determined using an ELISA kit for human IL-2. In brief, cell-free culture supernatants were obtained by centrifugation at 500 x g for 5 min. Samples were assayed according to the manufacturer's instructions and analyzed on an automated ELISA plate reader (MTP-120, Corona Electric). Data are expressed using international unit (I.U. , 1 I.U./ml = 0.2 ng/ml).

Measurement of $[Ca^{2+}]_i$: hPBL at 2×10^6 cells/ml in RPMI-1640 medium containing 0.3% BSA were incubated with 5 μ M Fura-2/AM at 37 °C for 20 min. To remove free Fura-2/AM, the cell suspension was diluted 10-fold in RPMI-1640 medium, incubated at 37 °C for 30 min, and centrifuged at 300 x g for 5 min. The cells were resuspended in Tyrode-Hepes buffer at 2×10^6 cells/ml and monitored for intracellular Fura-2 fluorescence by a fluorescence spectrophotometer (Hitachi F-2000) in a cuvette controlled at 37 °C over stirring. The fluorescence intensity in 1×10^6 cells was monitored at 510 nm after excitation at 340/380 nm. The equation $[Ca^{2+}]_i = K_d(R - R_{min}) / (R_{max} - R) \times F_{min}(340 \text{ nm}) / F_{max}(380 \text{ nm})$ was used, where R is the ratio in the fluorescence intensity of Fura-2-loaded cells at 340/380 nm, F_{min} is the fluorescence intensity of the Ca^{2+} -free dye at 340 nm, determined in a 10 mM EGTA solution of Triton X-100- lysed cells, and F_{max} is the fluorescence intensity of the Ca^{2+} -saturated dye at 380 nm, determined in a Triton X-100-lysed cell suspensions containing 1 mM $CaCl_2$. The limiting ratios, R_{min} (Ca^{2+} -free dye) and R_{max} (Ca^{2+} -saturated dye), were determined in Triton X-100-lysed cell suspensions using EGTA or Ca^{2+} buffers, respectively. The change in the ratio upon stimulation (R) was then used to calculate the change in $[Ca^{2+}]_i$. The published K_d value for the Fura-2/ Ca^{2+} interaction, 224 nm, was used for the calculations (9).

Flow cytometric analysis of CD3, CD2, CD4, CD8 and IL-2 receptor α subunit molecules on the cell surface: After incubation in PBS for 30 min at 4 °C in the presence of 1 μ g of murine mAb against CD3, CD2, CD4, CD8, or IL-2 receptor α subunit, 1×10^6 hPBL cells were reacted with 1.25 μ g of FITC-labeled secondary anti-mouse Ab for 30 min at 4 °C. The FITC-stained cells were analyzed with a flow cytometer (EPICS-CS, Coulter Electronics) to assess the expression of CD3, CD2, CD4,

CD8 or IL-2 receptor α subunit molecules on the cell surface.

RNA isolation and RT-PCR analysis: Total cellular RNA was extracted from hPBL and Jurkat cells with 4 M guanidine thiocyanate as described by Chomczynski and Sacchi (10). The cells were washed twice with ice-cold PBS prior to lysis. The RNA samples were spectrophotometrically quantified by absorbance at 260 and 280 nm. PCR primers of 21 nucleotides were designed for human muscarinic receptors m1-m5 (11). The sequences, corresponding base numbers relative to the coding initiation site, sizes of expected PCR products and the registered sequence numbers (GenBank) are shown in Table 1. 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. One μ g of the total RNA was incubated at 37 °C for 60 min with a mixture of 100 units of RT, 1 x first strand buffer, 10 mM DTT, 0.5 mM each dNTP, and 50 units RNase inhibitor in a final volume of 20 μ l. The reaction mixture was further incubated for 10 min at 70 °C to inactive RT. As control templates, human fetal brain MATCHMAKER cDNA library were digested with EcoRI and extracted. Two μ l of the RT products or the control templates were mixed with 1 mU of TUB DNA polymerase, 200 nM each of sense and antisense primers in a buffer containing 1 x TUB buffer and 0.2 mM each dNTP in final volume of 20 μ l. The mixture was overlaid with 30 μ l of liquid paraffin to prevent evaporation and then amplified by 40 cycles of PCR. The PCR products were separated by electrophoresis in a 1.5% agarose gel in 1 x TBE buffer. The gel was stained with ethidium bromide and photographed.

Cell culture and transient transfection: Human Jurkat cells were cultured

in IMDM supplemented with 10% FCS. Ten μg of the pIL-2LUC plasmid carrying the human IL-2 promoter of 568 bp linked to the luciferase cDNA was transfected to the cells (2×10^5 or 4×10^5 cells/ml) by the DEAE-dextran method (12) and the cells were incubated for 48 - 72 hr before harvest.

Luciferase assays: The transfected cells were harvested, washed with PBS, lysed using 200 μl of a detergent solution (lysis buffer), and centrifuged at 12,000 rpm for 10 min. The supernatants were standardized and subjected to the luciferase reaction in a total of 20 μl lysis buffer with 100 μl of luciferase substrates 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 pGV-B was assayed in parallel as a control.

Band shift assay: Nuclear extracts of Jurkat cells were prepared as described by Dignam *et al.* (13), with minor modifications (14). 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 MgCl_2 , 0.2 mM EDTA and 0.5 mM DTT. As probes, one μg of annealed oligonucleotides were end-labeled using $[\gamma\text{-}^{32}\text{P}]$ and T4 polynucleotide kinase, purified by gel filtration through Sephadex G50, separated in a 10% polyacrylamide gel, and eluted from the gel. Binding reactions were carried out by incubating 0.2 - 1 pmole of end-labeled DNA (10,000 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 mg/ml BSA in a final volume of 15 μl . After incubation, the reaction mixtures were loaded on a 4% polyacrylamide gel in 0.25 x 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 in this experiments were as follows, where small letters indicate additional linker sequence: AP-1,

gatcCATCGTGACTCAGCGATCTCGTGACTCAGCGG; NF- κ B,

GATCCGAGGGGACTTTCCGCTGGGGACTTTCCAGG; NF-AT,

ACGCCCAAAGAGGAAAATTTGTTTCATACA (15); Oct-1,

gatcGCATTTGCATGATCGATCGCATTTGCATGATC.

Western blot analysis: Cells were harvested, washed with 3 ml of ice-cold PBS, and lysed in 500 μ l of a lysis buffer containing 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin (pH 7.4 measured at 4 $^{\circ}$ C). The lysates were centrifuged at 12,000 rpm for 20 min at 4 $^{\circ}$ C and the supernatants were boiled for 5 min with the SDS sample buffer as previously described by Chen *et. al.* (16) and stored at -70 $^{\circ}$ C until use. The samples were re-boiled and subjected to a 7.5% SDS-PAGE followed by transfer on nitrocellulose filters at 100 V for 1.5 hr at 4 $^{\circ}$ C. The filters were then blocked with TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 1% BSA for 1 hr and incubated with the primary antibody in TBST for 1 hr at room temperature. After washing three times with TBST, the filters were incubated with the secondary antibody (an anti-mouse or an anti-rabbit IgG-horseradish peroxidase conjugate) for 30 min. After washing with TBST three times, antibody-reactive bands were visualized by a chemiluminescence detection kit (ECL Western detection kit).

p42/p44 MAP kinase assay: Cell lysates were prepared and the supernatants were stored as described above in Western blot analysis. The activity of p42/p44 MAP

kinase was assayed using the BIOTRAK™ MAP kinase assay system. Fifteen μ l of each sample were added to 10 μ l of a buffer containing HEPES, sodium orthovanadate, 0.05% sodium azide (pH 7.4) and the peptide corresponding to EGF receptor carrying the Thr669 phosphorylation site as a substrate. The reaction was triggered by adding 5 μ l of a magnesium- 32 P]ATP buffer (optimized solution of ATP in a buffer containing HEPES and magnesium chloride, pH 7.4), incubated for 30 min at 30 °C and terminated by the addition of 10 μ l of a stop reagent (solution of orthophosphoric acid containing carmosine red). Thirty μ l of each terminated reaction mixtures were applied on the center of each paper disc, and the discs were 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 discs were dried, put in a 20 ml scintillation vial, added 10 ml of liquid scintillation cocktail and counted in a scintillation counter for 32 P.

Protein kinase assay: Cell lysates were prepared as described above in Western blot analysis. The lysates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatants were applied to 50 ml of protein G-Sepharose beads (50% slurry) for 1 hr 4 °C to remove nonspecific immune complexes. Fifteen μ g of an anti-JNK-1 mAb were added to the flow through fractions, and the mixture was incubated for 4 hr at 4 °C. Specific immune complexes were precipitated with 30 μ l 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 *et. al.* (16) using GST-Jun (1-79) as a substrate. The reactions were carried out in 30 μ l at 30 °C for 30 min, stopped by the addition of 30 μ l of 2 x SDS sample buffer, and analyzed on an SDS-polyacrylamide gel. The gel was dried and autoradiographed.

Cell cycle analysis: Jurkat cells (2×10^6) were washed with PBS, then resuspended in 5 ml of 70% ethanol then incubated for 30 min at 4 °C. Cells were pelleted and resuspended in 0.4 ml PBS containing 20 µg RNase then incubated for 20 min at 37 °C. Cells were pelleted and resuspended in 0.5 ml of 0.1% sodium citrate containing 50 mg/ml propidium iodide, and incubated for 10 min on ice. The proportions of cells in G1, S and G2 phases of the cell cycle were analyzed by flow cytometry on a FACSort with CellQuest software (Becton Dickinson Advanced Cellular Biology, San Jose, CA).

Jurkat cells synchronization: Cells were synchronized at G1/S phase boundary by the double-thymidine block protocol (17). Briefly, Jurkat cells were incubated in IMDM containing 2.5 mM thymidine for 24 hr, washed 3 times, and incubated another 10 hr without thymidine. Thymidine was then added again at the same concentration, and the cells were incubated another 14 hr.

cAMP enzyme immunoassay (EIA) system: Jurkat cells (2×10^6) were washed with PBS, then resuspended in 5 ml of 70% ethanol then incubated for 30 min at 4 °C. The extracts were centrifuged at 5,000 rpm for 15 min at 4 °C and transferred the supernatants to fresh tubes. The combined extracts were dried in vacuum oven. The dried extracts were dissolved in 0.2 ml of assay buffer prior to analysis. The amount of cAMP was assessed using the BIOTRAK™ cAMP EIA system. Samples were assayed according to the manufacturer's instructions and analyzed on an automated ELISA plate reader (MTP-120, Corona Electric).

Table 1. PCR primers designed for human muscarinic receptors subtype m1-m5.

receptor subtype	PCR primers (upper/lower)	relative base # to initiation site	size of PCR product (bp)	Sequence # (GenBank)
m1	GAAAGAAGAGGAAGAGGACGAA CAGGAGAGGGGACTATCAGCA	826-847 1378-1399	573	X15263
m2	GGTCCTCTCTTTCATCCTCT TCCTGGGTATTTCATCATCT	443-464 891-912	469	X15264
m3	AGCCAAACGAACAACAAGAG TTGAAGGACAGAGGTAGAGTG	531-552 1356-1377	846	X15266
m4	CGCTATGAGACGGTGGAATG CGTCTTGGCTTCTCTCCTT	76-98 703-724	648	X15265
m5	GGA AACAGAGAAAGCGAACCAA AGCACAAACCAATAGCCCAAGT	654-675 1433-1454	800	M80333

Section 1

Roles of muscarinic receptors on T cell antigen receptor-mediated interleukin-2 production in human peripheral blood lymphocytes

1-1. Introduction

Activation of multicomponent TCR/CD3 complexes by antigens, lectins or anti-CD3 mAb results in activation of various signal transduction pathways, such as rapid PLC-mediated hydrolysis of PIP_2 into $\text{Ins}(1,4,5)\text{P}_3$ and DAG (18). They cause activation of $[\text{Ca}^{2+}]_i$ increase and PKC activation. Another rapid event that follows TCR/CD3 activation is phosphorylation of tyrosine residues of several proteins, including TCR ζ subunit and PLC- γ 1 (19), which are possibly involved in $\text{p}21^{\text{ras}}$ activation (20).

IL-2 is the lymphocytotropic hormones and has a pivotal role in the generation and regulation of immune responses. Activation of TCR/CD3 complexes induces IL-2 production in T cells, and the IL-2 production is inhibited by some immunosuppressants, such as CsA and FK-506 (21). CsA- and FK-506-binding proteins have been identified as immunophilins (cyclophilins and FKBP, respectively) (22), and are targets for Ca^{2+} /CaM-dependent phosphatase 2B which is also known as calcineurin (23). Detailed mechanisms of the IL-2 production and regulation are, however, not clear.

In this section, I found that muscarinic ACh receptors exist in human peripheral lymphocytes and that the activation of the receptors enhances the IL-2 production induced by TCR/CD3 complexes.

1-2. Results

Muscarinic agonist-induced IL-2 production in hPBL: Incubation of hPBL with 1 μ M ACh (in the presence of 100 μ M physostigmine, a cholinesterase inhibitor), 1 μ M Oxo-M, or 1 μ M nicotine for 24 hr did not induce IL-2 production (Fig. 1.1A). When the cells were incubated with ACh or Oxo-M as mentioned above, subsequent induction of IL-2 production by PHA was, however, enhanced (Fig. 1.1B). Preincubation with nicotine did not affect the PHA-induced IL-2 production (Fig. 1.1B). The IL-2 production induced by 1 μ g/ml of an anti-CD3 mAb was also enhanced by Oxo-M (162 %) to a similar level in case of the PHA-induced IL-2 production (169 %) (data not shown). The effects of Oxo-M on the IL-2 production induced by 10 μ g/ml PHA in CD3-positive T cells prepared by a positive panning selection from hPBL was also examined. As shown in Fig. 1.1C, the enhancement by Oxo-M pretreatment was similarly observed in hPBL. The PHA-induced IL-2 production was enhanced by preincubation with 1 μ M Oxo-M to 176 % in CD3-positive cells while 169 % in hPBL (Fig. 1.1B,C). Incubation with 1 μ M Oxo-M for 24 hr by itself did not induce IL-2 production in CD3-positive cells (data not shown). The enhancement of PHA-induced IL-2 production by Oxo-M pretreatment for 24 hr was observed with Oxo-M at various concentrations ranging from 10 nM to 10 μ M (data not shown). Addition of 0.1 μ M Oxo-M together with PHA did not enhance the IL-2 production, but pretreatment with Oxo-M longer than 1 hr prior to PHA addition resulted in the enhancement (Fig. 1.2A). Furthermore, the IL-2 production was induced by PHA much earlier (within 3 hr) in hPBL pretreated with Oxo-M for 24 hr than in untreated hPBL, and in 12 hr after PHA addition, the production in the pretreated cells was increased 2-3 fold compared to that in

the untreated cells (Fig. 1.2B). Addition of PHA at low concentrations (1-3 $\mu\text{g/ml}$) hardly induced IL-2 production in untreated hPBL, but induced in the cells pretreated with Oxo-M (Fig. 1.3).

Effects of FK-506 and CsA on IL-2 production from hPBL: Two immunosuppressant, FK-506 and CsA, are known to inhibit the production of several cytokines including IL-2 (21). Therefore, the effects of FK-506 and CsA on the PHA-induced IL-2 production as well as the enhancement of the IL-2 production by Oxo-M pretreatment in hPBL were examined. Both FK-506 and CsA significantly inhibited the IL-2 production either with or without Oxo-M pretreatment to similar extents (Fig. 1.4).

Effects of Oxo-M and PHA on $[\text{Ca}^{2+}]_i$ in hPBL: Incubation with 10 $\mu\text{g/ml}$ PHA induced and sustained a marked increase of $[\text{Ca}^{2+}]_i$ in hPBL (Fig. 1.5B). Treatment with an anti-CD3 mAb (NU-T3) also induced an increased of $[\text{Ca}^{2+}]_i$, to an extent similar to PHA-induced $[\text{Ca}^{2+}]_i$ (24). Oxo-M, on the other hand, hardly affected $[\text{Ca}^{2+}]_i$ in hPBL even at a high concentration (10 μM) (Fig. 1.5A). In Jurkat cells, Oxo-M at concentrations higher than 1 μM is required for $[\text{Ca}^{2+}]_i$ increase (25). The results suggest that $[\text{Ca}^{2+}]_i$ increase may not be involved in the enhancement of PHA-induced IL-2 production by Oxo-M.

Effects of Oxo-M treatment on expression of CD3, CD2, CD4, CD8 and IL-2 receptor α subunits on hPBL and T cells: To assess a possibility that Oxo-M pretreatment may upregulate the expression of cell surface molecules involved in the PHA response in T cells, flow cytometric analysis was performed. The fluorescence shifts with regards to CD3 and CD2 molecules were indistinguishable between untreated

hPBL and the cells treated with 0.1 μ M Oxo-M for 24 hr (Fig. 1.6A). Oxo-M did not affect the expression of CD4, CD8 or IL-2 receptor α subunit molecules, either. Similar results were obtained in CD3 positive T cells (Fig. 1.6B). Oxo-M thus did not upregulate the expression of the cell surface molecules such as CD3, CD2, CD4, CD8 and IL-2 receptor α subunit in T cells.

Expression of muscarinic receptor mRNA in hPBL: To investigate which subtypes of muscarinic receptor mRNA are expressed in hPBL, RT-PCR analyses were carried out on total RNA extracted from hPBL using primers for human muscarinic receptors m1-m5. As shown in Fig. 1.7, mRNA for muscarinic receptors m1 and m2 mRNA were detected, but the expression of the receptors m3-m5 was not observed in hPBL.

1-3. Discussion

Oxo-M, a nonselective muscarinic receptor agonist, was previously found to induce activation of PLC through different mechanisms from those by PHA in Jurkat cells (25). Here I examined the effects of Oxo-M on IL-2 production in hPBL.

I have shown that PHA-induced IL-2 production in hPBL was enhanced by pretreatment of hPBL with Oxo-M or ACh, but not with nicotine (Fig. 1.1). The enhancement of PHA-induced IL-2 production by Oxo-M required at least 1 hr preincubation of hPBL with Oxo-M before PHA addition, and the longer preincubation resulted in the stronger induction of IL-2 production (Fig. 1.2). These findings suggest that there are muscarinic receptors in hPBL and stimulation of these receptors enhanced IL-2 production.

FK-506 and CsA, immunosuppressive drugs which inhibit IL-2 production (21), form complexes with immunophilins (such as FKBP and cyclophilins) and block Ca^{2+} -pathways. A Ca^{2+} -pathway *via* calcineurin may be involved in IL-2 production by stimulating the translocation of transcriptional factors such as NF-AT from the cytoplasm into the nucleus. Here I showed that FK-506 and CsA inhibited the PHA-induced IL-2 production in Oxo-M-pretreated cells as well as in untreated cells. The decrease of the IL-2 production due to FK-506 or CsA was comparable between Oxo-M treated and untreated cells (Fig. 1.4). Oxo-M by itself caused a slight elevation of $[\text{Ca}^{2+}]_i$ in hPBL (Fig. 1.5A), but the elevation was much lower than that induced by PHA. The results indicate that Oxo-M-induced enhancement of IL-2 production is not, at least not largely, due to $[\text{Ca}^{2+}]_i$ elevation and thereby that increase of $[\text{Ca}^{2+}]_i$ may not be essential in the IL-2 production enhanced *via* muscarinic receptors in hPBL.

Another possible mechanism of the enhancement by Oxo-M was that Oxo-M might increase the expression of the surface molecules involved in PHA responses, including CD3, CD2, CD4, CD8 and IL-2R α . In FACS analyses using monoclonal antibodies

against each protein, no changes in their expressions were observed between the cells untreated and treated (Fig. 1.6). The results suggest that stimulatory effects of Oxo-M on IL-2 production might be due to activation of intracellular signaling mechanisms.

Muscarinic receptor subtype genes have been classified as m1 to m5 based on cDNA cloning (1). RT-PCR analyses revealed that only m1 and m2 receptors mRNA were expressed in hPBL (Fig. 1.7A). Subtype m1 couples with Gq protein which activates PLC- β (26). The signals from PLC- β may lead to activation of PKC through Ins(1,4,5)P₃ and DAG, and hence may enhance IL-2 production. The PKC family consists of heterogeneous PKC subspecies, including classical PKC group (α , β I, β II, γ), new PKC group (δ , ϵ , η , θ) and atypical PKC (ζ , λ), which are identified in different intracellular localization of particular cell types (27). A previous study indicated that Oxo-M induce the activation of PLC through the different mechanisms from those by PHA in Jurkat cells (25). The PLC and PKC activated by muscarinic receptors may be different species from those activated by TCR/CD3. Several studies showed that cAMP inhibits IL-2 production in the T cell line (28, 29, 30). Since m2 receptor couples with Gi protein which inhibits cAMP accumulation, signal transduction *via* m2 receptor may inhibit the cAMP accumulation, followed by disinhibition of IL-2 production. Muscarinic receptors m1 and m2 may thus cooperate in the enhancement of IL-2 production. The results I showed here suggest that the receptors m1 and m2 mRNA are expressed in human T cells and enhance the TCR/CD3-mediated IL-2 production, probably through G proteins and PLC, *via* pathways insensitive to both FK-506 and CsA.

1-4. Conclusion of Section 1

Muscarinic receptor m1 and m2 mRNAs were expressed in hPBL and pretreatment of these receptors with Oxo-M enhanced PHA-induced IL-2 production. Oxo-M-induced enhancement of IL-2 production may not be due to $[Ca^{2+}]_i$ elevation.

Stimulatory effects of Oxo-M on IL-2 production could be due to the intracellular signaling mechanism.

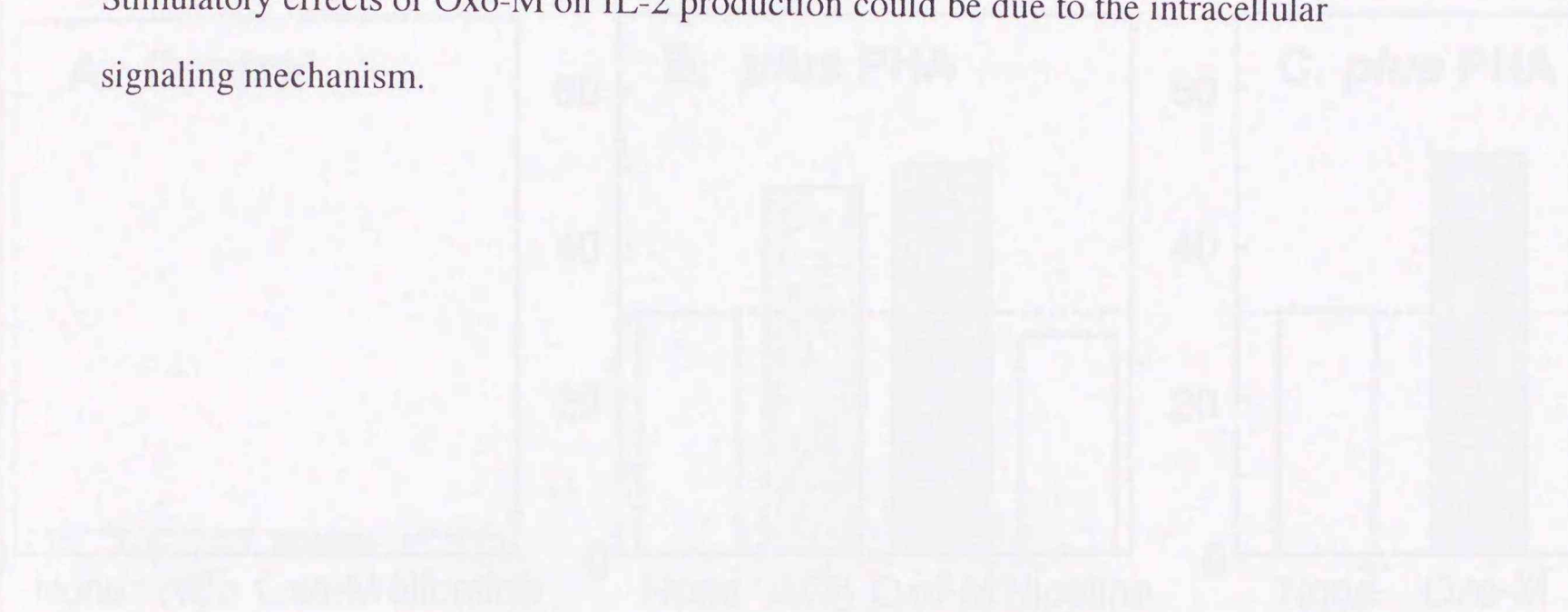


Fig. 1.1. Effect of muscarinic agonists on PHA-induced IL-2 production.

hPBL and C. plus cells were pretreated with Oxo-M (10⁻⁶ M) for 30 min.

PHA (10⁻⁵ M) was added to the cells and IL-2 production was measured after 24 h.

IL-2 production was measured by a standard IL-2 assay.

Results are expressed as mean ± SD of three experiments.

* p < 0.05 compared to control.

IL-2 production was measured by a standard IL-2 assay.

Results are expressed as mean ± SD of three experiments.

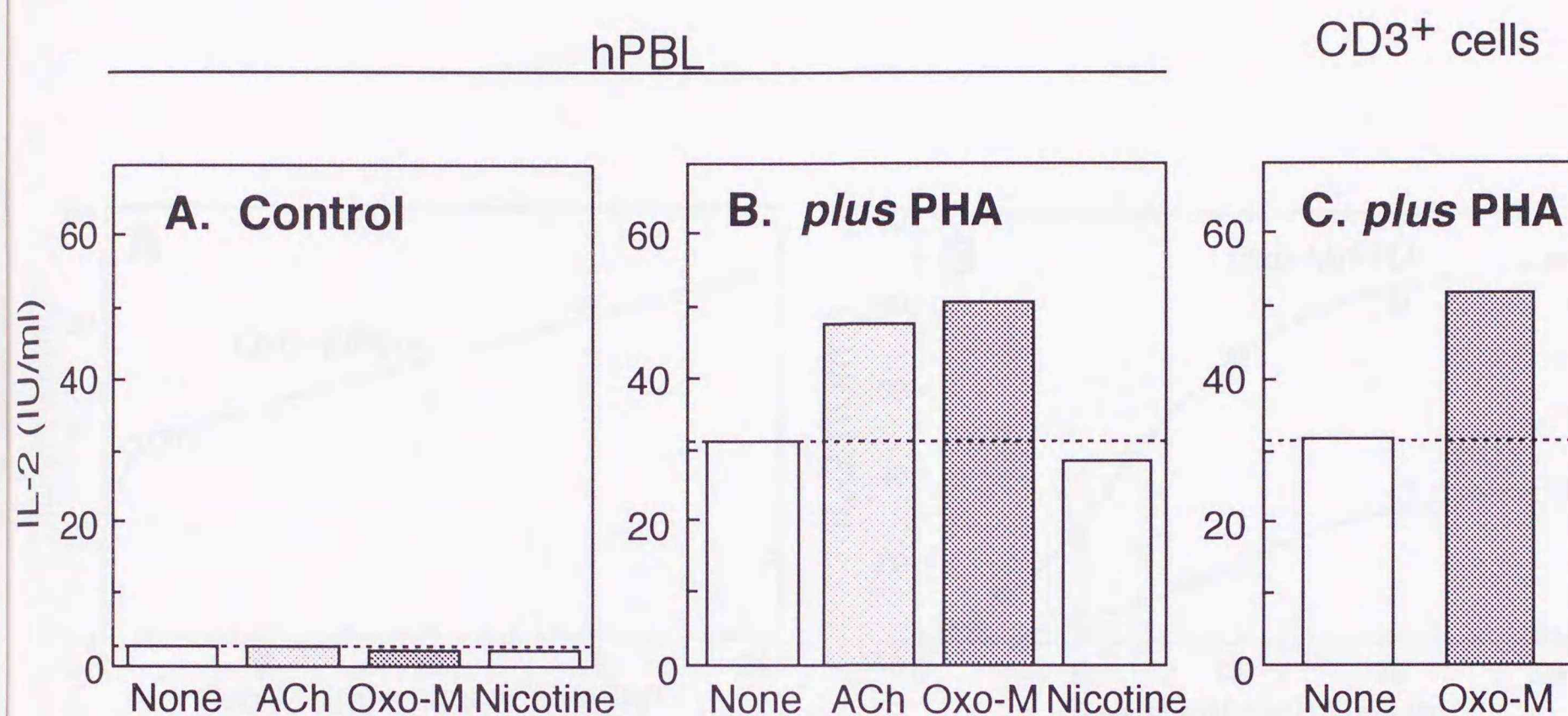


Fig. 1.1. Influences of cholinergic agonists on PHA-induced IL-2 production

from hPBL and CD3⁺ cells After hPBL were incubated with several cholinergic

agonists (1 μ M ACh in the presence of 100 μ M physostigmine, 1 μ M Oxo-M, 1 μ M nicotine)

for 24 hr, the vehicle (control) (A) or 10 μ g/ml PHA (B) was added into the culture medium

and further incubated for 24 hr, the IL-2 content in the medium was measured by ELISA for

human IL-2. C, CD3-positive T cells prepared from hPBL by a positive panning selection

method. Cells were pretreated with 1 μ M Oxo-M for 24 hr prior to 10 μ g/ml PHA treatment

and further incubated for 24 hr, the IL-2 content in the medium was measured. PHA-induced

IL-2 production and Oxo-M-induced enhancement were found in all four experiments.

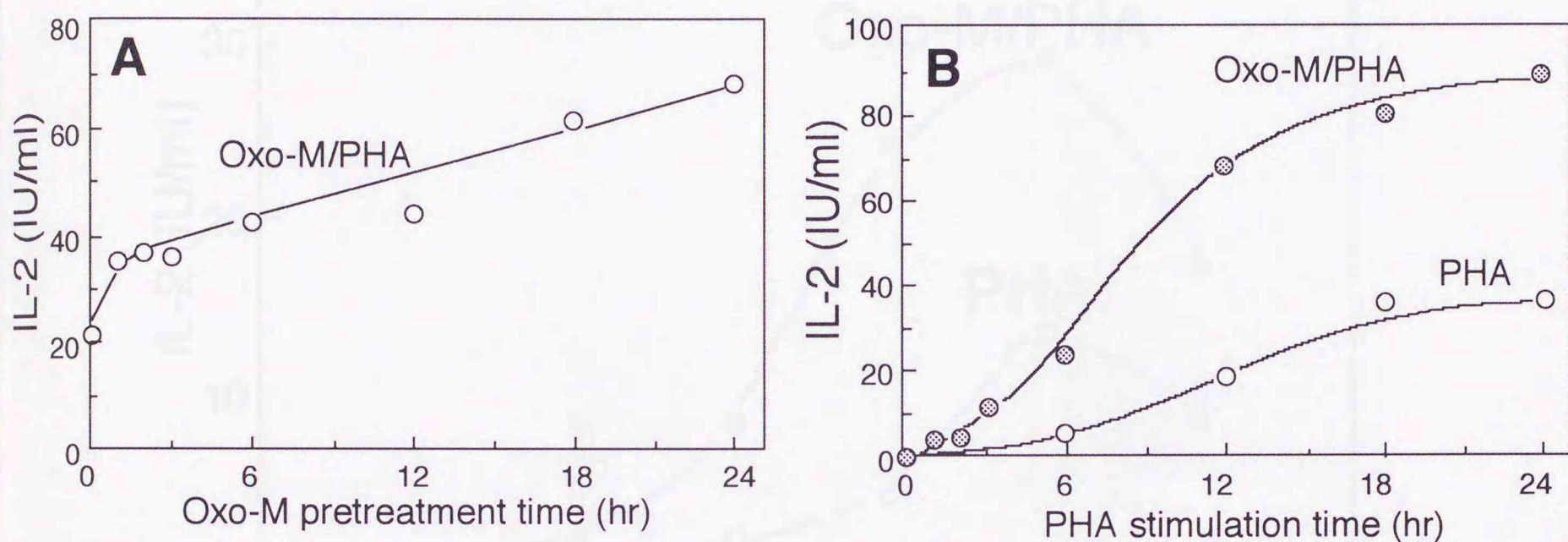


Fig. 1.2. Effect of Oxo-M on PHA-induced IL-2 production in hPBL A, hPBL

were pretreated with 0.1 μ M Oxo-M for 0 -24 hr prior to 10 μ g/ml PHA treatment for 24 hr,

subsequently the amount of IL-2 was measured. B, hPBL were pretreated with vehicle (O) or 0.

1 μ M Oxo-M (●) for 24 hr prior to 10 μ g/ml PHA treatment for a further 0 - 24 hr. Typical data

of four independent experiments are shown. Each point is the mean of 4 samples.

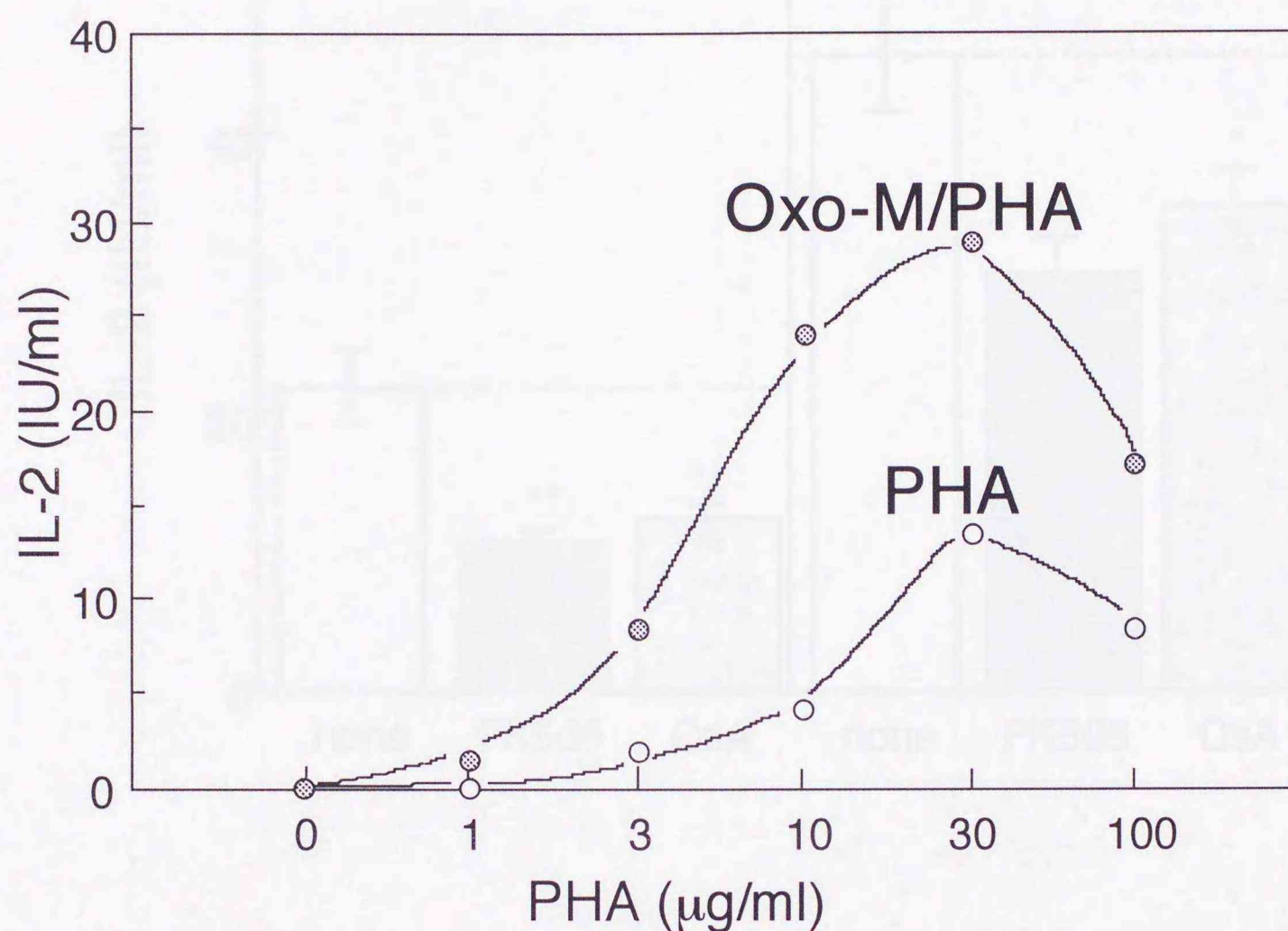


Fig. 1.3. Oxo-M enhanced PHA-induced IL-2 production in hPBL After

hPBL were pretreated with vehicle (O) or 0.1 mM Oxo-M (●) for 24 hr, PHA at 0 - 100

μg/ml was added and they were further incubated for 24 hr. Typical data of four independent

experiments are shown. Each point is the mean of 4 samples.

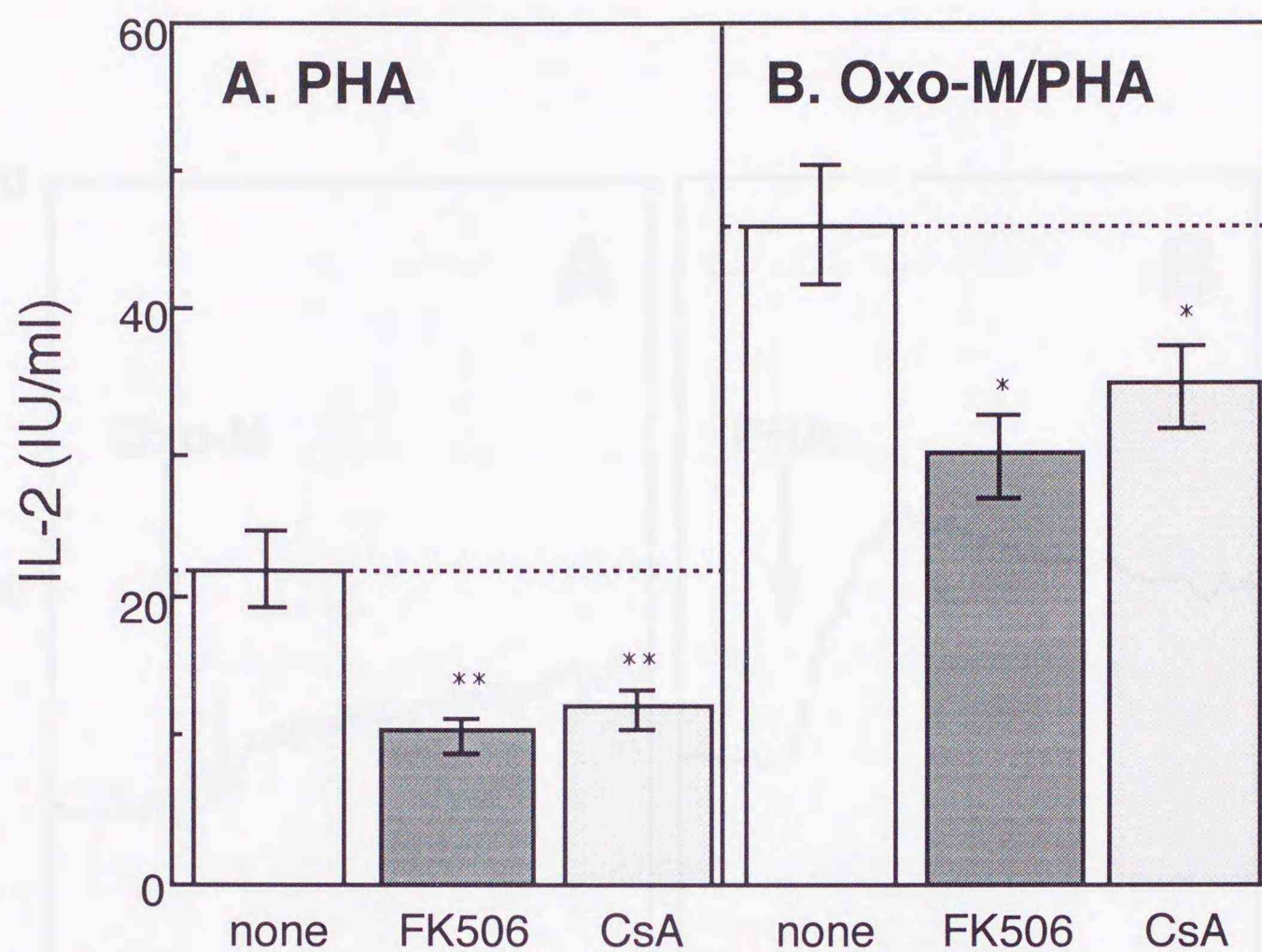


Fig. 1.4. Effects of FK506 and cyclosporin A on PHA-induced IL-2 production and the Oxo-M-induced enhancement of IL-2 production After hPBL were preincubated in the presence or absence of 10 nM FK506 or 100 nM CsA for 2 hr, hPBL were treated in the absence (A) or presence (B) of 0.1 mM Oxo-M for 24 hr, then added 10 μ g/ml PHA. They were incubated for a further 24 hr, the amount of IL-2 produced was measured in the medium. Each point is the mean of 4 samples.

*, $p < 0.05$, **, $p < 0.01$ versus none.

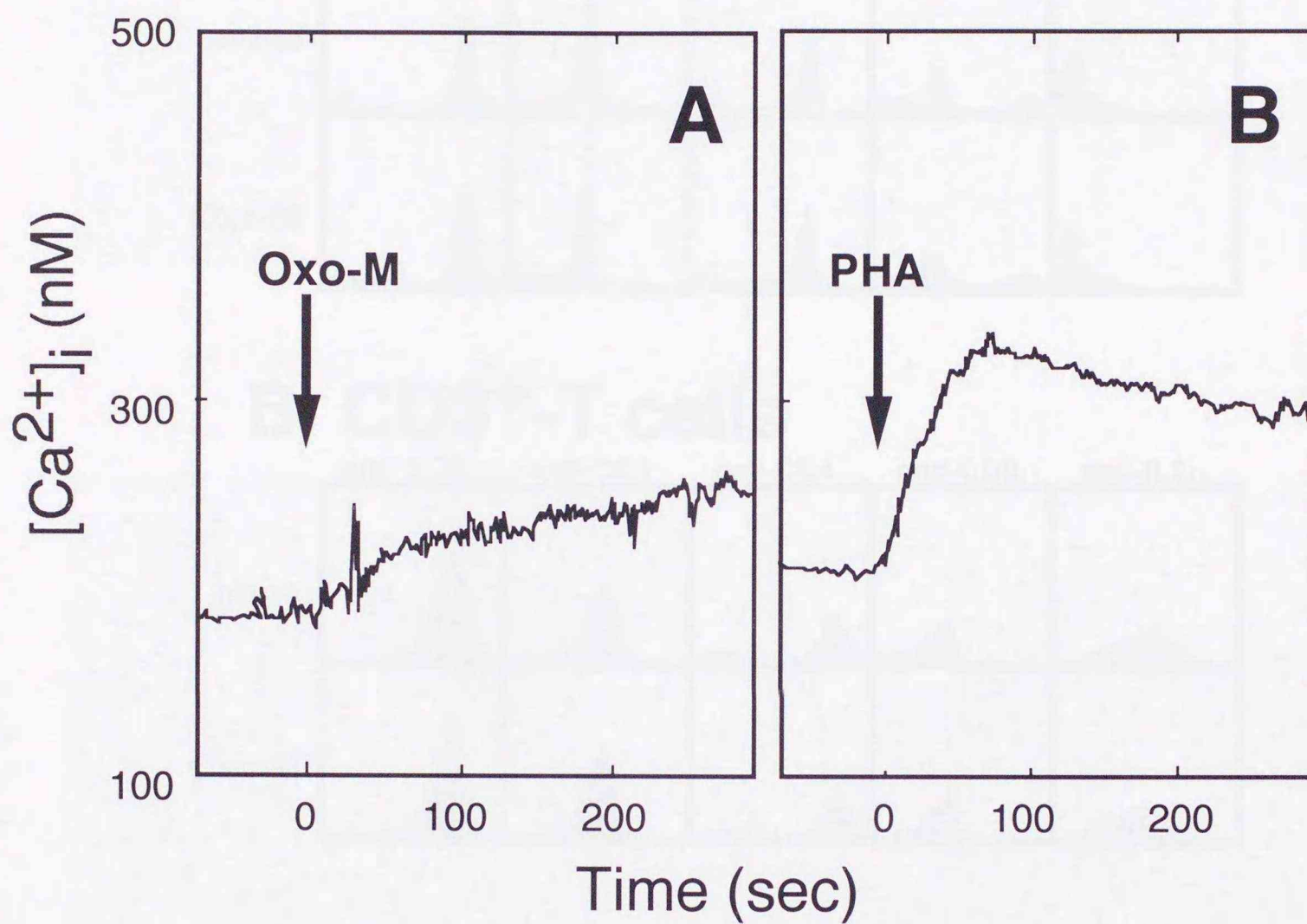


Fig. 1.5. Effects of Oxo-M and PHA on the $[Ca^{2+}]_i$ in hPBL hPBL were

preloaded with 5 μ M Fura-2/AM for 20 min and then washed. Time course of $[Ca^{2+}]_i$ rise

was examined after addition of 10 μ M Oxo-M (A) or 10 μ g/ml PHA (B). The fluorescence

intensity of Fura-2, $[Ca^{2+}]_i$ was determined using a Hitachi F-2000 spectrophotometer.

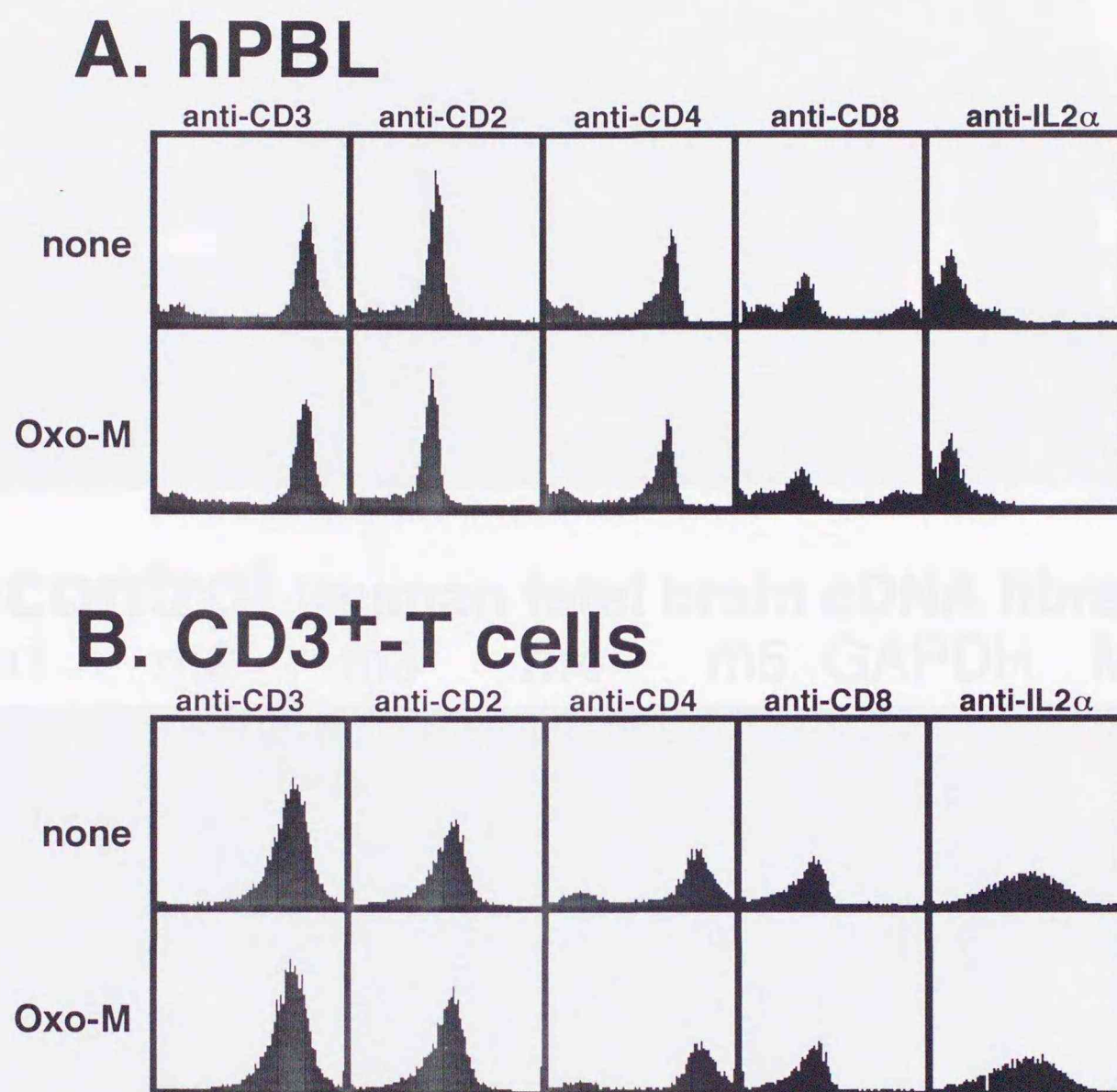
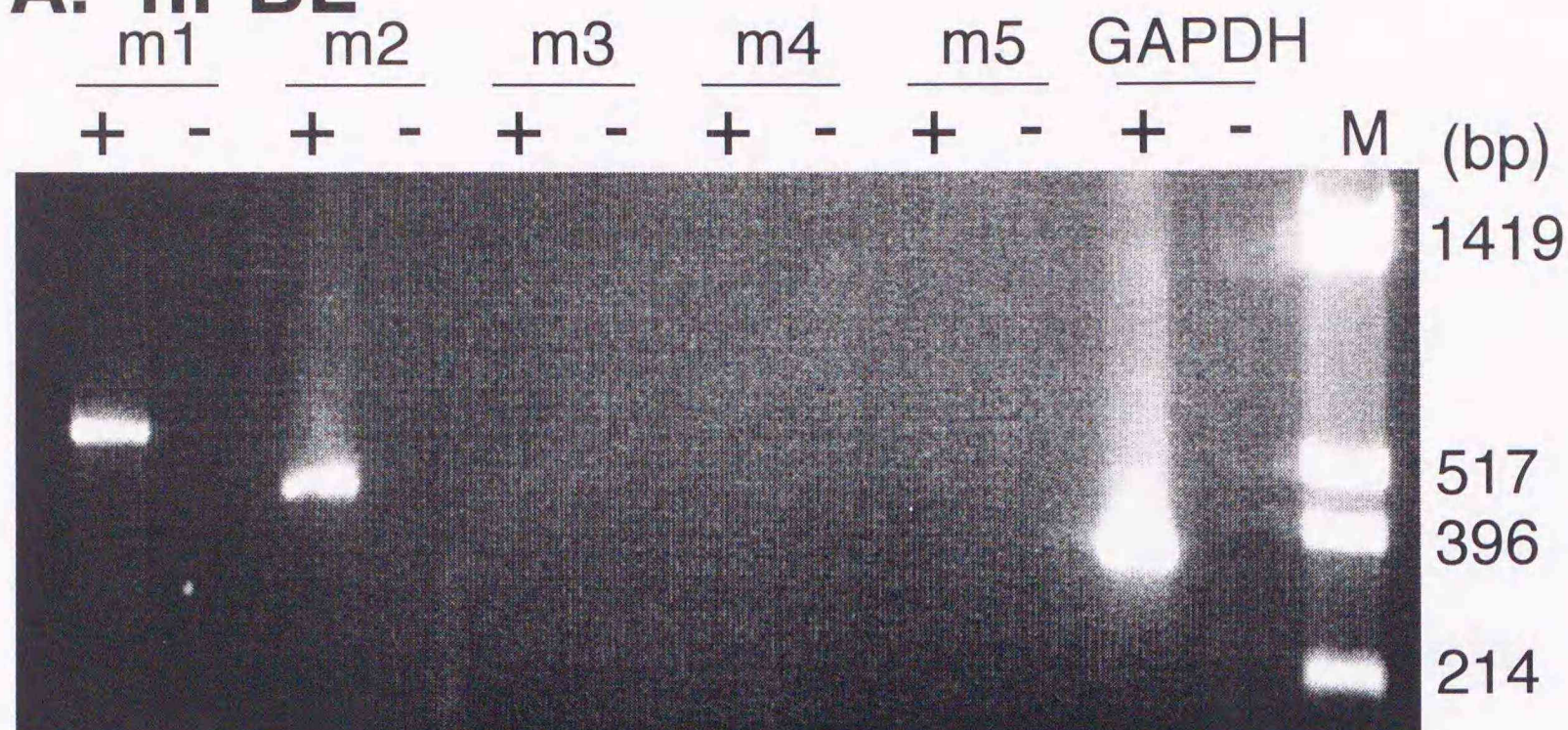


Fig. 1.6. Effects of Oxo-M on the expression of CD3, CD2, CD4, CD8 and α subunit of the IL-2 receptor in hPBL and T cells

hPBL (A) and T cells (B) were treated with or without 100 μ M Oxo-M for 24 hr and then their fluorescence intensities were analyzed by flow cytometry using anti-CD3 mAb, anti-CD2 mAb, anti-CD4 mAb, anti-CD8 mAb and anti-IL-2 receptor α subunit mAb. IL2R α : the α subunit of the IL-2 receptor.

A. hPBL



B. control (human fetal brain cDNA library)

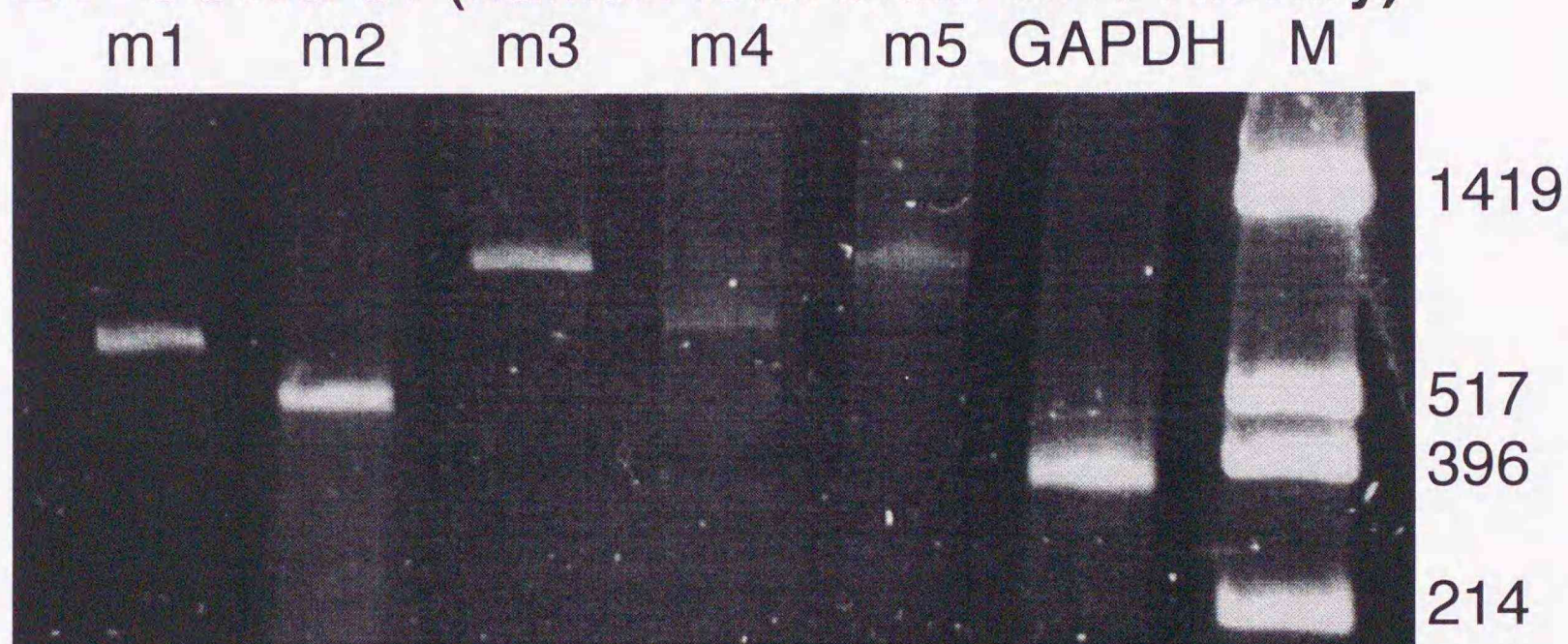


Fig. 1.7. Expression of muscarinic receptor mRNA in hPBL A, RT-PCR was used to amplify signals from isolated mRNA of hPBL. The size (bp) of the *Hinf*I cut pUC19 fragments in the last lane are indicated. For each set of subtype specific primers, mRNA was run in the presence (+) and absence (-) of reverse transcriptase. The blank lanes are the absence of reverse transcriptase (absence of contamination from cellular DNA or amplified products). B, control (human fetal brain cDNA library). Subsequently, the primer sequences and size of the PCR products were described in the "Experimental Procedures".

Section 2

Roles of muscarinic receptors on interleukin-2 production in Jurkat cells

3-1. Introduction

Part 1

Roles of m1 receptors

2-1. Introduction

IL-2 has a pivotal role in regulating the proliferation and differentiation of hematopoietic cells (31). In Section 1, I have shown that muscarinic receptors m1 and m2 are expressed on hPBL and that pretreatment of those receptors with Oxo-M enhanced IL-2 production. The IL-2 promoter activity is regulated by the 300 bp region adjacent to the transcription initiation site (32, 33, 34). This region contains specific binding sites for various transcription factors including AP-1, NF- κ B, NF-AT and Oct-1, and cooperation of these factors is required for maximal activation of the IL-2 promoter (35). Muscarinic receptors are typical G protein-coupled receptors. Each receptor contains seven transmembrane regions of helix structure and activates a specific class of trimeric G proteins, including Gq and Gi. Gq α and/or G $\beta\gamma$ subunits activate PLC- β , resulting in activation of PKC and an increase in the level of cytoplasmic Ca²⁺ by release of Ca²⁺ from intracellular storage. A tyrosine kinase, PYK2, has recently been reported to function as a switch which relays message from PKC and cytoplasmic Ca²⁺ to Shc and thus to activate Ras-mediated regulation systems in PC12 cells (36). Muscarinic receptor may thus stimulate the MAPK cascade *via* activation of PYK2 which phosphorylates Shc. Another report tells that G $\beta\gamma$ -mediated MAPK activation is initiated by a tyrosine phosphorylation event and proceeds by a pathway common to both G protein-coupled receptors and tyrosine kinase receptors in COS-7 cells (37). Furthermore, the signals from muscarinic receptors involve in the MAPKs in NIH-3T3 cells or Rat 1a fibroblast cells (38, 39).

In this section, I evaluated the effect of muscarinic receptors on regulatory elements of the IL-2 promoter and found the pathways mediated by those receptors in Jurkat cells.

2-2. Results

Muscarinic agonist enhanced IL-2 production in Jurkat cells:

Triggering of TCR has been shown to result in hydrolysis of PI(4, 5)P₂ to generate DAG and Ins(1, 4, 5)P₃ which induce Ca²⁺ mobilization. These events can be mimicked by phorbol ester PMA and calcium ionophore A23187 (40). To determine the optimal concentration of PMA for activation of human T cell line Jurkat cells, cells were treated for 24 hr with PMA at various concentrations ranging 0 - 20 nM in addition to a constant amount of A23187 (Fig. 2.1.1). In the presence of 500 nM A23187, IL-2 production increased with 1 - 5 nM PMA and reached plateau, while PMA by itself did not induce IL-2 production (Fig. 2.1.1). The IL-2 production induced by 10 nM PMA together with 500 nM A23187 was enhanced by pretreatment of the cells with Oxo-M for 24 hr in a dose dependent manner in the range of 0.1 - 100 μ M (Fig. 2.1.2). The enhancement was inhibited by cell treatment with an m1/m3 antagonist, 4-DAMP, or a non-selective antagonist, atropine for 20 min prior to the Oxo-M treatment (Fig. 2.1.3). Although Oxo-M by itself did not induce IL-2 production (data not shown).

Expression of muscarinic receptor mRNA in Jurkat cells: The results shown in Fig. 2.1.2 and Fig. 2.1.3 indicate that muscarinic receptors were present on Jurkat cells. To investigate which subtypes of muscarinic receptors are expressed in Jurkat cells, RT-PCR analyses were carried out using total RNA extracted from Jurkat cells as in the case of hPBL. As shown in Fig. 2.1.4, the expressions of subtypes m1 and m2, but not of m3 - m5, were detected in Jurkat cells, similarly to those in hPBL.

Roles of m2 receptors on IL-2 production: As shown in Fig. 2.1.3, an m1/m3 antagonist, 4-DAMP, inhibited the Oxo-M-mediated enhancement of IL-2 production. Since not only m1 but also m2 receptor was expected to express in Jurkat

cells, the involvement of m2 receptor in the IL-2 production in Jurkat cells was verified. When the cells were pretreated with an m2 antagonist, AFDX-116, the IL-2 production enhanced by Oxo-M was further stimulated (Fig. 2.1.5).

m1 receptor stimulation enhanced IL-2 promoter activity in Jurkat cells: To examine whether the enhancement of IL-2 production *via* m1 receptor is involved in the activation of the IL-2 promoter, pIL2-LUC, a plasmid carrying the human IL-2 promoter of 568 bp linked to the luciferase cDNA, was transfected into Jurkat cells and the cells were treated with PMA and A23187. The IL-2 promoter activity increased to the optimum by treatment with 1 nM PMA and 500 nM A23187 (Fig. 2.1.6A). The optimal activity was strongly enhanced by pretreatment with 100 μ M Oxo-M (Fig. 2.1.6A). The enhancement by Oxo-M was abolished when the cells were treated with an m1/m3 antagonist, 1 μ M 4-DAMP, for 20 min prior to the Oxo-M pretreatment (Fig. 2.1.6B). The results suggest that the IL-2 promoter activity was induced by PMA/A23187 in Jurkat cells and further enhanced *via* muscarinic receptor m1.

Effects of m1 receptor-enhanced IL-2 promoter activities on nuclear transcription factors' consensus sequence binding proteins in Jurkat cells: Induction of the IL-2 promoter activity depends on the region of 300 bp upstream from the transcription initiation site (32, 33, 34). The region contains the specific binding sites for AP-1, NF- κ B, NF-AT and Oct-1, and cooperation of these transcription factors is required for the maximal activation of the IL-2 promoter (35). Hence, I examined whether the binding of the transcription factors to the respective *cis* elements was affected by the m1 receptor-mediated signals on IL-2 production. Nuclear extracts were prepared from Jurkat cells which were treated or untreated with an m2 antagonist, 1 μ M AFDX-116, and 100 μ M Oxo-M prior to the stimulation with 1 nM PMA and 500 nM A23187

for 0 - 3 hr. I examined binding activity of the extracts to the target elements for AP-1, NF- κ B, NF-AT and Oct-1 by band shift assays. As shown in the right-end panel of Fig. 2.1.7A, the bands at the position indicated by arrowheads were due to the specific AP-1 nucleoprotein complex, because the bands disappeared when excess amount of unlabeled AP-1 oligonucleotides (x 5 or x 50 of the labeled probe) were added to the reactions as competitors. The AP-1 nucleoprotein complex increased along the incubation with PMA/A23187, and the stronger signals were detected in 2 - 2.5 hr for the extract prepared from the cells pretreated with AFDX-116/Oxo-M than that from the cells without pretreatment (Fig. 2.1.7A, B). In the Western blotting analyses, similar increases of c-Fos and c-Jun proteins were observed and the cells pretreated with AFDX-116/Oxo-M resulted in stronger signals than the cells without pretreatment (Fig. 2.1.7C).

Binding proteins to the consensus sequences for NF- κ B, NF-AT and Oct-1 in the cell extracts were assessed as described above for AP-1. The nucleoprotein complex on the NF- κ B element remarkably increased in 0.5 - 1 hr, but decreased sharply in the following half an hour, then increased gradually (Fig. 2.1.8A). No remarkable differences were observed in the NF- κ B complex between the cells pretreated with and without AFDX-116/Oxo-M (Fig. 2.1.8A). The proteins binding to the elements for NF-AT or Oct-1 were observed constantly during the PMA/A23187 induction and were not affected by the AFDX-116/Oxo-M pretreatment (Fig. 2.1.8B, C).

Effect of cyclosporin A on m1 receptor-mediated IL-2 promoter activity in Jurkat cells: Immunosuppressant, CsA, was examined for the effect on the PMA/A23187-induced IL-2 promoter activity and the m1-mediated enhancement of the promoter activity in Jurkat cells. The cells were treated with 1 nM CsA for 2 hr prior to the treatment with 1 μ M AFDX-116 (m2 receptor antagonist) and 100 μ M Oxo-M for

24 hr and then treated with 1 nM PMA and 500 nM A23187 for 24 hr. The CsA treatment remarkably decreased the IL-2 promoter activity induced by PMA/A23187 and there was no significant difference in the rates of inhibition, regardless of the treatment with AFDX-116/Oxo-M (Fig. 2.1.9).

Effect of herbimycin A on m1 receptor-mediated IL-2 promoter activity in Jurkat cells: Herbimycin A is known as a potent inhibitor of tyrosine kinase (41). To examine whether a signal transduction *via* tyrosine kinase was involved in the m1 receptor-mediated enhancement of the IL-2 promoter activity, the treatment with herbimycin A was carried out. The cells were treated with 0.01 - 1 µg/ml herbimycin A for 1 hr before the 24 hr treatment with 1 µM AFDX-116 and 100 µM Oxo-M pretreatment. Then the cells were stimulated with 1 nM PMA and 500 nM A23187 for 24 hr. The treatment with 0.01 - 0.1 µg/ml herbimycin A decreased the induction of IL-2 promoter activity and the induction was no longer observed after the treatment with 1 µg/ml herbimycin A (Fig. 2.1.10). The m1 receptor-mediated enhancement of IL-2 promoter activity was significantly decreased by the 0.01 µg/ml herbimycin A. The results suggest that signal transduction pathways *via* tyrosine kinase were involved in the m1 receptor-mediated enhancement of the IL-2 promoter activity.

Effects of MAP kinase superfamily on m1 receptor-mediated enhancement of IL-2 promoter activities in Jurkat cells: MAPKs belong to a group of serine/threonine kinases and are shown in various cell systems to be engaged in sequential phosphorylation cascades which transmit Ras activation signals to the nucleus. To examine the possible involvement of MAPK(ERK)/MEK pathways in the m1 receptor-mediated enhancement of IL-2 promoter activity, the cells were treated with

PD98059, a specific inhibitor of the MEK activation (42). Prior to the 24 hr treatment with 1 μ M AFDX-116 and 100 μ M Oxo-M followed by the treatment with 1 nM PMA and 500 nM A23187 for 24 hr, the cells were treated with 3 - 30 μ M PD98059 for 1 hr. The treatment with 3 μ M PD98059 decreased the m1 receptor-mediated enhancement significantly, and at higher concentrations of PD98059 enhancement by Oxo-M and the induction by PMA/A23187 decreased in a dose-dependent manner (Fig. 2.1.11). Another MAPK superfamily, termed p38 MAP kinase, was also examined for the m1 receptor-mediated enhancement of IL-2 promoter activity by use of a specific inhibitor of p38 MAPK, SB203580 (43). The cells were treated with 1 - 10 μ M SB203580 prior to the 24 hr treatment with 1 μ M AFDX-116 and 100 μ M Oxo-M followed by the treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Both the enhancement and the induction of IL-2 promoter activity were similarly inhibited by SB203580 in a dose dependent manner, and there was no significant differences among the rates of inhibition (Fig. 2.1.12). The results suggest that pathways *via* MAPK(ERK)/MEK, but not *via* p38 MAPK, were involved in the m1 receptor-mediated enhancement of IL-2 promoter activity.

Since the stimulation of muscarinic receptor by itself did not induce the IL-2 production, namely the IL-2 promoter activity, the induction of the production/promoter activity by PMA and A23187 was indispensable as far as approaching the m1 receptor-mediated enhancement by assaying IL-2 production or the promoter activity. To assess direct effects of the muscarinic receptor stimulation, MAP kinase assays were carried out. The cells were pretreated by 1 μ M AFDX-116 for 20 min to block m2 receptors and then treated with 100 μ M Oxo-M for 5 min. As shown in Fig. 2.1.13, the stimulation of m1 receptor by Oxo-M enhanced MAP kinase activity to about 130 % as compared to the control treatment with AFDX-116 alone. The enhancement was abolished by

pretreatment with 1 μ M 4-DAMP for 20 min. The results suggest that one of the effect of the m1 receptor stimulation was MAPK activation. PMA by itself also increased the MAPK activity.

JNK is a MAPK superfamily other than MAPK(ERK) and p38 MAPK. The JNK activity was also examined by an immunocomplex kinase assay method before and after the m1 receptor stimulation. The cells were pretreated with 1 μ M AFDX-116 for 20 min to block m2 receptors and then treated with 100 μ M Oxo-M for 0 - 60 min. The phosphorylation of GST-c-Jun was increased in 10 to 30 min after the stimulation (Fig. 2.1.14). The results suggest that another effect of the m1 receptor stimulation was activation of JNK. The treatment with PMA/A23187 also increased the JNK activity.

2-3. Discussion

Muscarinic receptors were expressed in hPBL and stimulation of the receptors resulted in enhancement of PHA-induced IL-2 production, as I showed in Section 1. To further examine the involvement of the receptors in IL-2 production, I used human T cell line Jurkat cells instead of hPBL.

Antigen-specific clonal proliferation of T cells is initiated through a process of signal transduction wherein the specific interaction of antigen-major histocompatibility complex (MHC) molecules and CD3/TCR complex triggers the expression of IL-2 and its homologous receptor. These events bring about not only the antigen-specific T cell proliferation but also a variety of cellular responses. Stimulation of the TCR by itself is insufficient to activate most T cells. In addition to TCR, a number of other T cell surface molecules such as CD28, a 44-kD glycoprotein, are required for induction of several cellular responses including IL-2 production. The phenomena can be mimicked by the treatment of the cells with a phorbol ester PMA and a calcium ionophore A23187. The treatment with PMA and A23187 may short-circuit unknown signals from TCR or CD28 towards IL-2 production. IL-2 production was induced in Jurkat cells by 1 nM PMA and 500 nM A23187 (Fig. 2.1.1). Pretreatment of the cells with Oxo-M enhanced the IL-2 production in a dose dependent manner (Fig. 2.1.2). The enhancement due to Oxo-M was eliminated when the cells were treated with an m1/m3 receptor antagonist, 4-DAMP, or a non-selective muscarinic receptor antagonist, atropine, prior to the Oxo-M addition (Fig. 2.1.3). The data indicate that there were muscarinic receptors on Jurkat cells and stimulation of the receptors enhanced IL-2 production in the cells.

The RT-PCR analyses revealed that muscarinic receptors m1 and m2, but not m3 - m5, were also expressed on Jurkat cells as well as on hPBL (Fig. 2.1.4). As shown in Fig. 2.1.3, 4-DAMP inhibited the Oxo-M-mediated enhancement of IL-2 production in Jurkat cells. The pretreatment with an m2 receptor antagonist, 1 μ M AFDX-116, on the

other hand, further enhanced the Oxo-M-mediated enhancement of IL-2 production (Fig. 2.1.5). A positive effect *via* m1 receptor and a negative effect *via* m2 receptor were thus overlapping in the enhancement as a whole by the stimulation of muscarinic receptors by Oxo-M.

To examine the IL-2 promoter activity in response to various treatments, transcriptional assays were performed using a plasmid which contained the human IL-2 promoter of 568 bp linked to the luciferase cDNA. The transcription, i.e. the IL-2 promoter activity, was enhanced by pretreatment with 100 μ M Oxo-M of the cells transfected with the plasmid and the enhancement was inhibited by treatment with 4-DAMP prior to the Oxo-M addition (Fig. 2.1.6A,B).

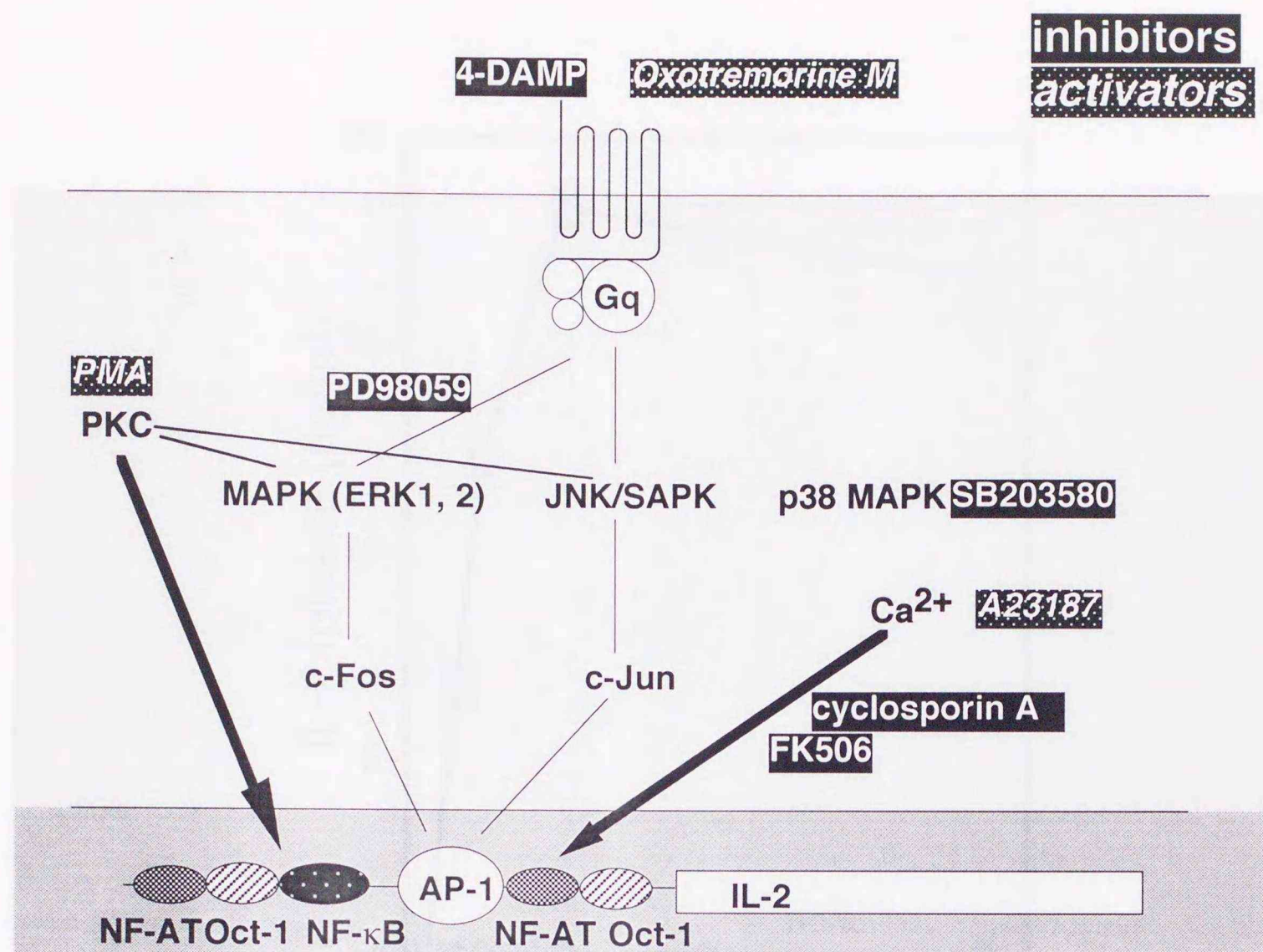
Induction of IL-2 promoter activity depends on the 300 bp region upstream from the transcription initiation site. This region contains the binding sites for AP-1, NF- κ B, NF-AT and Oct-1, and cooperation of these transcription factors induces the maximal activation of the IL-2 promoter. Band shift assays were therefore carried out to determine which transcriptional factors were involved in the m1 receptor-mediated enhancement of IL-2 production. Increase of the nucleoprotein complex on the AP-1 consensus sequence was observed upon induction of the IL-2 production and the increase was enhanced when the m1 receptors were stimulated before the induction (Fig. 2.1.7). Western blotting analyses showed that similar increase and enhancement were observed in the c-Fos and c-Jun proteins which specifically recognize the AP-1 sequence (Fig. 2.1.7, upper panel). The NF- κ B-like complex was also increased by the induction, but not affected by the m1 pretreatment (Fig. 2.1.8A). The specific complex on either the NF-AT or the Oct-1 consensus sequence were observed constantly, regardless of the IL-2 induction or the m1 stimulations (Fig. 2.1.8B,C). The results suggest that the AP-1 element and its binding proteins were the major factors involved in the m1 receptor-mediated enhancement of IL-2 production.

To investigate by which pathways the m1 receptors enhanced the IL-2 promoter activity, the cells were pretreated with several inhibitors specific for various signal transduction pathways prior to the m1 receptors stimulation. Pretreatment with cyclosporin A, which blocks translocation of NF-AT from cytoplasm to nucleus by inhibiting the activity of calcineurin (44, 45), significantly diminished the IL-2 promoter activity regardless of the m1 receptor stimulation (Fig. 2.1.9). The results indicate that m1 receptor-mediated enhancement of IL-2 promoter activity may not be due to $[Ca^{2+}]_i$ elevation, as suggested in hPBL (Figs. 1.4 and 1.5). NF-AT was actually detected constantly in NE before and after the m1 receptor stimulation (Fig. 2.1.8B).

Various growth factors and cytokines as well as stresses activate several signal transduction pathways with specific physiological roles. The pathways contain at least three different MAPK homologues. Among them, ERK and JNK play key roles in the transmission of extracellular signals of T cells to the AP-1-binding sequence in T cell nuclei. MAPK kinases (MAPKK) has a dual-specificity: it phosphorylates Ser/Thr and Tyr of MAPK. Herbimycin A, a potent tyrosine kinase inhibitor, significantly decreased the m1 receptor-mediated enhancement of IL-2 promoter activity (Fig. 2.1.10). PD98059 has previously been shown to inhibit the phosphorylation form of MEK1 and thus inhibit the activation of MAPK/ERK through the inactivation of MEK1. In Jurkat cells, pretreatment of PD98059 significantly decreased the m1 receptor-mediated enhancement of IL-2 promoter activity (Fig. 2.1.11). The results indicate that a tyrosine kinases and/or MEK1 and MAPK/ERK are involved in the transduction pathway from m1 receptor to the IL-2 promoter. Another MAPK superfamily termed p38 MAPK is specifically inhibited by SB203580. Pretreatment with SB203580 did not affect the m1 receptor-mediated enhancement of IL-2 promoter activity (Fig. 2.1.12). The results suggest that p38 MAPK was not involved in this enhancement. To assess the direct effects of m1 receptor stimulation in absence of PMA/A23187, MAP kinase assays were performed. I found that the m1 receptor stimulation enhanced MAPK/ERK activity (Fig.

2.1.13). Immunocomplex kinase assays revealed that the stimulation of m1 receptors also enhanced the activity of JNK, another MAPK superfamily (Fig. 2.1.14). The results indicate that MAPK/ERK and JNK are involved in the m1 receptor-mediated enhancement. MAPK/ERK and JNK may activate AP-1 proteins. Since the m1 receptor stimulation by itself did not induce IL-2 production or promoter activity, AP-1 for itself may not induce the production and the promoter activity, either, but the pre-activated AP-1 may function rapidly to enhance them in response to the T cell stimulation by PMA/A23187.

2.4. Conclusion of in Section 2, Part 1



m1 system on IL-2 production

Jurkat cells expressed m1 and m2 receptor mRNAs.

Stimulation of m1 receptors enhanced PMA/A23187-induced IL-2 production and IL-2 promoter activity. AP-1 could be involved in this enhancement and both MAPK and JNK also seem to be involved.

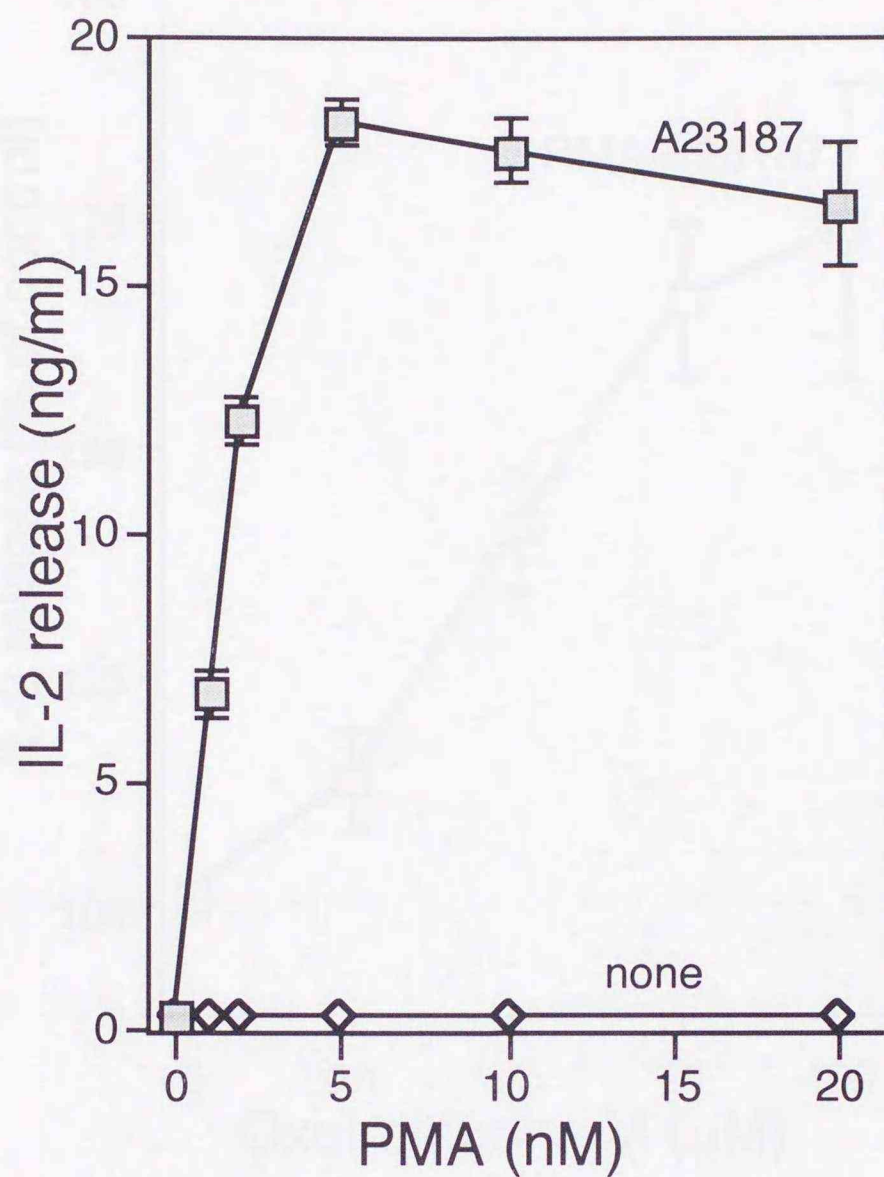


Fig. 2.1.1. Effects of PMA and A23187 on IL-2 production in Jurkat cells

Jurkat cells (4×10^5 cells/ml) were treated with PMA (0, 1, 2, 5, 10, 20 nM, respectively) and in the presence or absence of A23187 (0, 500 nM). Cells were further incubated for 24 hr and the amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays.

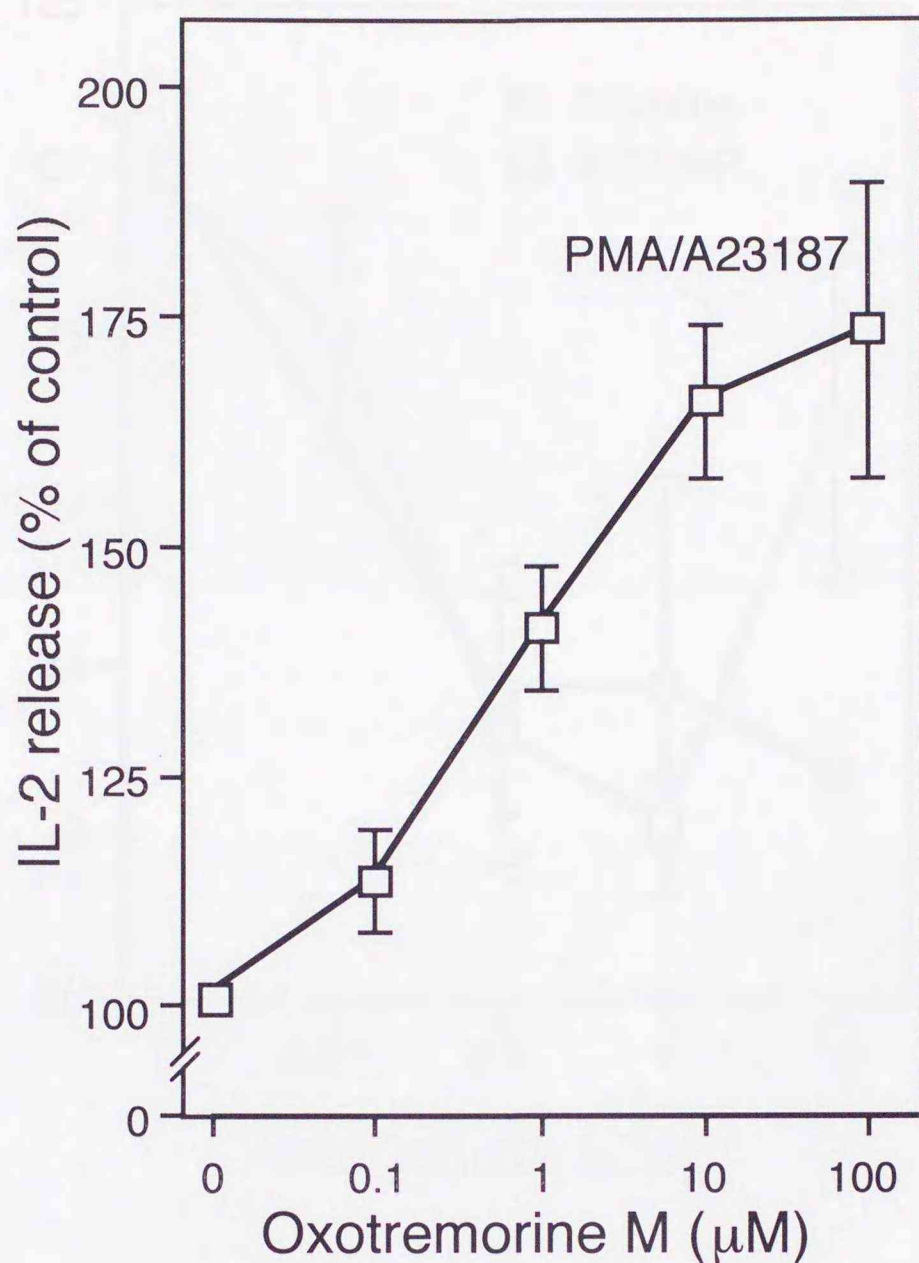


Fig. 2.1.2. Effect of Oxo-M on PMA/A23187-induced IL-2 production in

Jurkat cells Jurkat cells (4×10^5 cells/ml) were pretreated with Oxo-M (0, 0.1, 1, 10, 100 μM) for 24 hr, 10 nM PMA and 500 nM A23187 were added and cells were further incubated for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The amount of PMA/A23187-induced IL-2 production was estimated as 100%.

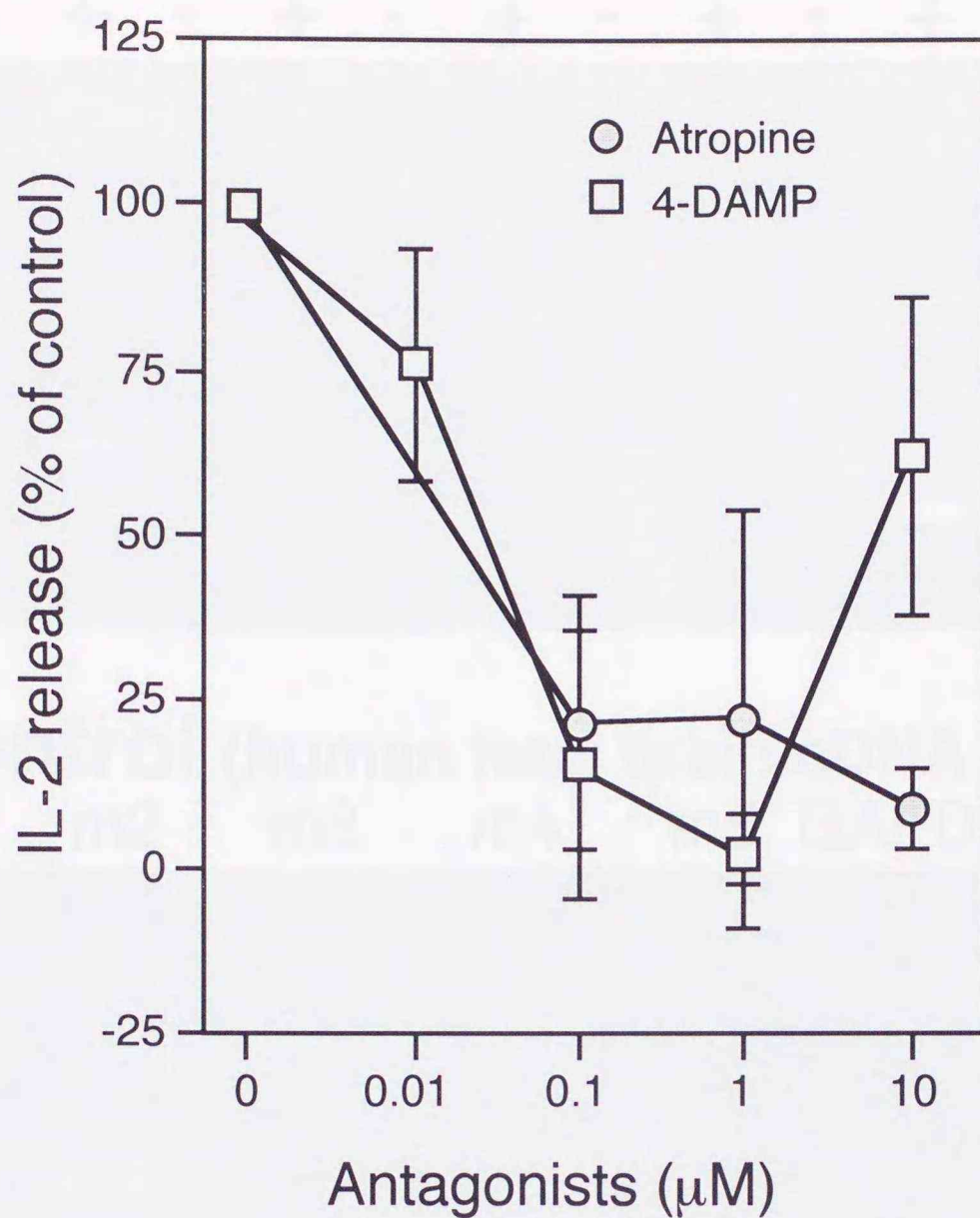
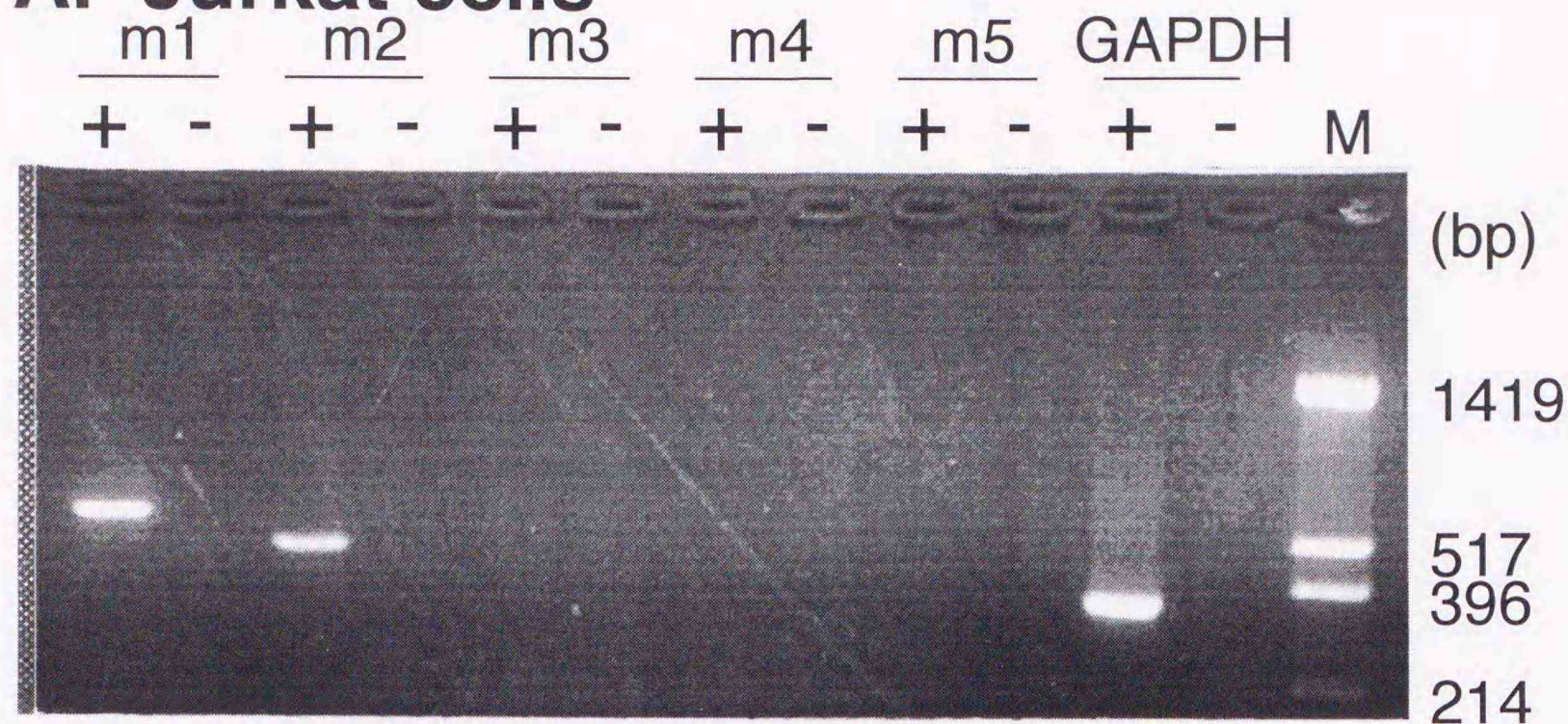


Fig. 2.1.3. Effects of muscarinic receptor antagonists on Oxo-M enhanced PMA/A23187-induced IL-2 production in Jurkat cells

4-DAMP or atropine (0.01 - 10 μM) were treated 30 min before the Oxo-M treatment. Cells were treated with 100 μM Oxo-M for 24 hr prior to 10 nM PMA and 500 nM A23187 treatment for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays.

A. Jurkat cells



B. control (human fetal brain cDNA library)

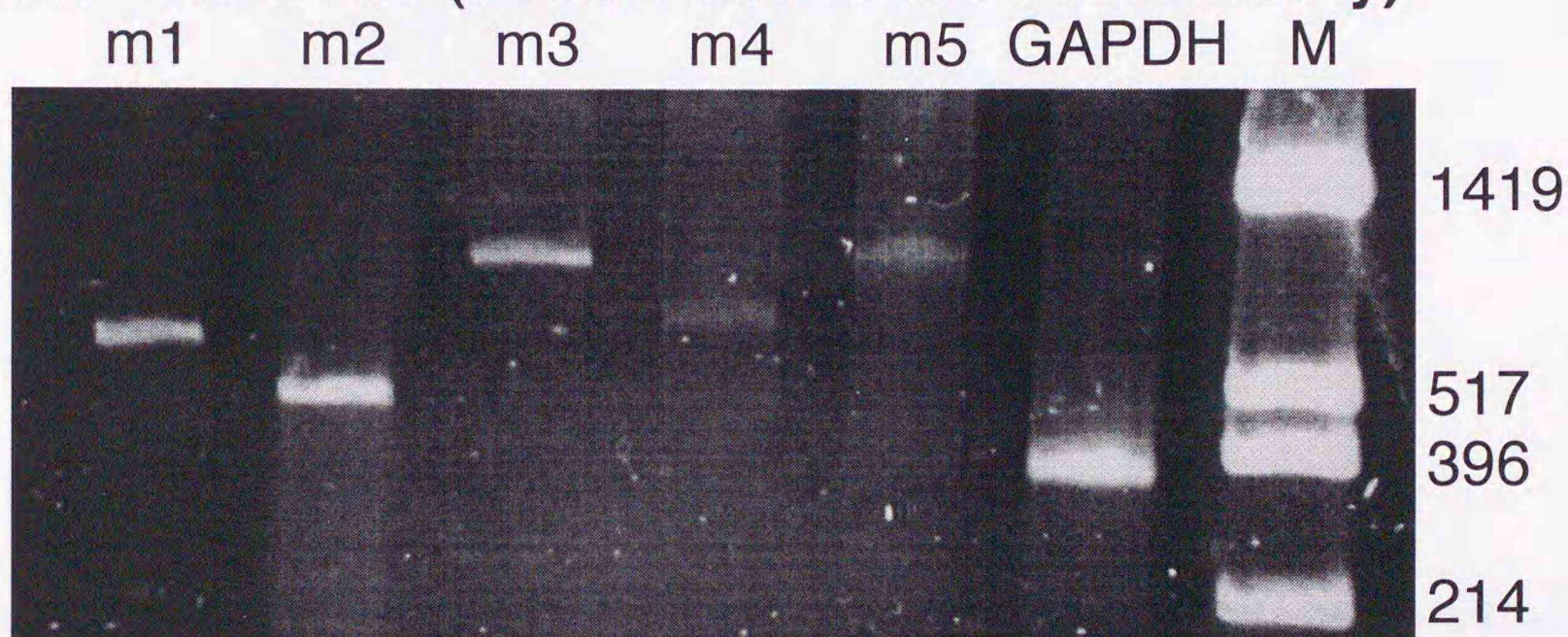


Fig. 2.1.4 Expression of muscarinic receptor mRNA in Jurkat cells

A, RT-PCR was used to amplify signals from isolated mRNA of Jurkat cells. The size (bp) of the *Hinf*I cut pUC19 fragments in the last lane are indicated. For each set of subtype specific primers, mRNA was run in the presence (+) and absence (-) of reverse transcriptase. The blank lanes are the absence of reverse transcriptase (absence of contamination from cellular DNA or amplified products). B, control (human fetal brain cDNA library). Subsequently, the primer sequences and size of the PCR products were described in the "Experimental Procedures".

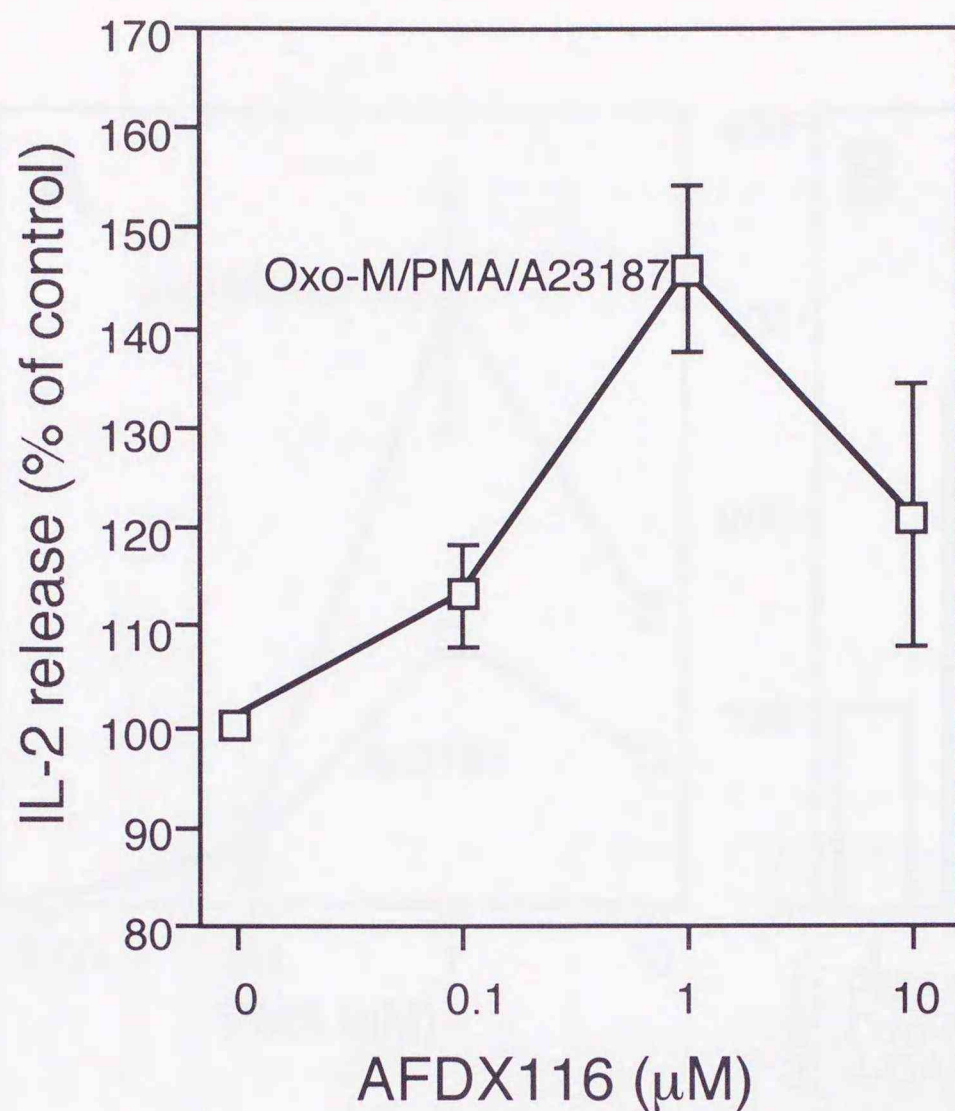


Fig. 2.1.5. Effect of AFDX-116 on Oxo-M enhanced PMA/A23187-induced IL-2 production in Jurkat cells AFDX-116 (0.1 - 10 μM) was treated 30 min before the Oxo-M treatment. Cells were pretreated with 100 μM Oxo-M for 24 hr prior to 10 nM PMA and 500 nM A23187 treatment for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean ± SE of three assays.

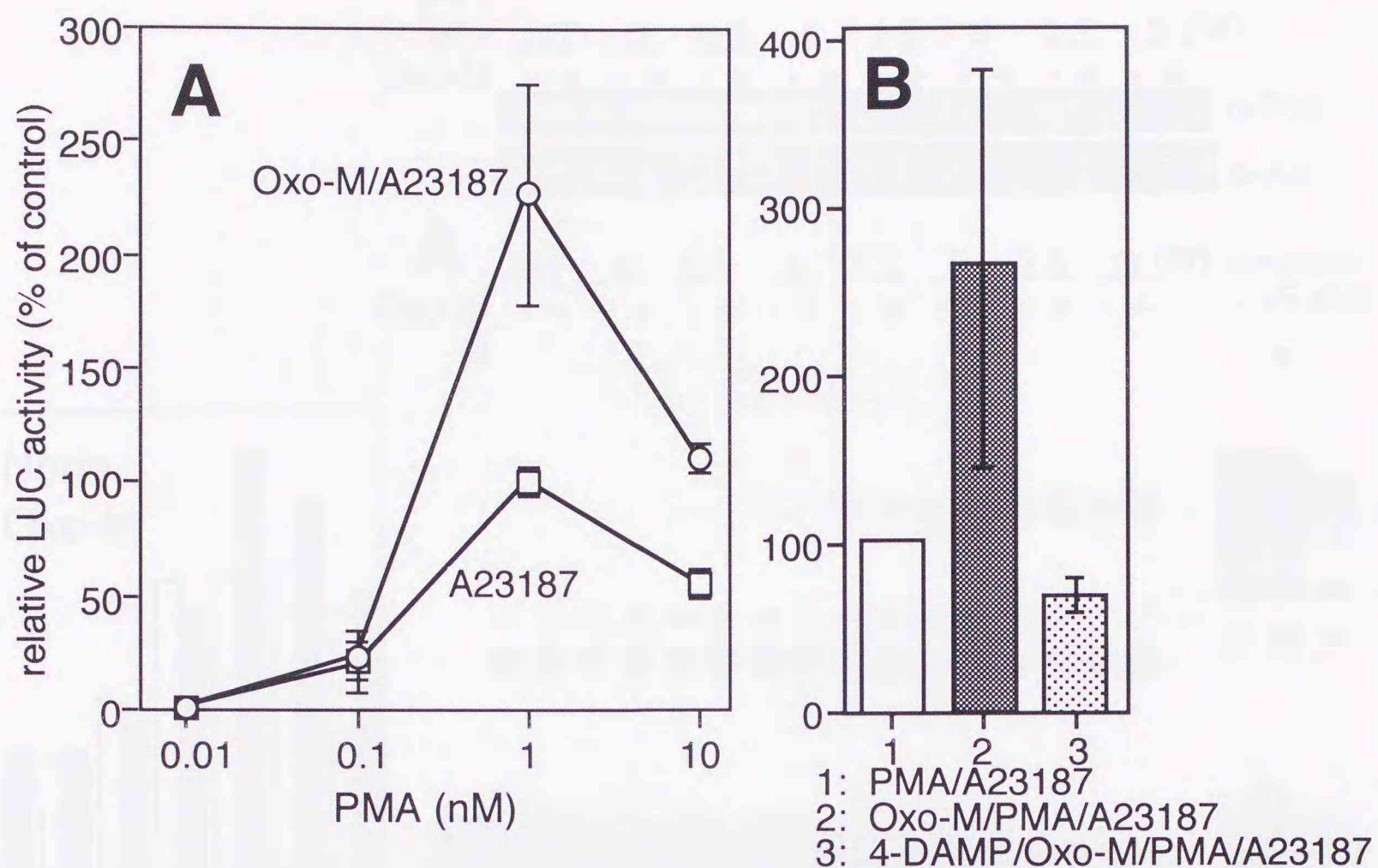


Fig. 2.1.6. Effects of Oxo-M and 4-DAMP on PMA/A23187-induced IL-2

promoter activity in Jurkat cells A, Jurkat cells were transfected with pIL2-LUC and were treated with 100 mM Oxo-M for 24 hr prior to PMA and 500 nM A23187 treatment for 24 hr. B, Cell were pretreated with 4-DAMP (1 μ M) for 30 min prior to Oxo-M treatment for 24 hr then treated with PMA/A23187 for 24 hr. Cells were transfected at least three times, and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity as 100% ($\pm$ SE).

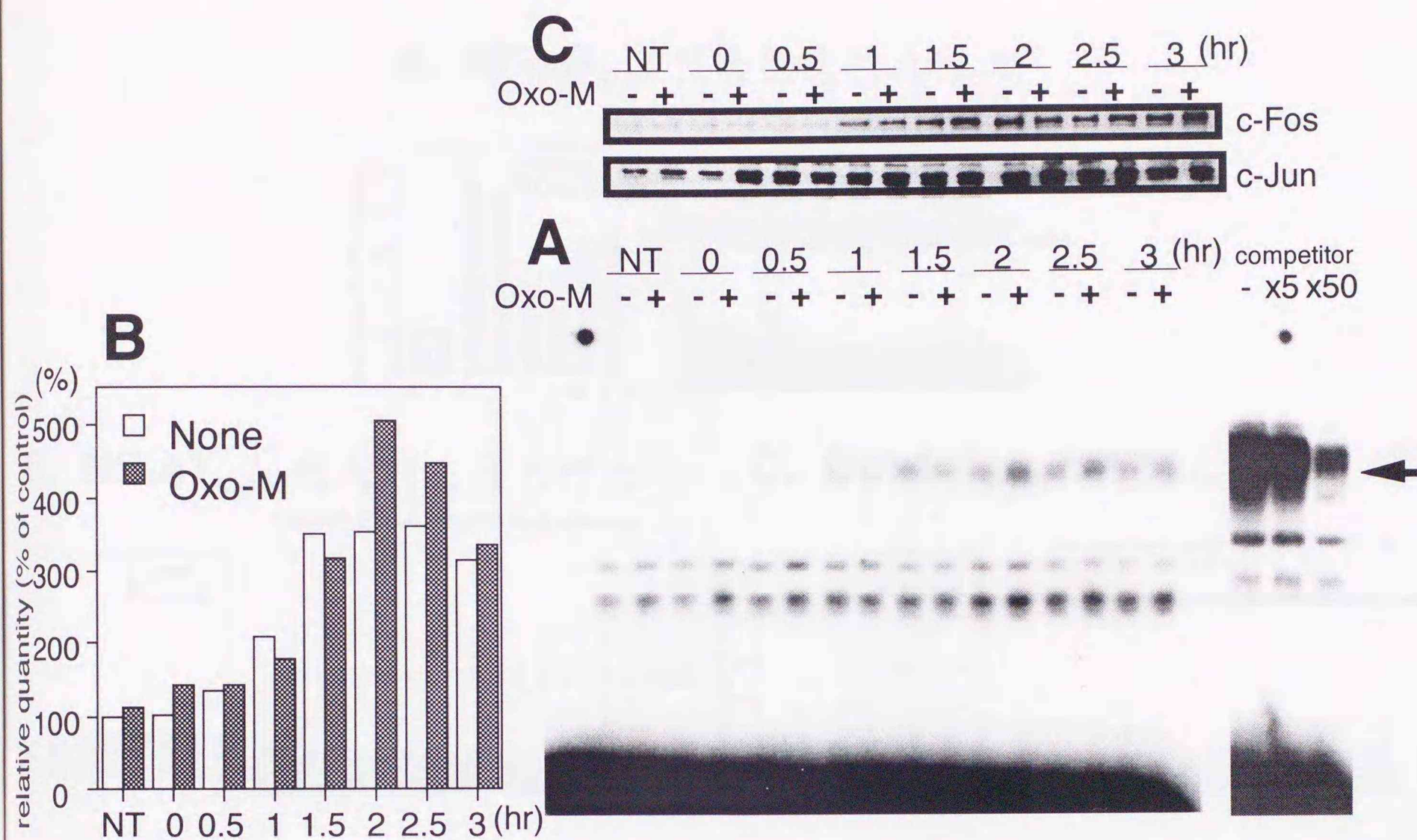


Fig. 2.1.7. Effect of m1 receptor stimulation on PMA/A23187-induced AP-1

consensus sequence binding proteins in Jurkat cells

Jurkat cells were treated or untreated with 1 μ M AFDX-116 and 100 μ M Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract (A). The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (x 5 or x 50 of the labeled probe). The quantities of each band were shown in a bar chart (B). The amount of c-Fos and c-Jun was measured by Western blotting analysis (C).

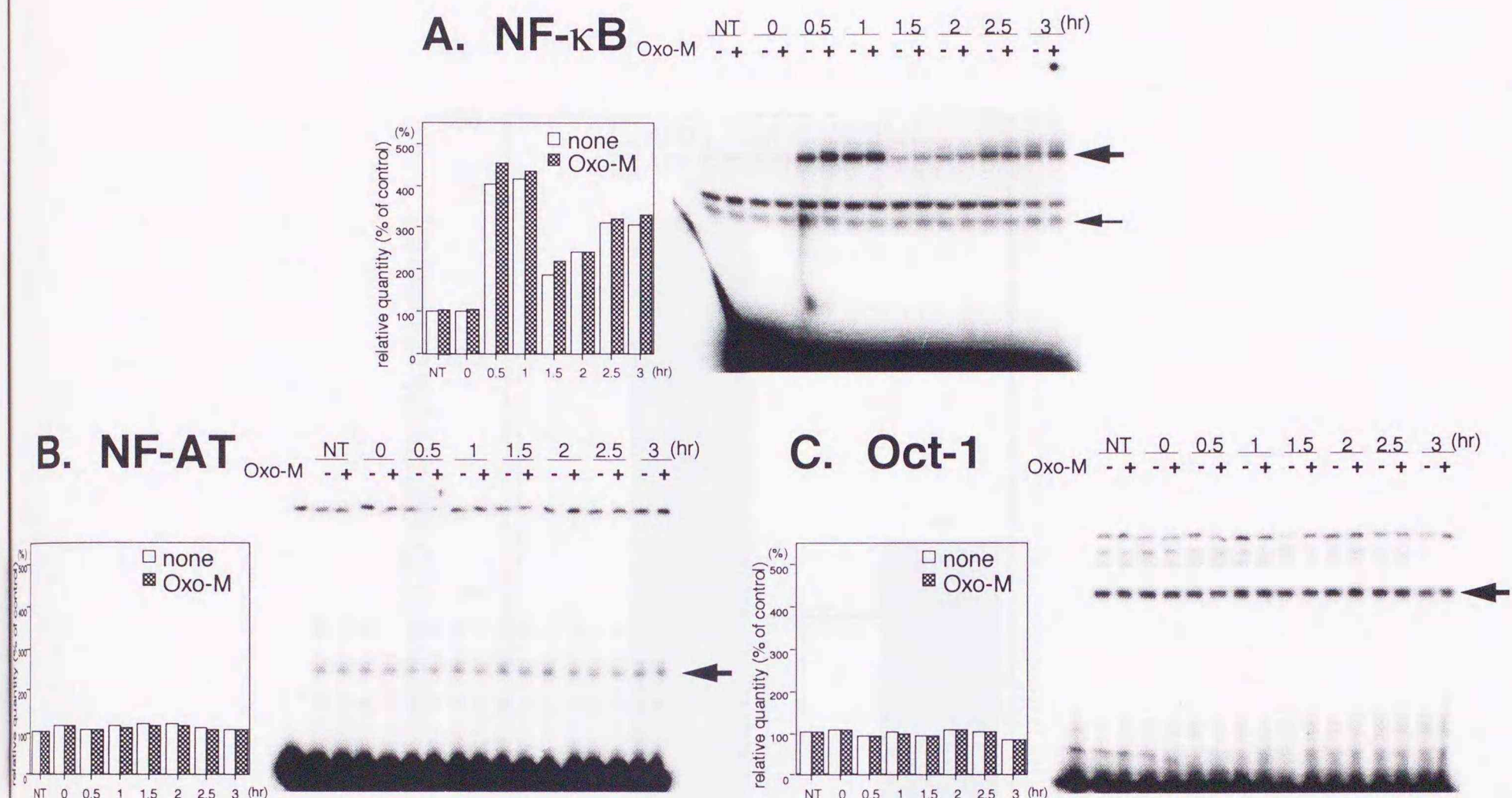


Fig. 2.1.8. Effect of m1 receptor stimulation on PMA/A23187-induced NF- κ B, NF-AT and Oct-1 consensus sequence binding proteins in Jurkat cells Jurkat cells were treated or untreated with 1 μ M AFDX-116 and 100 μ M Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract. The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (data not shown). The quantities of each band were shown in a bar chart. A, nuclear complexes binding to the NF- κ B site. B, nuclear complexes binding to the NF-AT site. C, nuclear complexes binding to the Oct-1 site.

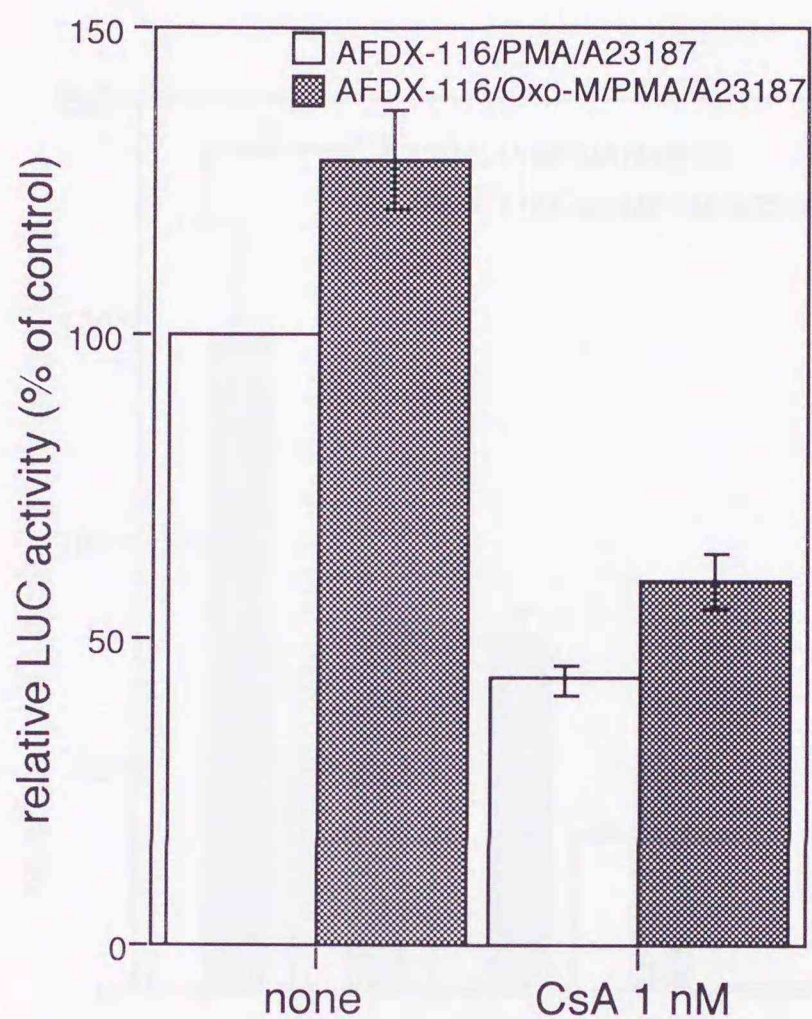


Fig. 2.1.9. Effect of cyclosporin A on m1 receptor-mediated IL-2 promoter

activity in Jurkat cells Cells were pretreated with 1 nM CsA for 2 hr prior to treatment with 1 μ M AFDX-116 and 100 μ M Oxo-M for 24 hr, and then treated 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

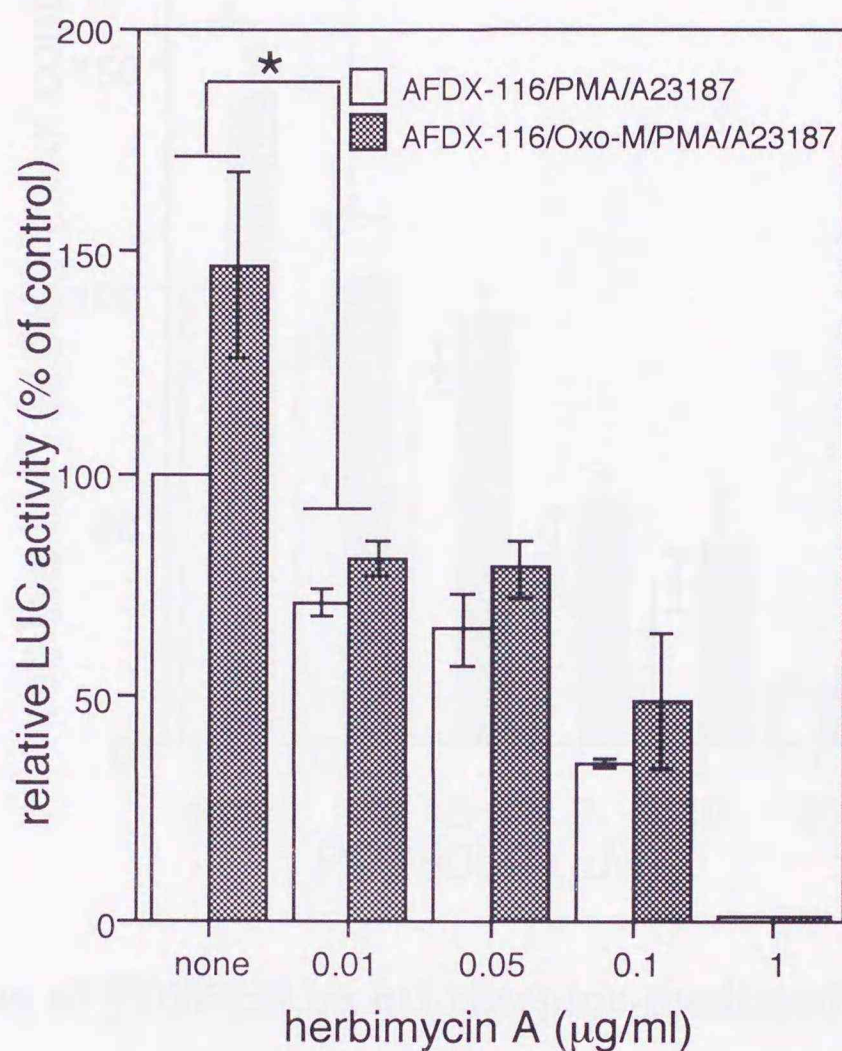


Fig. 2.1.10. Effects of herbimycin A on m1 receptor-mediated IL-2 promoter

activity in Jurkat cells Cells were treated with 0.01 to 1 µg/ml herbimycin A for 1 hr prior to treatment with 1 µM AFDX-116 and 100 µM Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity (± SE). *, $p < 0.05$ versus the enhancement rate of none.

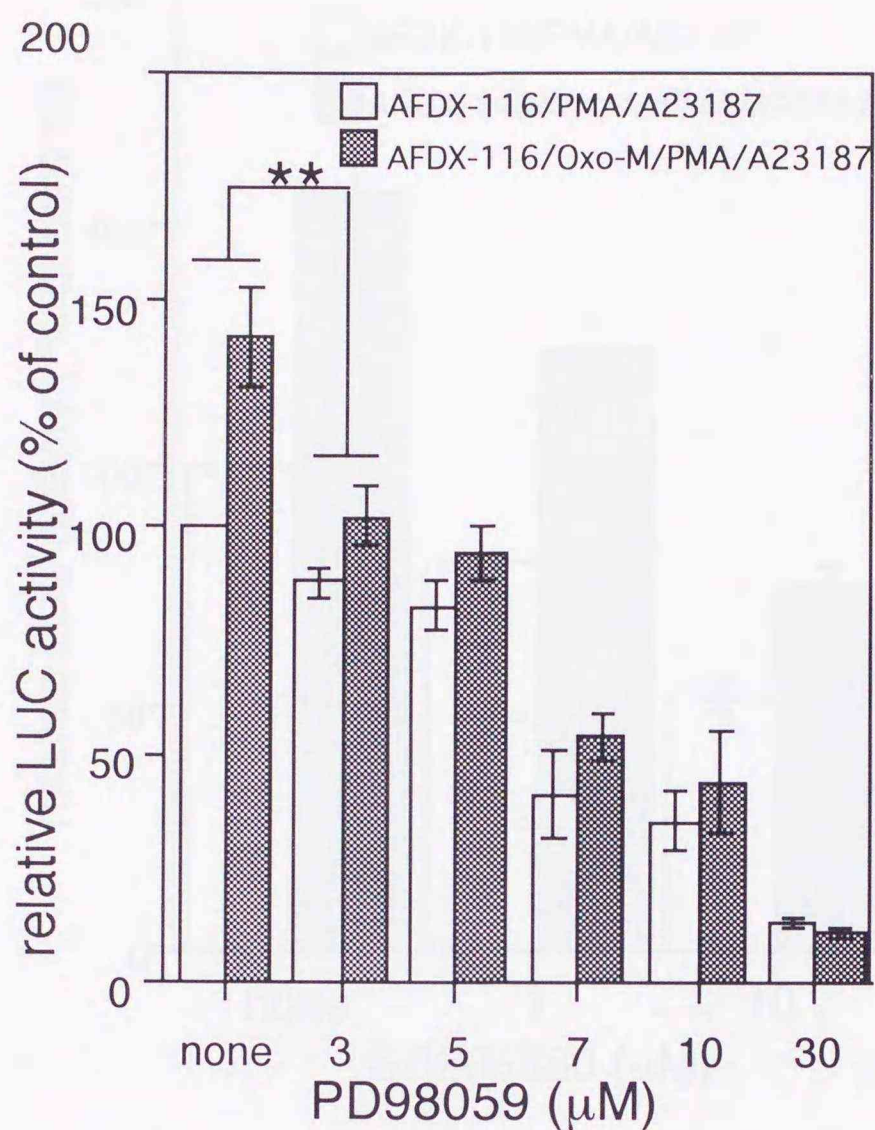


Fig. 2.1.11. Effect of PD98059 on m1 receptor-mediated IL-2 promoter activity

in Jurkat cells Cells were pretreated with 3 to 30 μ M PD98059 for 1 hr prior to treatment with 1 μ M AFDX-116 and 100 μ M Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE). **, $p < 0.01$ versus the enhancement rate of none.

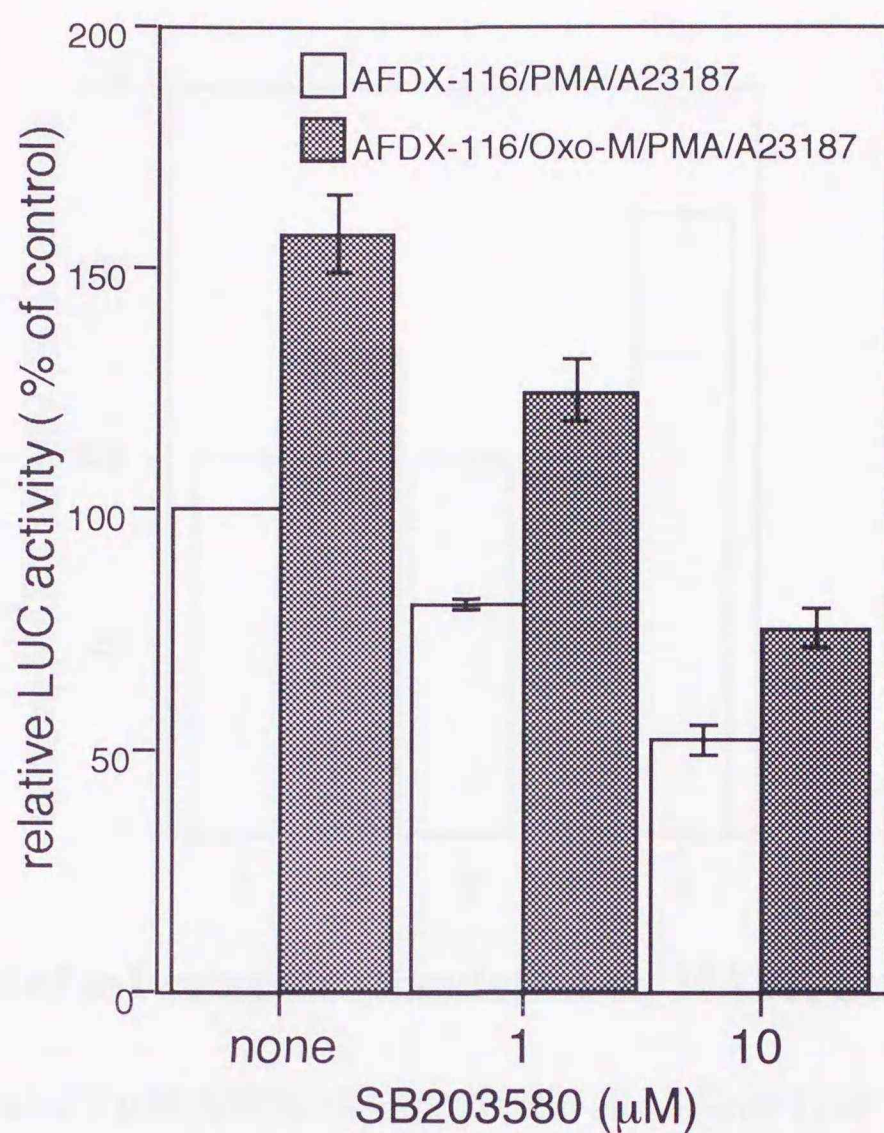


Fig. 2.1.12. Effect of SB203580 on m1 receptor-mediated IL-2 promoter activity

in Jurkat cells Cells were pretreated with 1 or 10 μ M SB203580 for 1 hr prior to treatment with 1 μ M AFDX-116 and 100 μ M Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

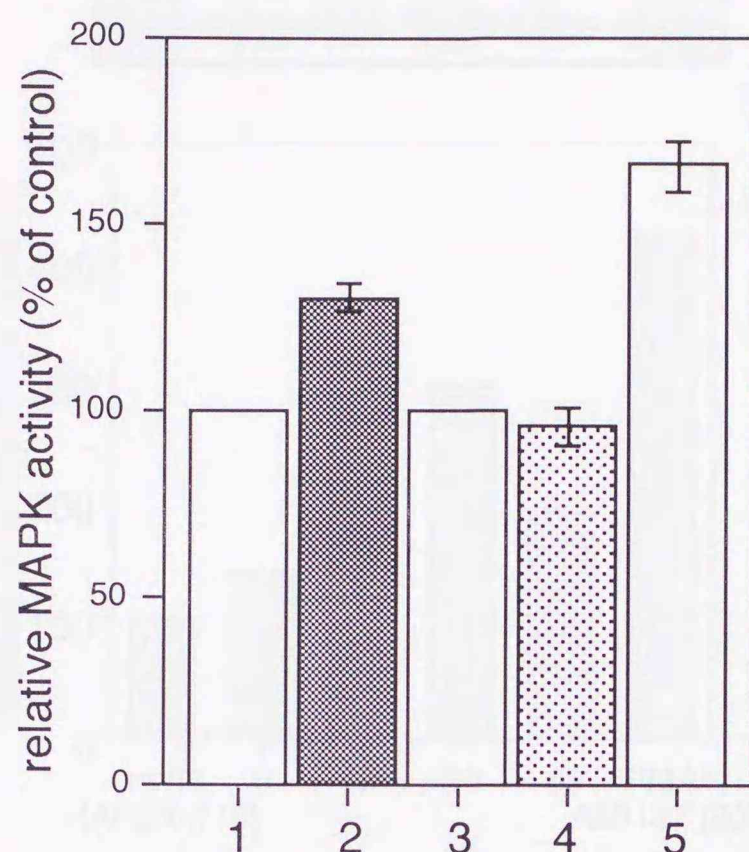


Fig. 2.1.13. Effect of m1 receptor stimulation on MAPK activities in Jurkat

cells Cells were treated 1 μ M AFDX-116 for 20 min alone (lane 1) or pretreated 1 μ M AFDX-116 for 20 min then treated 100 μ M Oxo-M for 5 min (lane 2). Cells were treated 1 μ M of AFDX-116 and 4-DAMP for 20 min (lane 3) or pretreated 1 μ M of AFDX-116 and 4-DAMP for 20 min then treated 100 μ M Oxo-M for 5 min (lane 4). Cells were treated 1 μ M PMA for 5 min (lane 5). MAPK activities were assessed as described in the "Experimental procedures". Each point was assayed three times. The activity of AFDX-116 was the same value as vehicle and estimated as 100%.

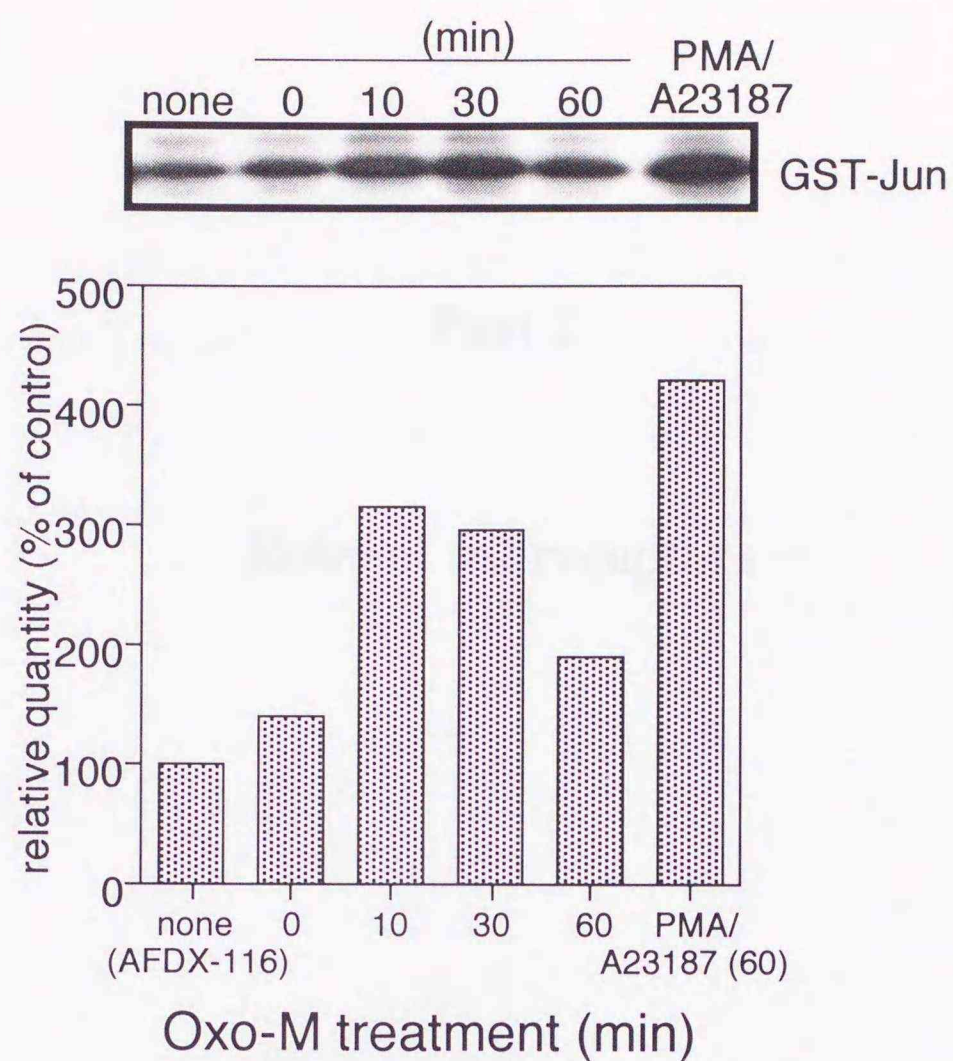


Fig. 2.1.14. Effect of m1 receptor stimulation on JNK activity in Jurkat cells

Cells were treated 1 μ M AFDX-116 for 20 min alone (none) or pretreated 1 μ M AFDX-116 for 20 min then treated 100 μ M Oxo-M for 0 to 60 min. Cells were treated 1 nM PMA and 500 nM A23187 for 60 min as the positive control. JNK activities were assessed as described in the "Experimental procedures". The quantities of each band were shown in a bar chart. The activity of AFDX-116 was the same value as vehicle and estimated as 100%.

2.3. Introduction

Since the introduction of the m2 receptor, AFDA-1 (4), further released by Gao
M. et al. (2004) (5), the m2 receptor has been found to be involved in the regulation of IL-2 production in T cells (6,7,8). The m2 receptor plays a key
role in the regulation of IL-2 production in T cells. To further study the role of m2
receptor in the regulation of IL-2 production, several experiments were performed using the m2 receptor agonist, AFDA-1 (4) and
antagonist, AFDA-2 (5). The results of these experiments are presented in this paper.

Part 2

Roles of m2 receptors

2-5. Introduction

Since the pretreatment with an m2 antagonist, AFDX-116, further enhanced the Oxo-M-mediated enhancement of IL-2 production (Fig. 2.1.5), the m2 receptor signals may function to decrease the IL-2 production in Jurkat cells. To evaluate this hypothesis, several experiments such as ELISA, luciferase assay, band shift assay, MAPK assay and immunocomplex kinase assay were performed using the same procedures as in the analyses of m1 receptors.

2-6. Results

Effect of m2 receptor stimulation on PMA/A23187-induced IL-2 production and promoter activity in Jurkat cells: To stimulate m2 receptors, Oxo-M was added in the presence of an m1/m3 receptor antagonist, 4-DAMP. The cells were pretreated with 1 μ M 4-DAMP for 20 min prior to the treatment of 100 μ M Oxo-M for 0 - 24 hr and then treated with 1 nM PMA and 500 nM A23187 for 24 hr. The m2 receptor stimulation decreased the IL-2 production although the decrease was biphasic (Fig. 2.2.1). Similar decrease by the m2 stimulation was also observed for the IL-2 promoter activity (Fig. 2.2.2).

Effects of m2 receptor-mediated IL-2 promoter activity on transcription factors in Jurkat cells: To examine whether the m2 receptor-mediated signals would affect the binding of *cis* regulatory elements to the IL-2 promoter, band shift assays were performed. Nuclear extracts were prepared from Jurkat cells stimulated by 1 nM PMA and 500 nM A23187 with or without pretreatment with 1 μ M 4-DAMP and 100 μ M Oxo-M. The extracts were tested for their potential to bind the AP-1, NF- κ B, NF-AT and Oct-1 sequences by band shift assays. In the presence of 1 μ M 4-DAMP, the increase by Oxo-M of the induction of AP-1-like proteins (Fig. 2.1.7A) was not observed and inhibited the induction occurred at 2 - 2.5 hr (Fig. 2.2.3A,B). Western blot analysis revealed that similar inhibition of the induction was also observed in the c-Fos and c-Jun proteins (Fig. 2.2.3C).

The stimulation of m2 receptors did not affect the increase of the nucleoprotein complexes on the NF- κ B consensus sequence (Fig. 2.2.4A) nor the constant complex formation on the NF-AT and Oct-1 sequences (Fig. 2.2.4B,C).

Effect of cyclosporin A on m2 receptor-mediated inhibition of IL-2 promoter activity in Jurkat cells: The effect of CsA on the induction of IL-2 promoter activity by PMA and A23187 as well as on the m2 receptor-mediated inhibition of the promoter activity in Jurkat cells was examined. The cells were pretreated with 1 nM CsA for 2 hr prior to the treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr, and then treated with 1 nM PMA and 500 nM A23187 for 24 hr. CsA decreased the IL-2 promoter activity, although there was no significant difference between the rates of inhibition, regardless to the inhibition by 4-DAMP/Oxo-M (Fig. 2.2.5).

Effect of herbimycin A on m2 receptor-mediated inhibition of IL-2 promoter activity in Jurkat cells: To examine whether tyrosine kinase is involved in m2 receptor-mediated decrease of the IL-2 promoter activity, the cells were pretreated with herbimycin A. After the pretreatment with 10 ng/ml herbimycin A followed by the treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr, the cells were treated with 1 nM PMA and 500 nM A23187 for 24 hr. Herbimycin A did not affect the IL-2 promoter activity either with or without the treatment with 4-DAMP and Oxo-M (Fig. 2.2.6).

Effects of MAP kinase superfamily on m2 receptor-mediated inhibition of IL-2 promoter activity in Jurkat cells: To investigate which pathways underlie the m2 receptor-mediated decrease of IL-2 promoter activity, the cells were pretreated with PD98059 and SB203580 as in the analyses of m1 receptors. To examine the involvement of MEK through MAPK (ERK) or p38 MAPK, the cells were pretreated with 3 μ M PD98059 or 1 μ M SB203580 for 1 hr prior to the treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr then treated 1 nM PMA and 500 nM A23187 for 24 hr. PD98059 or SB203580 decreased IL-2 promoter activity, although there was no

significant difference between the rates of inhibition, regardless of the inhibition by 4-DAMP/Oxo-M (Figs. 2.2.7 and 2.2.8).

To assess the effects of m2 receptor stimulation, MAP kinase assays were also performed. The cells were pretreated with 1 μ M 4-DAMP for 20 min to block m1 receptors, and then treated with 100 μ M Oxo-M for 5 min. The m2 receptor stimulation decreased MAP kinase activity to about 75% as compared to the control cells treated with 4-DAMP alone (Fig. 2.2.9). The decrease was blocked by pretreatment with an m2 receptor antagonist, 1 μ M AFDX-116, for 20 min (Fig. 2.2.9).

To examine the effects of m2 receptors on JNK activities, immunocomplex kinase assays were performed. The cells were pretreated with 1 μ M 4-DAMP for 20 min to block m1 receptors, and then treated with 100 μ M Oxo-M for 0 - 60 min. The phosphorylation of GST-c-Jun by the cell extracts was decreased in 30 - 60 min after the stimulation of m2 receptors (Fig. 2.2.10).

Effects of simultaneous m2 receptor stimulation with IL-2 promoter induction on transcription factors in Jurkat cells: As shown in Figs. 2.2.1 and 2.2.2, simultaneous stimulation of m2 receptor with the IL-2 promoter induction resulted in the decrease of the promoter activity. The mechanism of the decrease might be different from that mediated by the long-term (18 hr) stimulation of m2 receptor prior to the induction. Therefore, to examine whether the simultaneous m2 receptor stimulation would affect the binding activity of *cis* regulatory elements to the IL-2 promoter, band shift assays were performed. Nuclear extracts were prepared from Jurkat cells treated with 1 nM PMA and 500 nM A23187 for 0 - 3 hr either with or without the 1 μ M 4-DAMP and 100 μ M Oxo-M, and tested for potential binding to the AP-1, NF- κ B, NF-AT and Oct-1 sequences. The nucleoprotein complex on the AP-1 consensus sequence

increased in 2 - 3 hr after the PMA/A23187 induction, but the increase was diminished in the cells simultaneously treated with 4-DAMP and Oxo-M (Fig. 2.2.11A). The proteins bound to the NF- κ B consensus sequence were also increased, by the stimulation of PMA/A23187 on the IL-2 promoter induction. The simultaneous stimulation of m2 receptor inhibited the increase as well as in the case of AP-1 (Fig. 2.2.11B). The DNA/protein complexes on the NF-AT and Oct-1 sequences were observed constantly before and after the IL-2 promoter induction and were not affected by the simultaneous m2 receptor stimulation (Fig. 2.2.12A,B).

2-7. Discussion

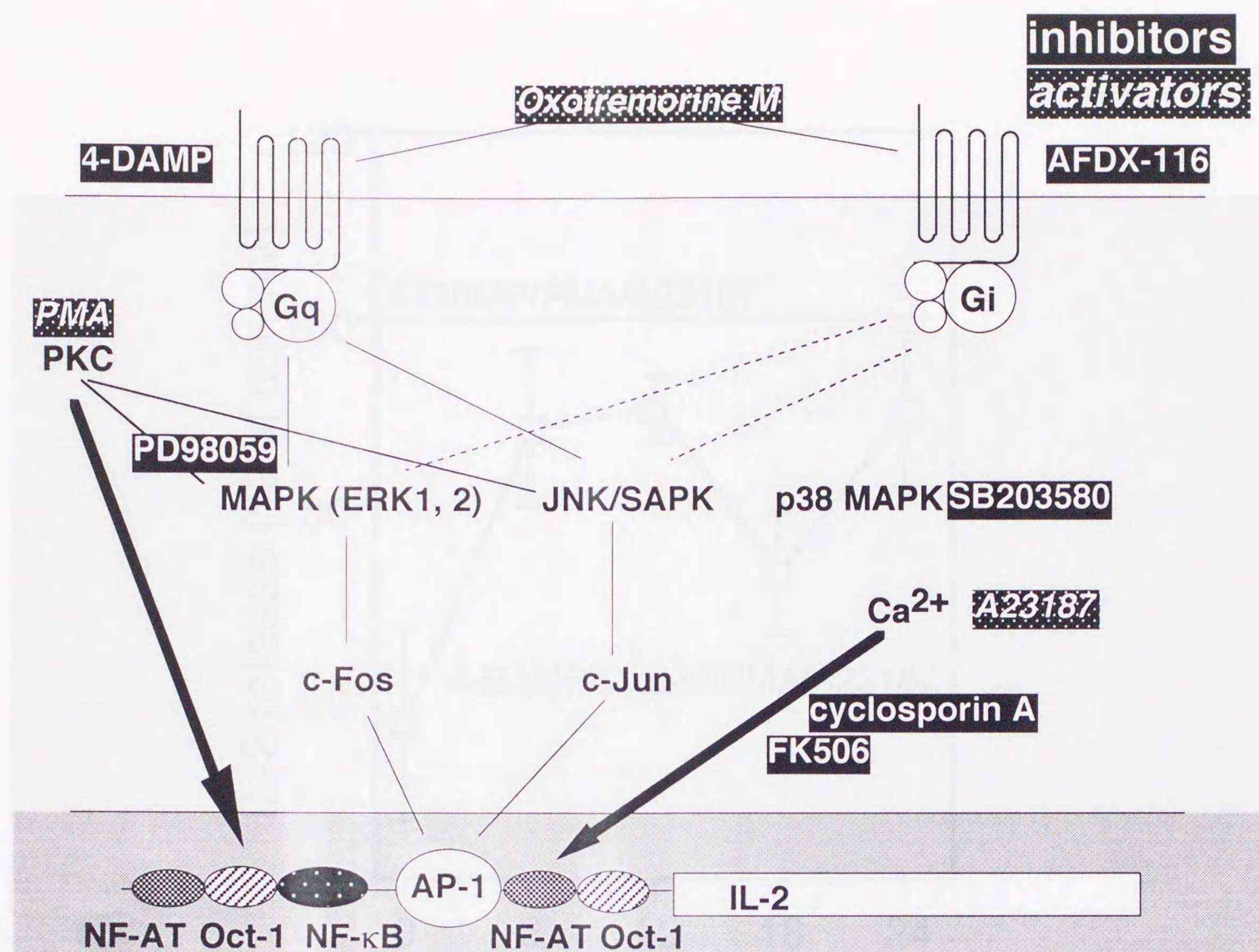
Since m2 receptors were also expressed on Jurkat cells, I examined the effect of signals *via* m2 receptors on IL-2 production in Jurkat cells. As shown in Fig. 2.1.5, pretreatment of the cells with an m2 receptor antagonist, AFDX-116, further enhanced the Oxo-M-mediated enhancement of IL-2 production. The result indicates that m2 receptors negatively functions on IL-2 production. To investigate the mechanism of the inhibition, the cells were treated with an m1/m3 antagonist, 4-DAMP, to block m1 receptors and to detect the phenomena only *via* m2 receptors. Pretreatment of the cells with 4-DAMP and Oxo-M, i.e. stimulation of m2 receptors, decreased IL-2 production and the promoter activity (Figs. 2.2.1 and 2.2.2). To examine whether the m2 receptor-mediated signals affect the binding activity of specific proteins to various *cis* regulatory elements in the IL-2 promoter, band shift assays were performed. The nucleoprotein complex formed on the AP-1 consensus sequence as well as c-Fos and c-Jun proteins increased on the induction of IL-2 production, but the increases were diminished by the m2 receptor stimulation (Fig. 2.2.3). The biphasic increase of the complex on the NF- κ B element, on the other hand, was not affected by the m2 receptor stimulation (Fig. 2.2.4A). The specific complexes on the NF-AT and Oct-1 elements were constant: neither the m2 receptor stimulation nor the IL-2 induction affected the DNA/protein complexes (Fig. 2.2.4B,C). The results suggest that AP-1 is involved in the m2 receptor-mediated decrease of IL-2 production in Jurkat cells.

To investigate by which pathways m2 receptors mediated the inhibition of the IL-2 promoter activity, the cells were pretreated with various inhibitors before the m2 receptor stimulation. Pretreatment with CsA did not affect the decrease of IL-2 promoter activity (Fig. 2.2.5). The result indicated that the m2 receptor-mediated decrease of IL-2 promoter activity may not due to the CsA-mediated pathways. The constant binding of NF-AT (Fig. 2.2.4B) also supported a speculation. Herbimycin A, a potent tyrosine

kinase inhibitor, did not affect the m2 receptor-mediated decrease of IL-2 promoter activity (Fig. 2.2.6). The result indicates that activation of tyrosine kinase is not involved in the inhibition.

A previous report showed that stimulation of the m2 receptors resulted in the activation of cytoplasmic MAPK in Rat 1a fibroblast cells (39). To examine whether the m2 receptors expressed on Jurkat cells also activate MAPKs, the cells were pretreated with a MEK inhibitor, PD98059, or a p38 MAPK inhibitor, SB203580, prior to the m2 receptor stimulation. The inhibitors blocked the enzyme activity regardless of the m2 receptor stimulation (Figs. 2.2.7 and 2.2.8). The results indicates that the decrease of the IL-2 promoter activity by m2 receptors is not mediated by the activation of MAPK/ERK and p38 MAPK. To assess the possibility that these MAPKs may be inhibited by the m2 receptor stimulation, MAPK assays and JNK immunocomplex kinase assays were performed. The m2 receptor stimulation resulted in the decrease of both MAPK/ERK and JNK activities (Figs. 2.2.9 and 2.2.10). The data suggest that the induction of the IL-2 promoter activity upon the m2 receptor stimulation is negatively regulated by AP-1 through MAPK/ERK and JNK.

IL-2 production induced by simultaneous treatment with 4-DAMP/Oxo-M (stimulation of m2 receptors) and PMA/A23187 resulted in the inhibition of the induction. Band shift assays revealed that, in contrast with the case of the 18 hr pretreatment, not only AP-1 but also NF- κ B complexes were involved in the inhibition of IL-2 production upon the simultaneous stimulation/induction (Fig. 2.2.11). Further clarification of the different mechanisms of both 18 hr pretreatment and simultaneous stimulation of m2 receptors may provide a key of the general understanding of the muscarinic receptor-mediated regulation of IL-2 production.



m1 and m2 systems on IL-2 production

Pretreatment of m2 receptors for 18 hr decreased PMA/A23187-induced IL-2 production and promoter activity. AP-1 binding activity and MAPK and JNK activities were decreased by m2 receptor stimulation compared to those in the control cells. m2 receptors stimulation with simultaneous PMA/A23187 treatment decreased AP-1 and NF-κB binding activities.

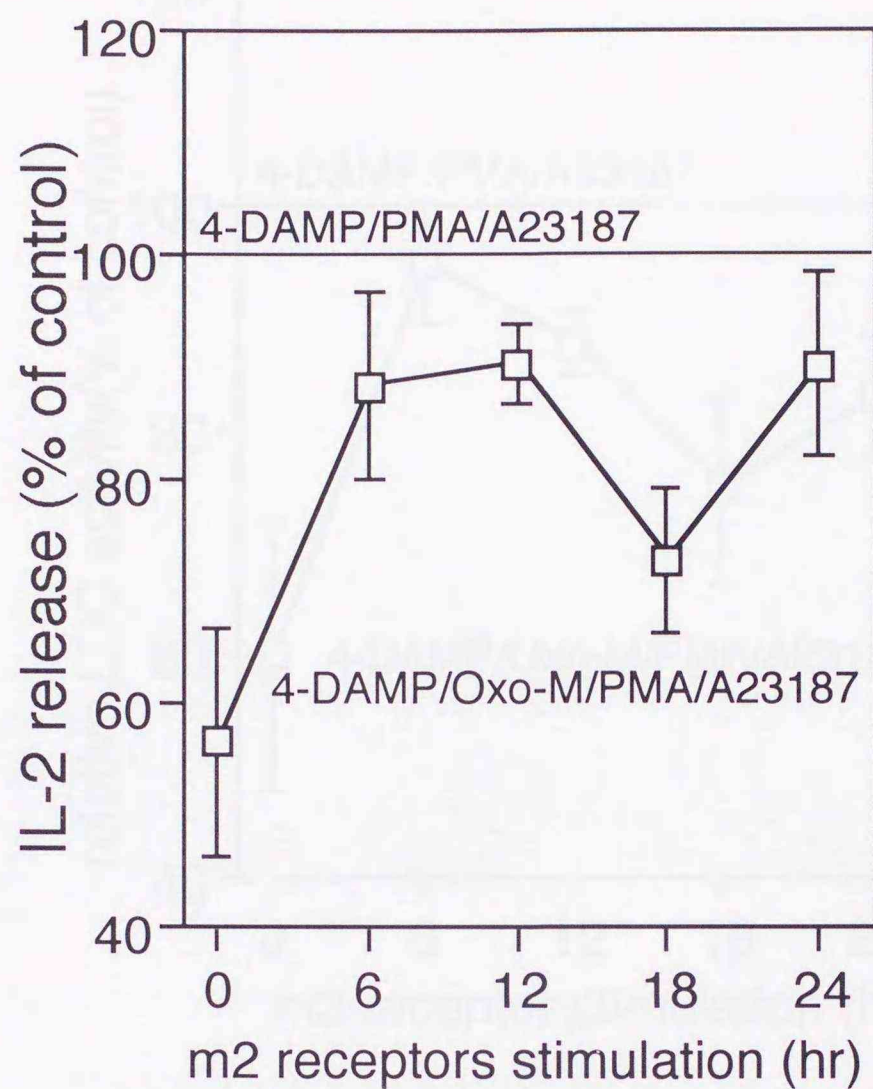


Fig. 2.2.1. Effect of m2 receptor stimulation on PMA/A23187-

induced IL-2 production in Jurkat cells Jurkat cells (2×10^5 cells/ml)

were pretreated with $1 \mu\text{M}$ 4-DAMP for 20 min prior to $100 \mu\text{M}$ Oxo-M for 0 to 24 hr followed by 1 nM PMA and 500 nM A23187 treatment for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The amount of 4-DAMP/PMA/A23187-induced IL-2 production was estimated as 100%.

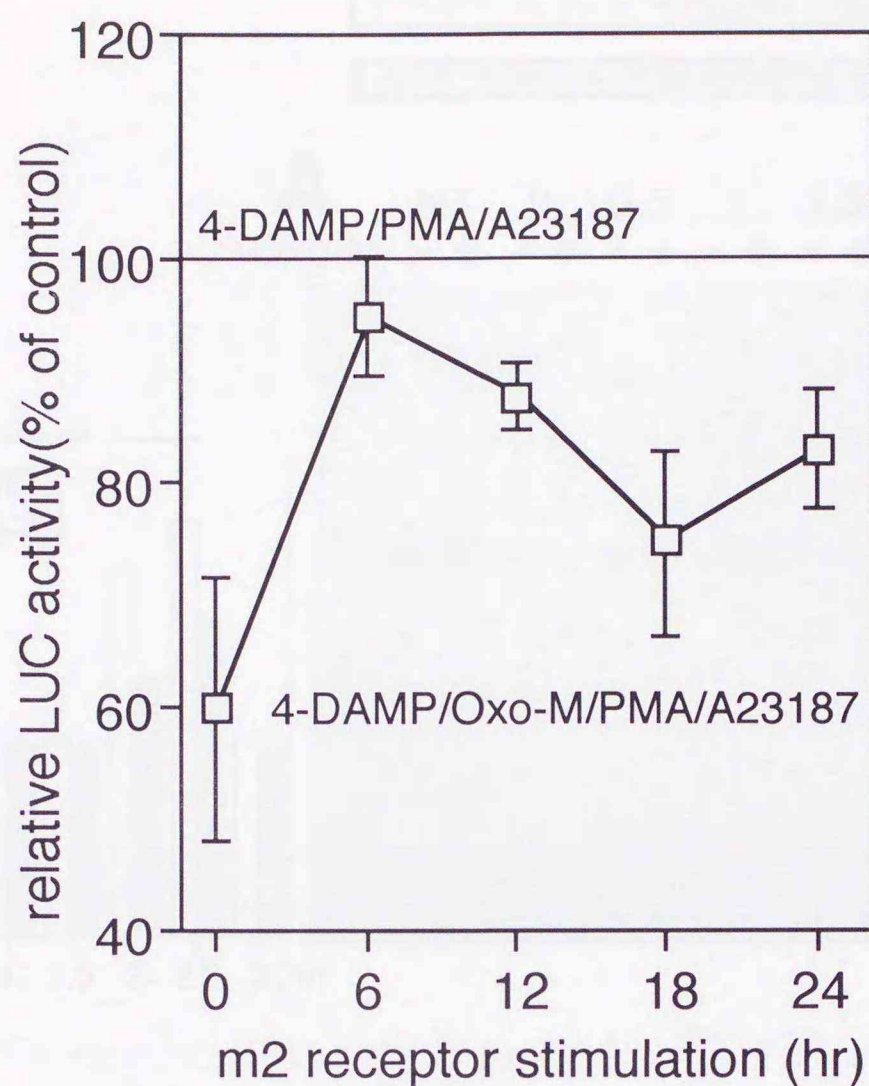


Fig. 2.2.2. Effect of m2 receptor stimulation on PMA/A23187-induced IL-2 promoter activity in Jurkat cells Jurkat cells were transfected with pIL2-LUC and pretreated with 1 μ M 4-DAMP for 20 min prior to 100 μ M Oxo-M for 0 to 24 hr followed by 1 nM PMA and 500 nM A23187 treatment for 24 hr. Cells were transfected at least three times (3 - 5). The value of 4-DAMP/PMA/A23187-induced IL-2 promoter activity was estimated as 100%.

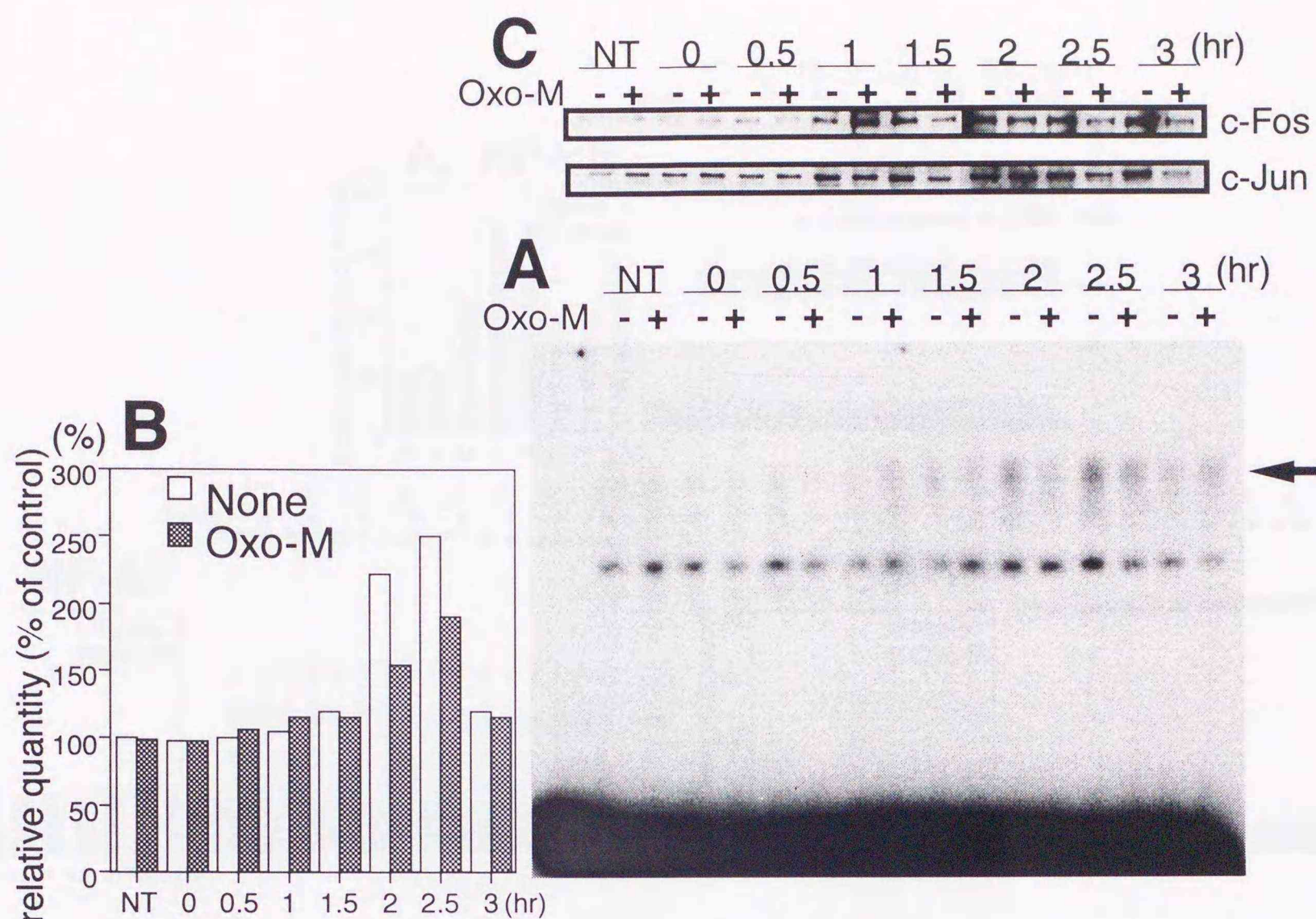


Fig. 2.2.3. Effect of m2 receptor stimulation on PMA/A23187-induced AP-1

consensus sequence binding proteins in Jurkat cells

Jurkat cells were treated or untreated with 1 μ M 4-DAMP and 100 μ M Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract (A). The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (data not shown). The quantities of each band were shown in a bar chart (B). The amount of c-Fos and c-Jun was measured by Western blotting analysis (C).

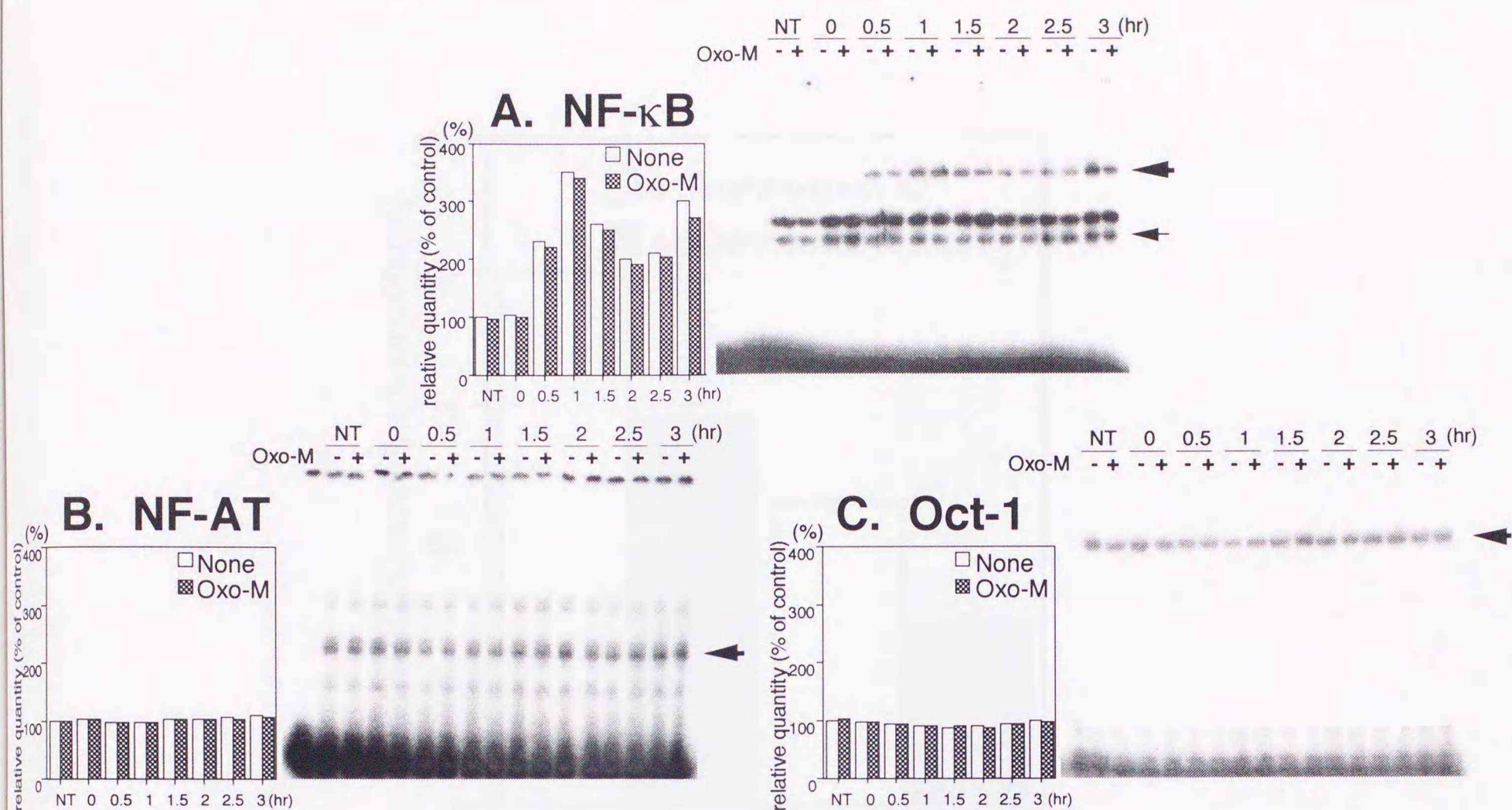


Fig. 2.2.4. Effect of m2 receptor stimulation on PMA/A23187-induced NF-κB, NF-AT and Oct-1 consensus sequence binding proteins in Jurkat cells Jurkat cells were treated or

untreated with 1 μM 4-DAMP and 100 μM Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract. The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (data not shown). The quantities of each band were shown in a bar chart. A, nuclear complexes binding to the NF-κB site. B, nuclear complexes binding to the NF-AT site. C, nuclear complexes binding to the Oct-1 site.

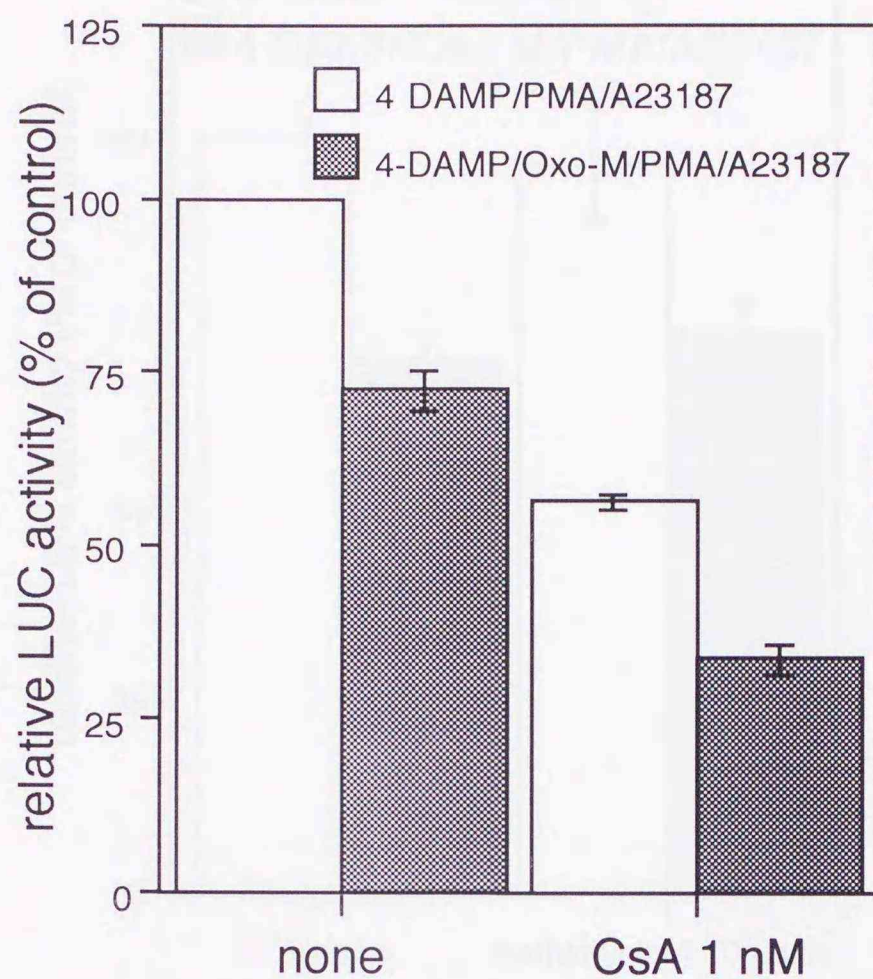


Fig. 2.2.5. Effect of cyclosporin A on m2 receptor-mediated inhibition of IL-2

promoter activity in Jurkat cells

Cells were pretreated with 1 nM CsA for 2 hr prior to treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

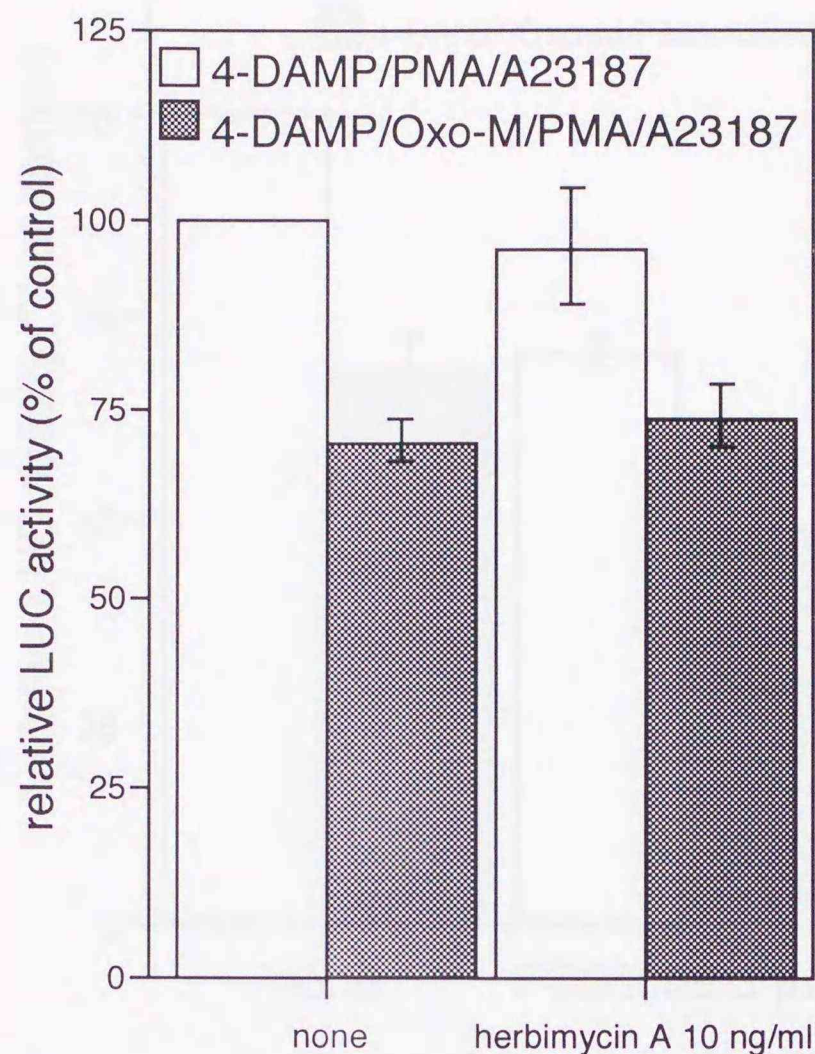


Fig. 2.2.6. Effect of herbimycin A on m2 receptor-mediated inhibition of IL-2

promoter activity Cells were pretreated with 10 ng/ml herbimycin A for 1 hr prior to treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

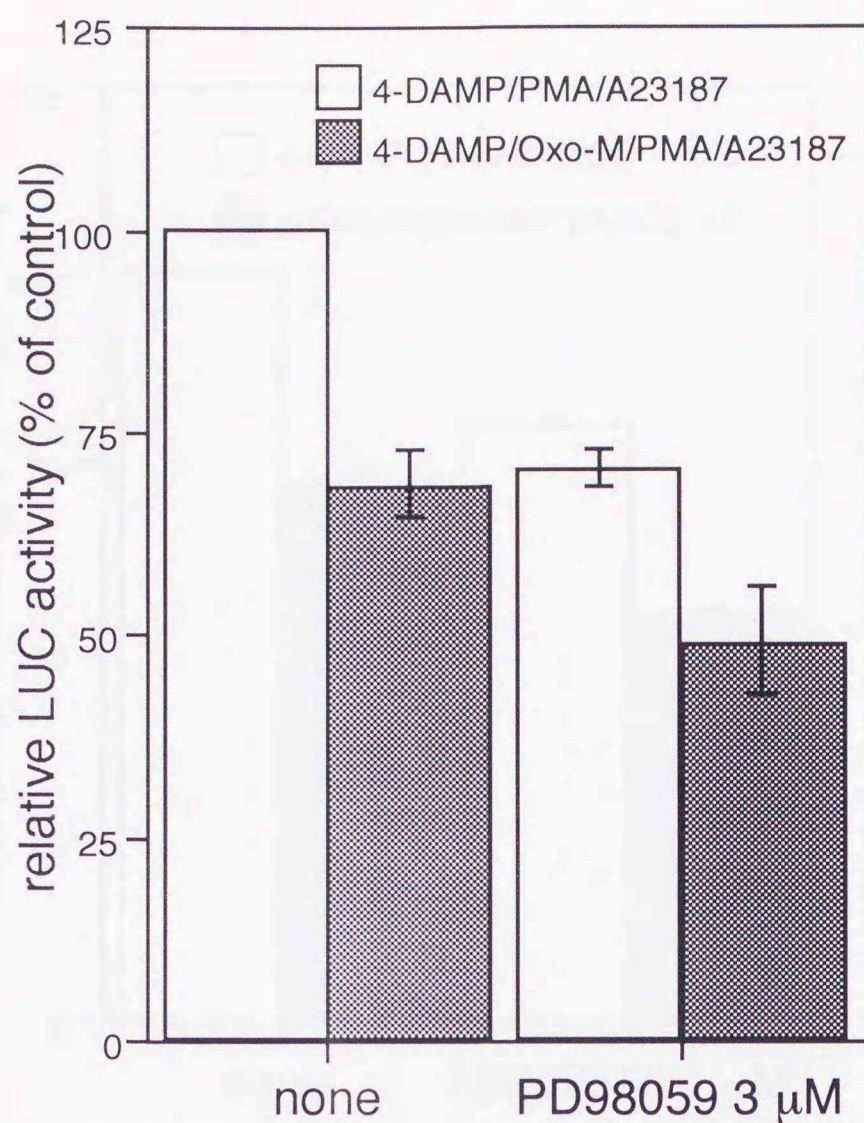


Fig. 2.2.7. Effect of PD98059 on m2 receptor-mediated inhibition of IL-2 promoter activity in Jurkat cells Cells were pretreated with 3 μ M PD98059 for 1 hr prior to treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr followed by treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

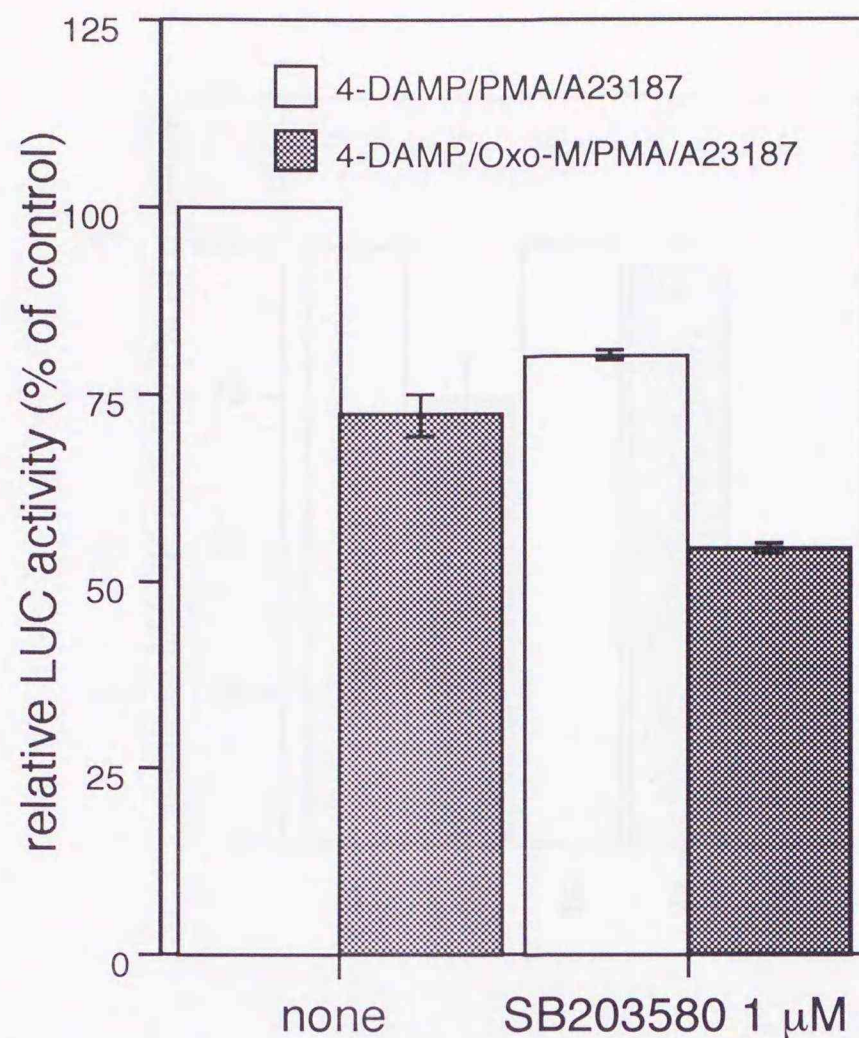


Fig. 2.2.8. Effect of SB203580 on m2 receptor-mediated inhibition of IL-2

promoter activity in Jurkat cells Cells were pretreated with 1 μ M SB203580 for 1 hr prior to treatment with 1 μ M 4-DAMP and 100 μ M Oxo-M for 24 hr followed by treatment 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5), and the average of data normalized to the value of PMA/A23187-stimulated pIL2-LUC is shown as relative luciferase activity ($\pm$ SE).

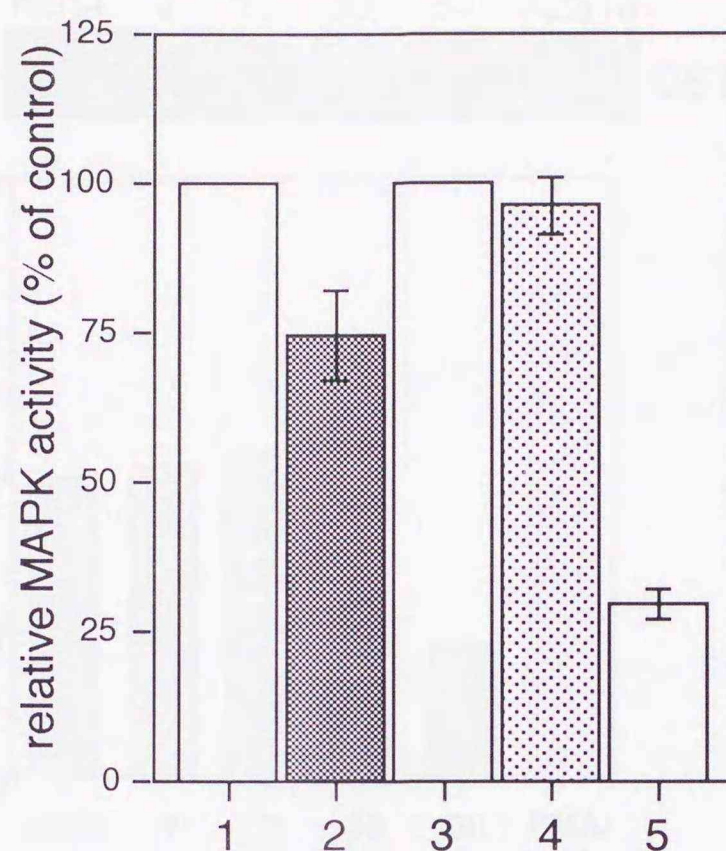


Fig. 2.2.9. Effect of m2 receptor stimulation on MAPK activity in Jurkat cells

Cells were treated 1 μ M 4-DAMP for 20 min alone (lane 1) or pretreated 1 μ M 4-DAMP for 20 min then treated 100 μ M Oxo-M for 5 min (lane 2). Cells were treated 1 μ M of 4-DAMP and AFDX-116 for 20 min (lane 3) or pretreated 1 μ M of 4-DAMP and AFDX-116 for 20 min then treated 100 μ M Oxo-M for 5 min (lane 4). Cells were treated 5 μ M PD98059 for 5 min (lane 5). MAPK activities were assessed as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The activity of 4-DAMP was the same value as vehicle and estimated as 100%.

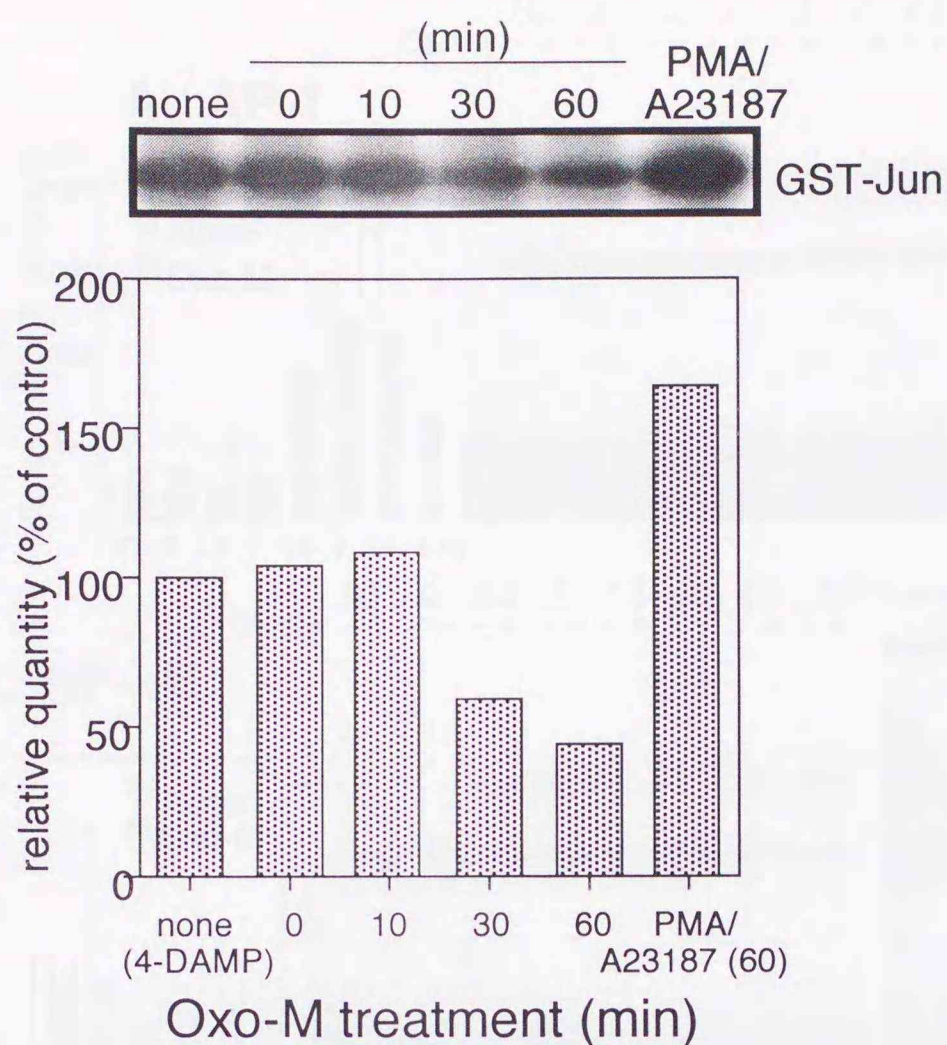


Fig. 2.2.10. Effect of m2 receptor stimulation on JNK activity in Jurkat cells

Cells were treated 1 μ M 4-DAMP for 20 min alone (none) or pretreated 1 μ M 4-DAMP for 20 min then treated 100 μ M Oxo-M for 0 to 60 min. Cells were treated 1 nM PMA and 500 nM A23187 for 60 min as the positive control. JNK activities were assessed as described in the "Experimental procedures". The quantities of each band were shown in a bar chart. The activity of 4-DAMP was the same value as vehicle and estimated as 100%.

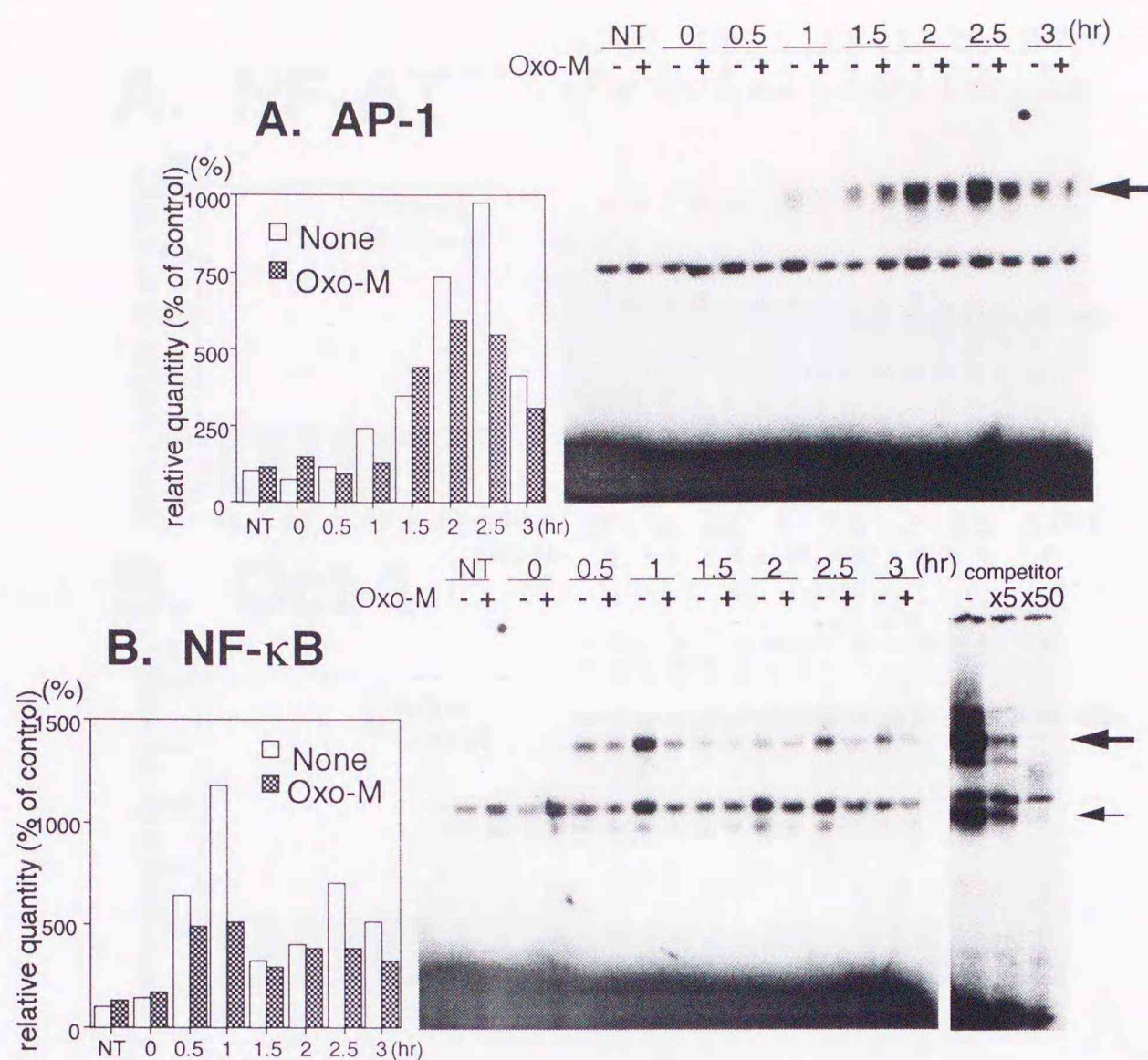


Fig. 2.2.11. Effects of simultaneous m2 receptor stimulation on PMA/A23187-induced AP-1 and NF-κB consensus sequence binding proteins in Jurkat cells

Jurkat cells were treated or untreated with 1 μM 4-DAMP and 100 μM Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract. The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (x 5 or x 50 of the labeled probe). The quantities of each band were shown in a bar chart. *A*, nuclear complexes binding to the AP-1 site. *B*, nuclear complexes binding to the NF-κB site.

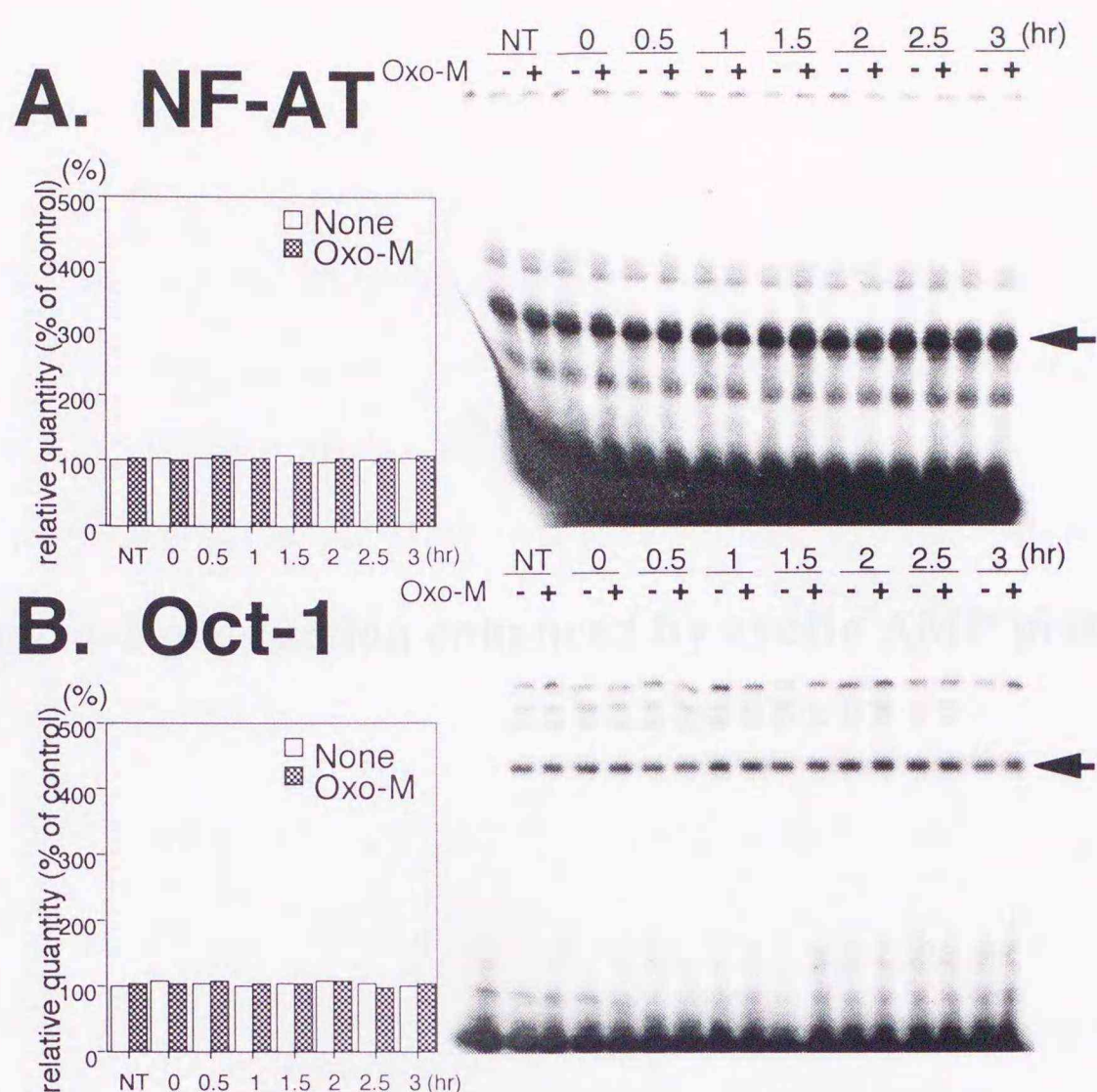


Fig. 2.2.12. Effects of simultaneous m2 receptor stimulation on PMA/A23187-induced NF-AT and Oct-1 consensus sequence binding proteins in Jurkat cells

Jurkat cells were treated or untreated with 1 μ M 4-DAMP and 100 μ M Oxo-M prior to treatment with 1 nM PMA and 500 nM A23187 for 0 to 3 hr then band shift assays were performed with using each nuclear extract. The bands indicated by arrowheads were shown to be specific by competition analysis, using homologous unlabeled oligonucleotides (data not shown). The quantities of each band were shown in a bar chart. *A*, nuclear complexes binding to the NF-AT site. *B*, nuclear complexes binding to the Oct-1 site.

3-1. Introduction

Section 3

Interleukin-2 production enhanced by cyclic AMP pretreatment

Induced T_H1 response (32, 33) as well as the induction of PGE₂ (34) involves phosphorylation and the subsequent activity (35) and the regulation of gene expression (36, 37) and a TCR gene product (38, 39). CD4⁺ T_H1 cell activation involves a variety of cytokines including IL-1 (40) and IL-2 (41, 42). In contrast, another group showed a significant decrease in the production of cytokines (43) which measured the T cell stimulation and thus suggested that cAMP might positively regulate T cell proliferation (44). The effect of cAMP on T cell proliferation is dependent on the different states of the cells (45).

Analysis of cAMP in mouse cells in the early phase of the cell cycle (46). The results showed that the T cell cycle was cAMP dependent. CD4⁺ T_H1 cells and CD4⁺ T_H2 cells have different cAMP levels (47). CD4⁺ T_H1 cells have higher cAMP levels than CD4⁺ T_H2 cells. The results showed that the T cell cycle was cAMP dependent. CD4⁺ T_H1 cells and CD4⁺ T_H2 cells have different cAMP levels (47). CD4⁺ T_H1 cells have higher cAMP levels than CD4⁺ T_H2 cells.

Pharmacological regulation of cAMP in T cells (48). The results showed that the T cell cycle was cAMP dependent. CD4⁺ T_H1 cells and CD4⁺ T_H2 cells have different cAMP levels (47). CD4⁺ T_H1 cells have higher cAMP levels than CD4⁺ T_H2 cells. The results showed that the T cell cycle was cAMP dependent. CD4⁺ T_H1 cells and CD4⁺ T_H2 cells have different cAMP levels (47). CD4⁺ T_H1 cells have higher cAMP levels than CD4⁺ T_H2 cells.

3-1. Introduction

In T lymphocytes, activation of PKC and increased $[Ca^{2+}]_i$ promote mitogenic signals. T cell proliferation is, however, inhibited by agents such as prostaglandin E1 and E2, cholera toxin, all of which increase intracellular cAMP levels (46 - 48), or cell-permeable cAMP analogues (49 - 51). Initial attempts to characterize the mechanism of cAMP-mediated inhibition of T cell mitogenesis have shown the interference with antigen-induced PI turnover (52, 53) as well as the inhibition of PLC γ 1 tyrosine phosphorylation and the enzymatic activity (54) and the inhibition of phosphorylation of the γ and ϵ TCR polypeptides (55, 56). cAMP inhibits T cell activation mediated by a variety of mitogens, including IL-2 (57) and PMA plus ionomycin (58 - 61). In contrast, another report showed a significant increase in the accumulation of intracellular cAMP within minutes of the T cell stimulation and thus suggested that cAMP may positively regulate T cell proliferation (62). The effect of cAMP has been suggested to depend on the differentiation state of the cells (63).

Another effect of cAMP is to arrest cells in the G1 phase of the cell cycle (64 - 66). The mechanism of the G1 arrest was cAMP indirectly, *via* p27^{kip1}, prevents the cdk-activating kinase (CAK) from phosphorylating cdk4 (67). Levels of the cdk inhibitor p27^{kip1} increase in cAMP-treated cells. Kip1 does not bind to CAK, but its association with cyclin D-cdk4 prevents CAK from phosphorylating and activating the holoenzyme.

Human normal T lymphocytes are an ideal system for studies of growth regulation, because the cells are in the G0 phase and semisynchronously enter the cell cycle simply upon the activation of the TCR/CD3 complex (68). A number of experiments have now established that the cell cycle progression in T cells occurs as a result of the sequential expression of a series of the genes. In particular, stimulation of the TCR/CD3 complex initiates the transition from G0 to G1, whereby the gene encoding

the receptor for the T cell growth factor, IL-2, is expressed and thus the cells become competent to proliferate (68 - 72). In addition, the IL-2 gene itself is simultaneously transcribed and the interaction with IL-2 receptor promotes the G1 progression towards DNA replication.

The m2 receptor is predominantly coupled with Gi, the pertussis toxin-sensitive G protein (73, 74). Gi protein is ubiquitously expressed and is abundant G protein found in fibroblasts and many other cell types. Inhibition of adenylyl cyclase activity has been classically defined as a Gi-regulated response (75). Since Gi protein is expressed at sufficient levels in many cell types, dissociation of the $\beta\gamma$ dimers subunits from α_i2 /GTP upon activation may also contribute to the regulation of effectors including the specific isoforms of adenylyl cyclase and PLC (76, 77).

Since the m2 receptor-mediated signals decreased IL-2 production in Jurkat cells (see Section 2), I examined here whether the m2 receptor-mediated inhibition of IL-2 production is mediated by the cAMP and/or the cell cycle regulations.

3-2. Results

Effects of cAMP on PMA/A23187-induced IL-2 production and the promoter activity in Jurkat cells: The IL-2 production induced by 10 nM PMA and 500 nM A23187 was enhanced by pretreatment for 24 hr with forskolin at the concentrations ranging 0.1 - 10 μ M (Fig. 3.1A) or DBcAMP at the concentrations ranging 0.01 - 1 mM (Fig. 3.1B) in a dose-dependent manner. When the cells were pretreated for 24 hr with 0.01 - 1 mM DBcAMP, similar enhancement was also observed in the IL-2 promoter activity linked to the luciferase gene (Fig. 3.2).

To determine the optimal pretreatment time, the cells were pretreated with 1 mM DBcAMP for 0 - 24 hr and then treated with 1 nM PMA and 500 nM A23187 for 24 hr. Short-term treatment (0 - 6 hr) with DBcAMP markedly inhibited the IL-2 production, up to 75 % inhibition, but the long-term treatment (6 - 24 hr) resulted in the larger production than short-term treatment. In 24 hr, the 25 % enhancement was observed (Fig. 3.3). Similar effects of DBcAMP, namely decrease in short-term and then increase in long-term, were observed on the IL-2 promoter activity (Fig. 3.4), except that the activity stayed higher than the control at all the time for 24 hr.

Effects of H-89 on DBcAMP-enhanced IL-2 production and the promoter activity in Jurkat cells: To examine the effect of PKA mediated by cAMP on the IL-2 production and the promoter activity, the cells were pretreated with a PKA inhibitor, H-89, at the concentrations ranging 0.1 - 10 μ M for 3 hr, prior to the treatment with 1 nM PMA and 500 nM A23187 for 24 hr. H-89 inhibited the PMA/A23187-induced IL-2 production (Fig. 3.5A) and the promoter activity (Fig. 3.5B) in a dose dependent manner, regardless of the enhancement by DBcAMP.

Effects of DBcAMP on the cell cycle in Jurkat cells: Flow cytometric analysis of the cell cycle indicated that the treatment with 1 mM DBcAMP for 24 hr resulted in the cell cycle arrest in the G1 phase (Fig. 3.6). The number of the cells in the G1 phase started to decrease at 6 hr after the DBcAMP addition, whereas the cells in the G2 phase increased (Fig. 3.6). The results indicate that the regulation of IL-2 production is correlated with the cell cycle. To examine the speculation, the cells were synchronized using the double-thymidine block protocol to trap in the G1/S phase and assayed for the IL-2 production. The IL-2 production was higher in the cells trapped in the G1/S phase (0 hr) than the randomly cultured cells (control). In the cells in the G2/M phase (18 hr), on the contrary, the IL-2 production was decreased (Fig. 3.7).

Effects of m2 receptor stimulation on the cell cycle in Jurkat cells: If the IL-2 production is correlated with the cell cycle regulation, stimulation of m2 receptor might affect the cell cycle progression. The results of cell cycle analysis, however, suggested that the treatment with 4-DAMP/Oxo-M for 0 - 24 hr hardly affected the cell cycle in Jurkat cells (Fig. 3.8). The following PMA/A23187 treatment did not affect the cell cycle, either (data not shown).

Effects of m2 receptor stimulation on cAMP accumulation in Jurkat cells: Muscarinic m2 receptors are known to inhibit cAMP accumulation. The intracellular amount of cAMP of the cells treated with 1 μ M 4-DAMP for 20 min was decreased by the following treatment of the cells with 100 μ M Oxo-M for 30 min (Fig. 3.9, lanes 1 and 2). The PMA/A23187-induction for 30 min, on the other hand, increased the cAMP accumulation of the 4-DAMP-treated cells (Fig. 3.9, lane 3). When the cells were simultaneously treated with 4-DAMP/Oxo-M and PMA/A23187, the cAMP amount was decreased, but the PMA/A23187-induction after the 18 hr incubation with 4-DAMP/Oxo-M the decrease was abolished (Fig. 3.9, lanes 4 and 5). The results

suggest that the m2 receptor stimulation by 4-DAMP/Oxo-M transiently inhibited the cAMP accumulation, but this inhibition was disappeared when m2 receptors were stimulated for 18 hr.

3-3. Discussion

A cell-permeable cAMP analogue, DBcAMP, has been shown to inhibit T cell mitogenesis induced by various agonists including alloantigen, IL-2 and PMA plus ionomycin. The mechanism of inhibition was initially assumed to reflect interference with the TCR-coupled PI turnover (52, 53, 54).

Pretreatment of Jurkat cells with forskolin or DBcAMP for 24 hr enhanced the PMA/A23187-induced IL-2 production (Fig. 3.1) and the promoter activity (Fig. 3.2), but the short-term treatment with DBcAMP inhibited both the IL-2 production and the promoter activity (Figs. 3.3 and 3.4). It has been suggested that the effect of cAMP may depend on the differentiation state of cells (63). These data indicate that cells may enter into the differentiation state, for example, different stage of the cell cycle, after DBcAMP treatment. The major function of cAMP is to regulate the activity of PKA as a second messenger. When Jurkat cells were treated with H-89, a PKA inhibitor, prior to the DBcAMP treatment, the PMA/A23187-induced IL-2 production as well as the promoter activity was inhibited, but the inhibition was similarly observed regardless of the DBcAMP treatment (Fig. 3.5). Previous study showed the increase in the accumulation of cAMP within minutes after the induction in lymphocyte (62). Increase in intracellular cAMP may result in activation of PKA. PMA/A23187-induced IL-2 may bind to the IL-2 receptor expressed in Jurkat cells. The signals from IL-2 receptor may activate autocrine system and induce further IL-2 production and whole cell activation. PKA may be involved in this autocrine system and H-89 may partially inhibit this activation by inhibition of PKA. The results suggest that PKA is involved in the induction of IL-2 production, but not in the enhancement by the DBcAMP pretreatment for 24 hr.

cAMP blocks mitogenic effects in lymphocytes and induces a cell cycle arrest in the mid-G1 phase and indirectly prevents the cdk-activating kinase from phosphorylating

cdk4. The inhibition is mediated by p27^{kip1} (67). IL-2 plays a pivotal role in the immune response by inducing TCR-activated lymphocytes in the G1 phase to progress into the replicative phase of the cell cycle (68). Correlation among cAMP, IL-2 production and the cell cycle have been thus suggested. FACS analysis revealed that the treatment with DBcAMP for 24 hr arrested the cells in the G1 phase, but that for 6 hr, Jurkat cells were arrested in the G2 phase (Fig. 3.6). The results indicate that Jurkat cells produce IL-2 and proliferate in response to PMA/A23187 treatment while they are in the G1 phase, but that once they are committed to replicate DNA, they may mostly ignore the signals until they complete mitosis and re-enter the G1 phase. The pretreatment with DBcAMP for 6 hr (i.e. the short-term) hence inhibited the IL-2 production, because the cells were in the G2 phase. When the cells were synchronized and trapped in the G1/S phase by the double-thymidine block protocol, the IL-2 production was enhanced in the cells in the G1/S phase, while decreased in the cells in the G2 phase (Fig. 3.7). The results indicate that the IL-2 production depends on the cell cycle.

I have shown that the pretreatment of m2 receptor decreased IL-2 production in Jurkat cells. Since m2 receptor is reported to couple with Gi protein and to inhibit cAMP accumulation, FACS analyses were performed to examine the correlation of the cAMP accumulation with the inhibitory effect of m2 receptor on the IL-2 production and the cell cycle progression. Neither the stimulation of m2 receptor (Fig. 3.8) nor the PMA/A23187 induction (data not shown) affected the cell cycle in Jurkat cells. The results suggest that the inhibition of IL-2 production by the m2 receptor stimulation is not corresponding to the cell cycle regulation *via* cAMP. As for the intracellular amount of cAMP, the short term stimulation (30 min) of m2 receptor inhibited the cAMP accumulation, but the long term stimulation (18 hr) did not affect the cAMP amount in Jurkat cells (Fig. 3.9). The results indicate that the decrease in IL-2 production mediated by m2 receptor may be corresponding to the transient decrease in the cAMP amount, but not to the cell cycle progression mediated by cAMP.

3-4. Conclusion of Section 3

Pretreatment with 1 mM DBcAMP for 24 hr enhanced the IL-2 production, in contrast, pretreatment for 6 hr inhibited it in Jurkat cells. In addition, the DBcAMP treatment arrested the cell cycle. The mechanism of these biphasic IL-2 productions by DBcAMP may be due to the cell cycle. The m2 receptor stimulation, however, did not affect the cell cycle, but transiently affected the cAMP accumulation. The inhibition of IL-2 production by the m2 receptor stimulation is thus suggested to be mediated by the suppression of cAMP pathways, but not by the cell cycle progression mediated by cAMP.

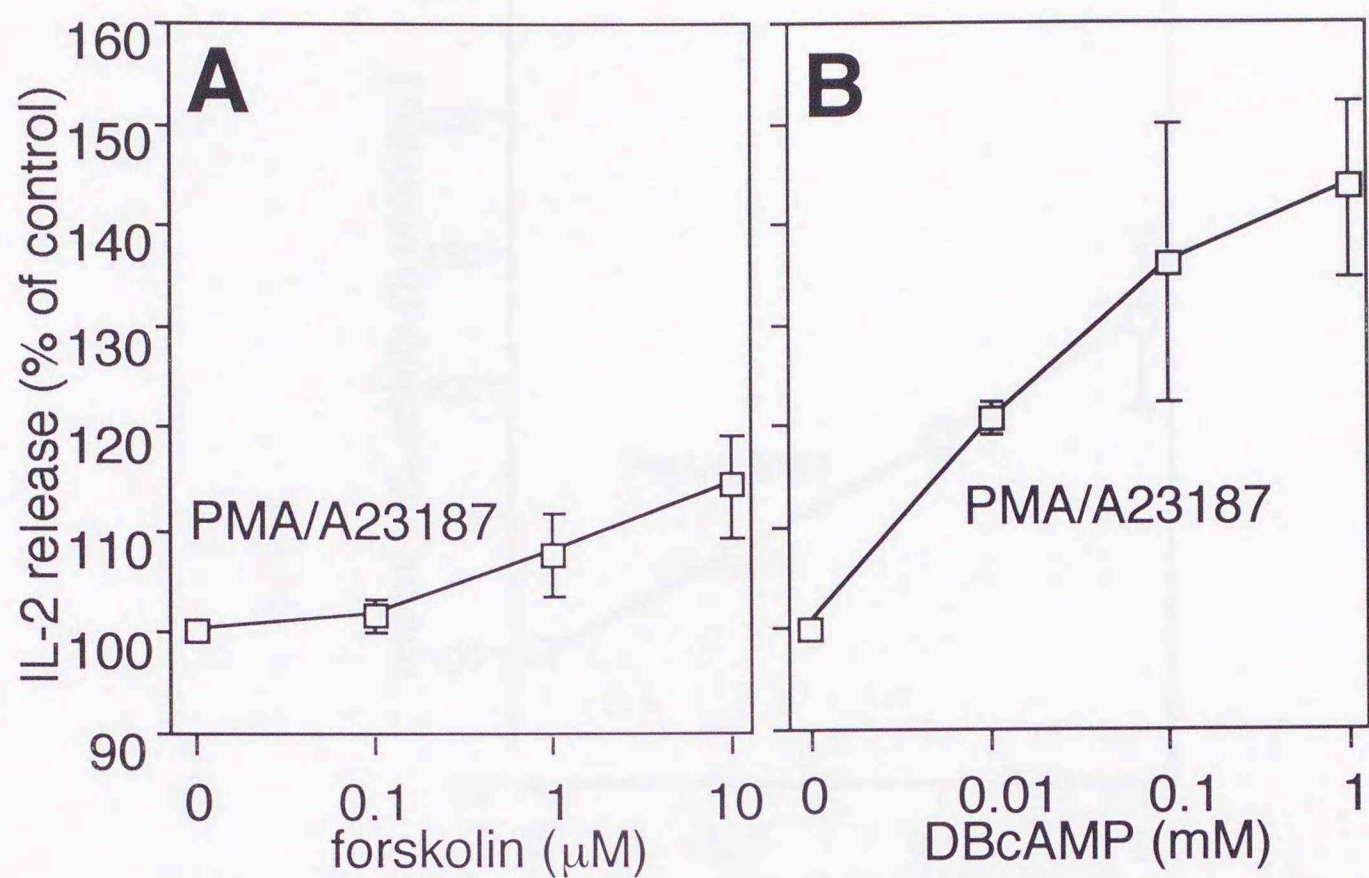


Fig. 3.1. Effects of cAMP on PMA/A23187-induced IL-2 production in

Jurkat cells Jurkat cells were pretreated with forskolin (0.1 to 10 μ M, in panel A) or DBcAMP (0.01 to 1 mM, in panel B) for 24 hr, 10 nM PMA and 500 nM A23187 were added and they were further incubated for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The amount of PMA/A23187-induced IL-2 production was estimated as 100%

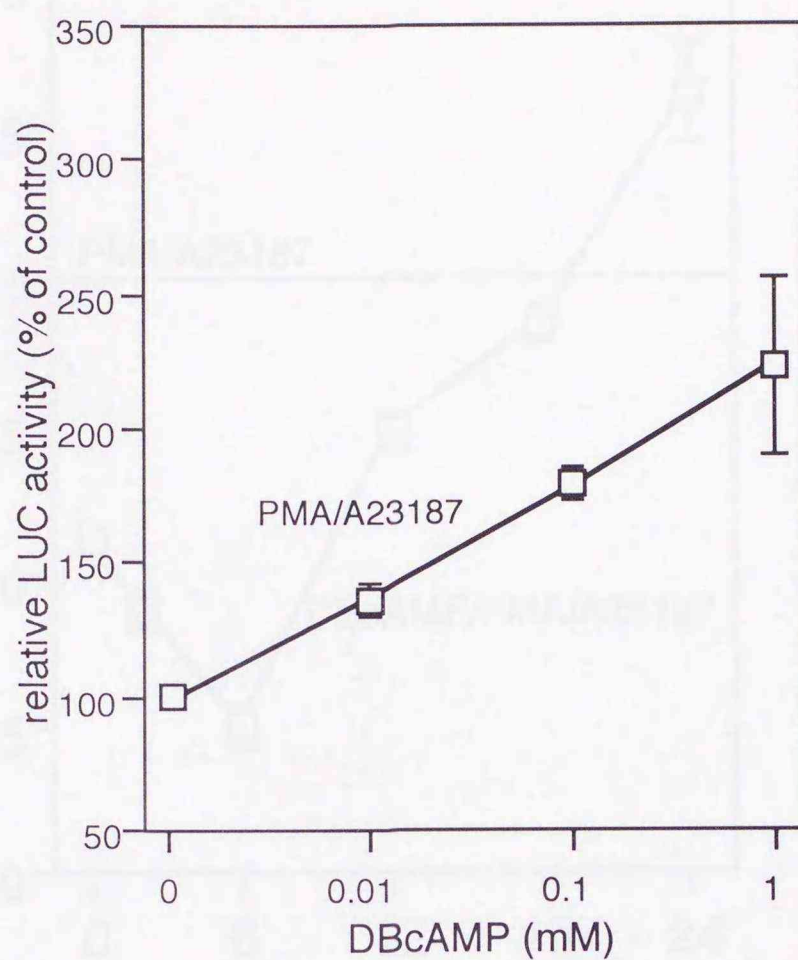


Fig. 3.2. Effect of DBcAMP on PMA/A23187-induced IL-2 promoter

activity in Jurkat cells

Jurkat cells were transfected with pIL2-LUC and were pretreated with DBcAMP (0.01 to 1 mM) for 24 hr prior to treatment with 1 nM PMA and 500 nM A23187 for 24 hr. Cells were transfected at least three times (3 - 5). The value of PMA/A23187-induced IL-2 promoter activity was estimated as 100%.

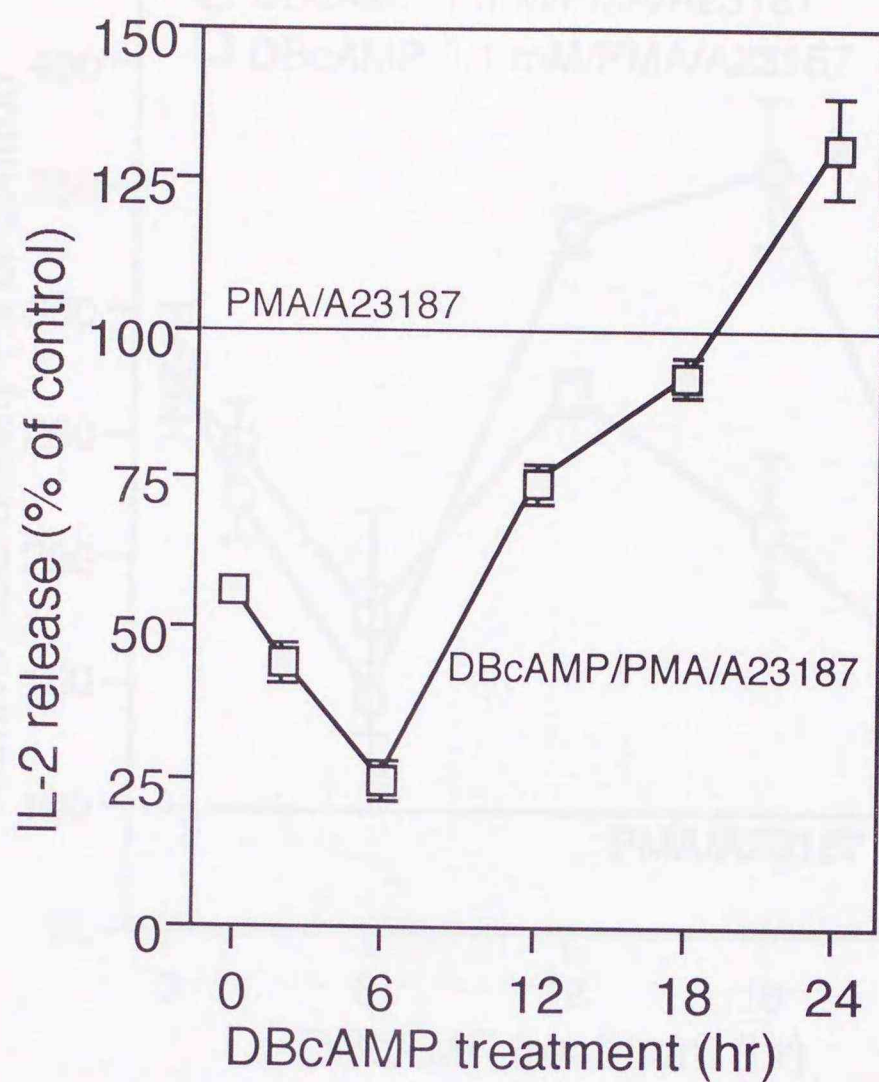


Fig. 3.3. Effect of DBcAMP on PMA/A23187-induced IL-2 production

Jurkat cells were pretreated with 1 mM DBcAMP for 0 to 24 hr prior to 10 nM PMA and 500 nM A23187 treatment for 24 hr. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The amount of PMA/A23187-induced IL-2 production was estimated as 100%

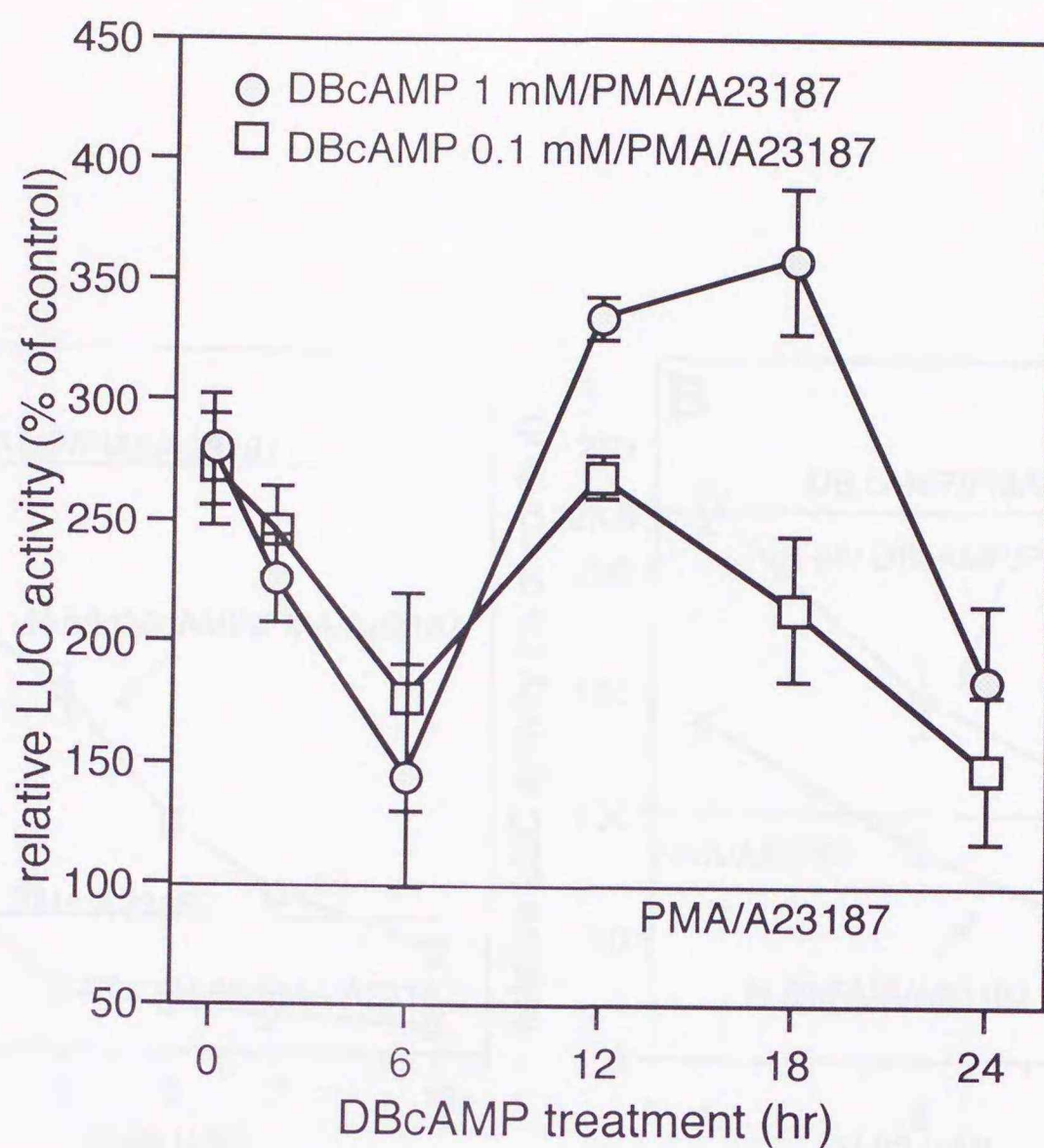


Fig. 3.4. Effects of DBcAMP on PMA/A23187-induced IL-2 promoter

activities Jurkat cells were transfected with pIL2-LUC and pretreated with 0.1 mM (■) 1 mM (●) DBcAMP for 0 to 24 hr prior to 1 nM PMA and 500 nM A23187 treatment for 24 hr. Cells were transfected three times. The value of PMA/A23187-induced IL-2 promoter activity was estimated as 100%.

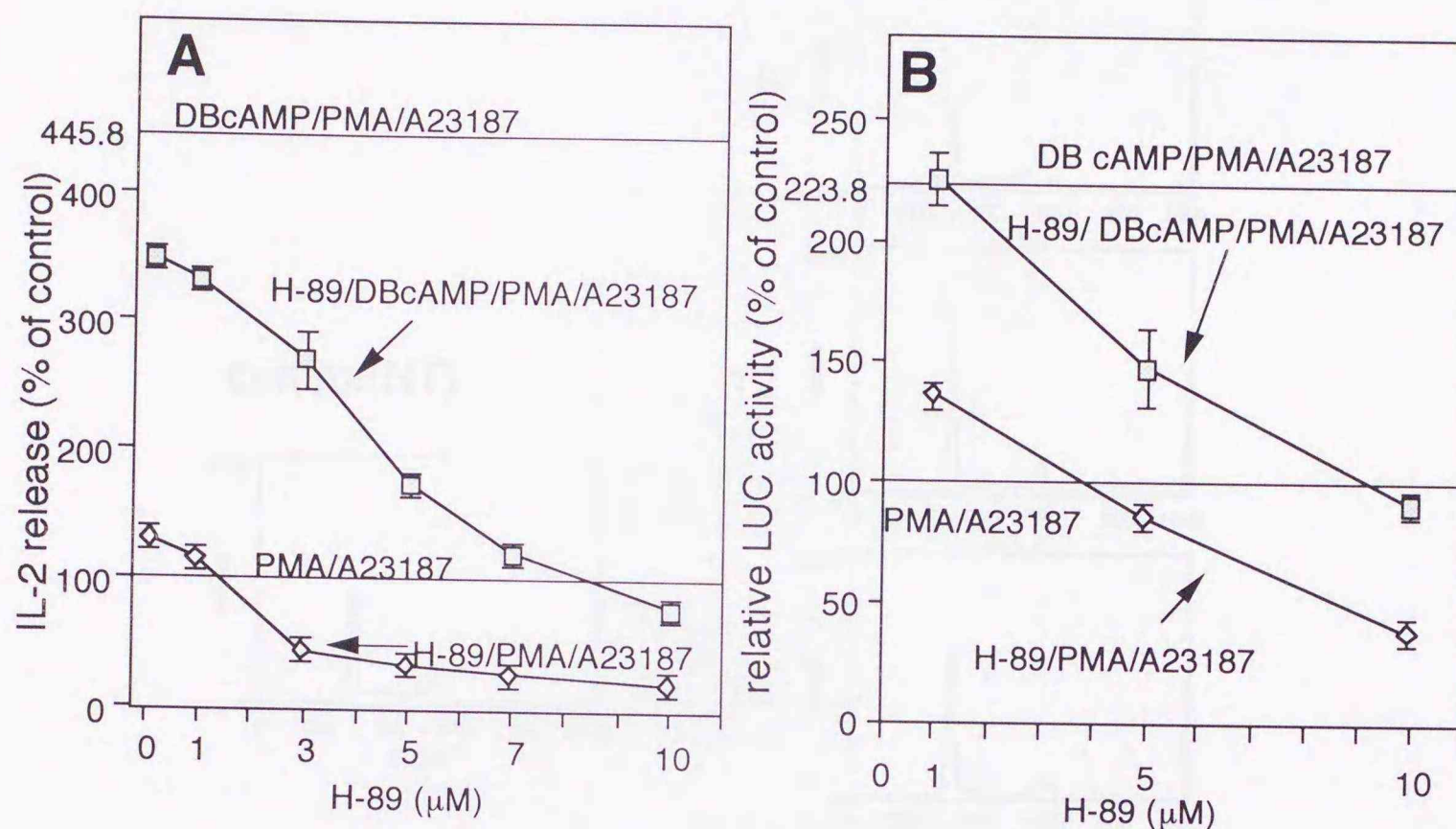


Fig. 3.5. Effects of H-89 on DBcAMP-enhanced IL-2 production and

promoter activity in Jurkat cells Cells were pretreated with H-89 (0.1 to 10

mM) for 3 hr prior to presence or absence of 1 mM DBcAMP for 24 hr followed by 1 nM

PMA and 500 nM A23187 treatment for 24 hr. A, IL-2 productions assessed by ELISA.

B, IL-2 promoter activities assessed by luciferase assay. Each point is the mean $\pm$ SE of

three assays. The amount of PMA/A23187-induced IL-2 production or the value of

PMA/A23187-induced IL-2 promoter activity was estimated as 100%.

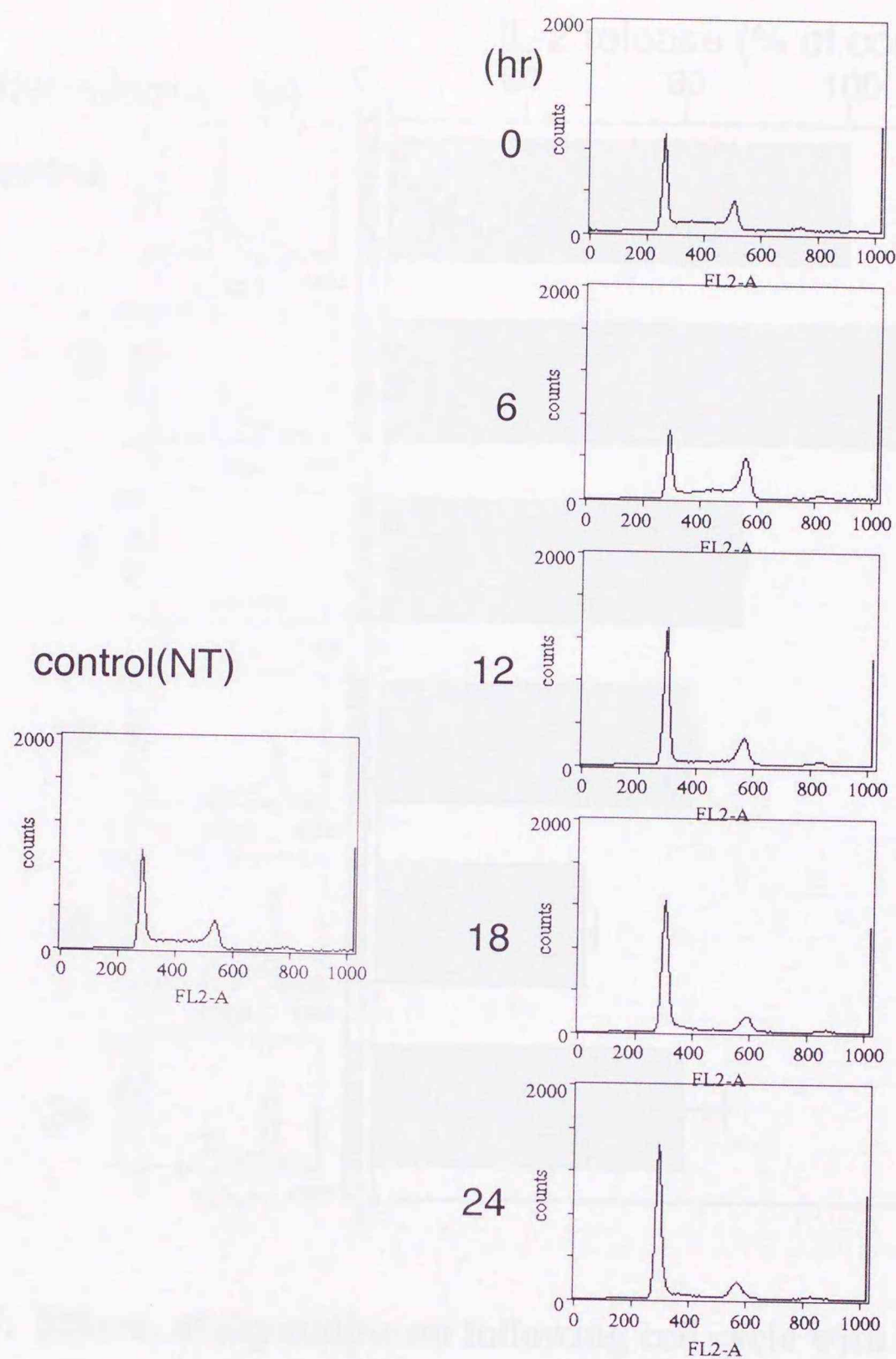


Fig. 3.6. Effect of DBcAMP on cell cycle in Jurkat cells Cells were treated with 1 mM DBcAMP for 0 to 24 hr, then flow cytometric analysis of cell cycle were done as described in the "Experimental procedures".

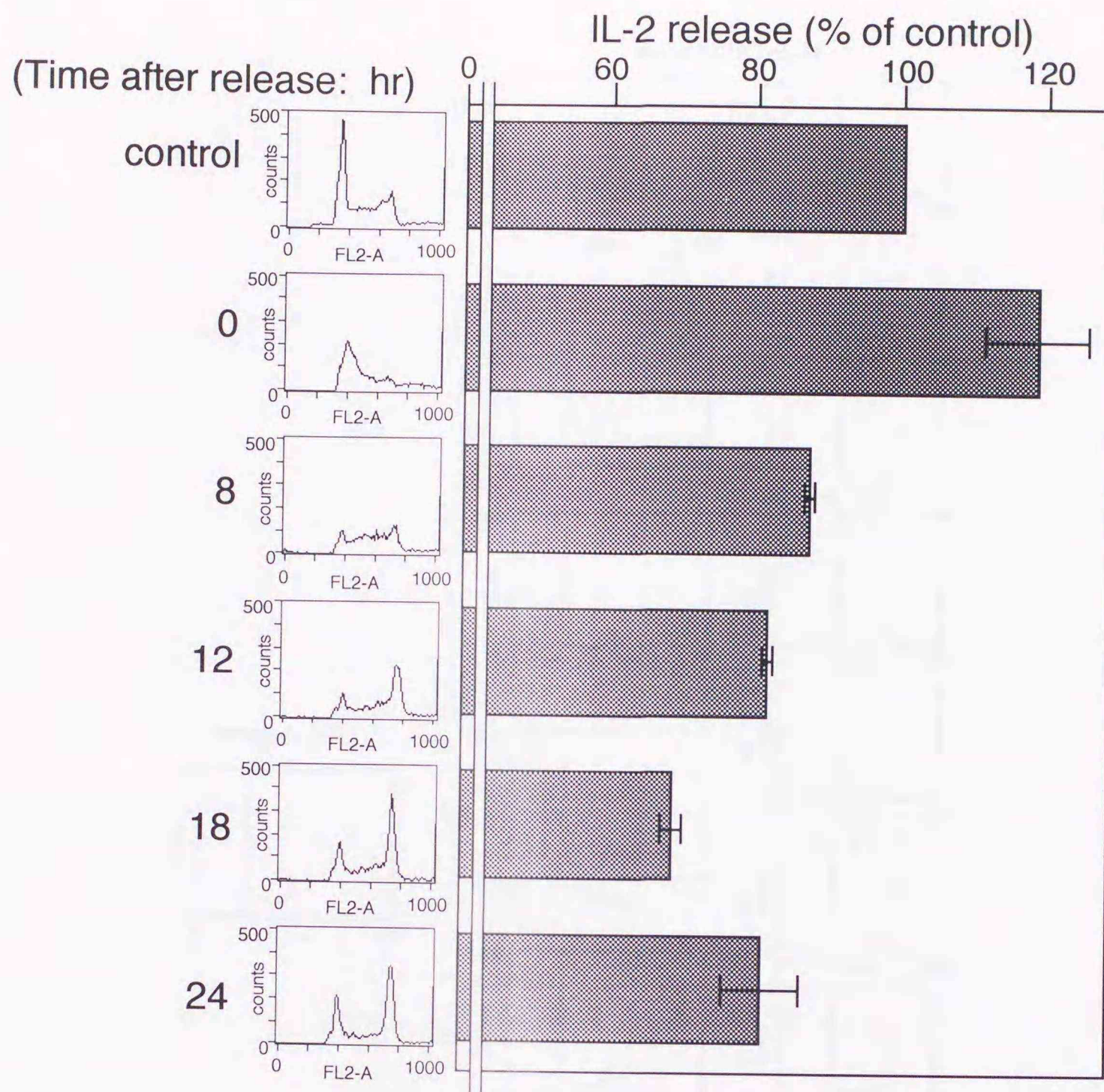


Fig. 3.7. Effects of thymidine on following cell cycle with PMA/A23187-induced IL-2 promoter activity in Jurkat cells Cells were synchronized using double-thymidine block protocol as described in the "Experimental procedures". After synchronized, cells were treated with 1 nM PMA and 500 nM A23187 for 24 hr in the several phases. The amount of IL-2 was estimated as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. Control: the randomly cultured Jurkat cells.

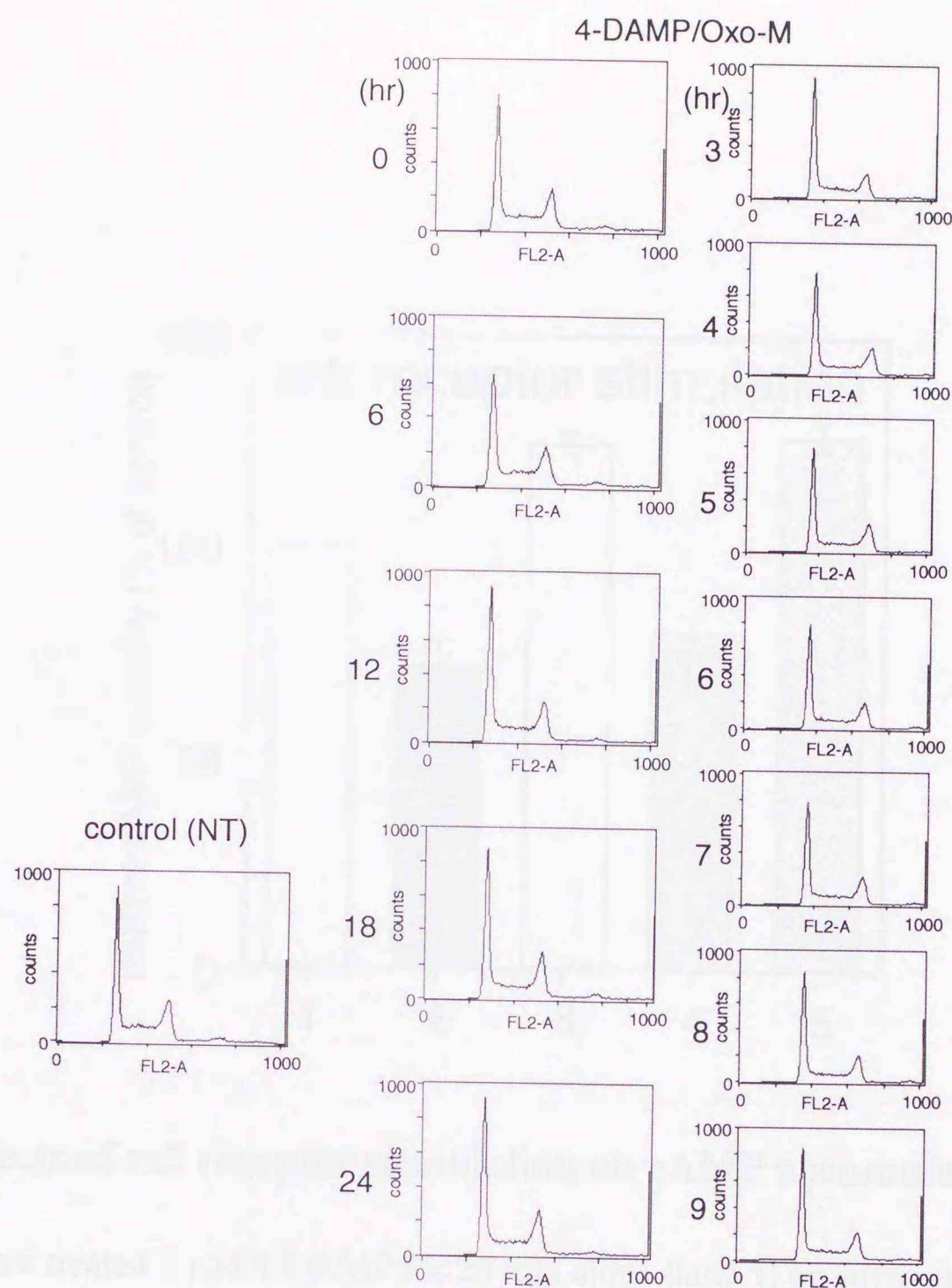


Fig. 3.8. Effect of m2 receptor stimulation on cell cycle in Jurkat cells

Cells were treated with 1 μ M 4-DAMP for 20 min then 100 μ M Oxo-M for 0 to 24 hr, then flow cytometric analysis of cell cycle were done as described in the "Experimental procedures".

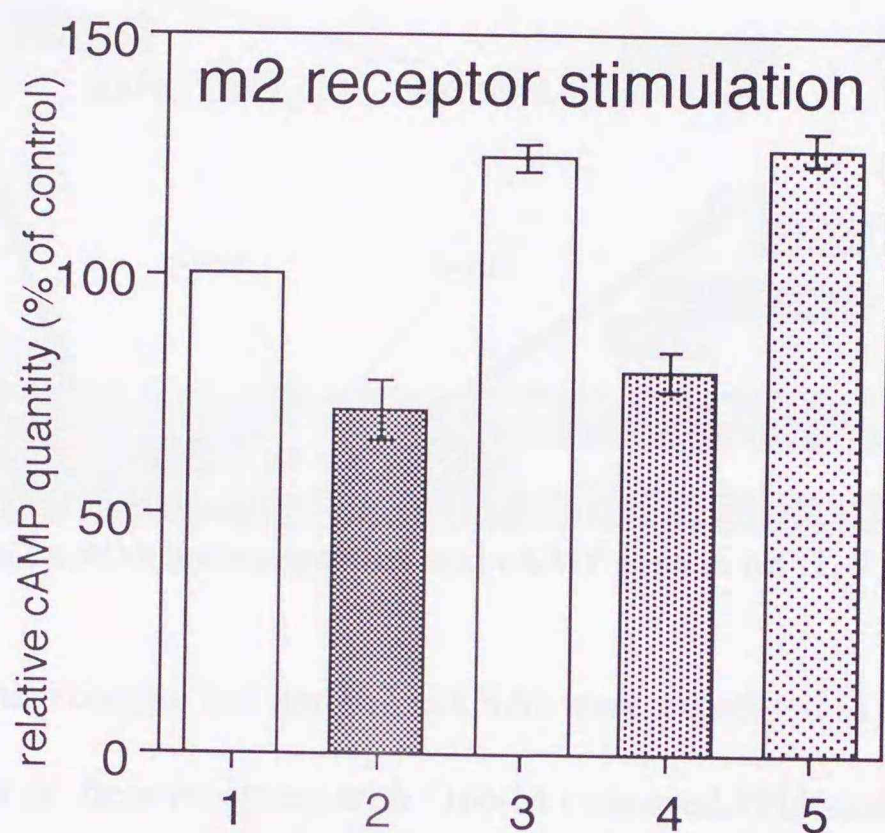
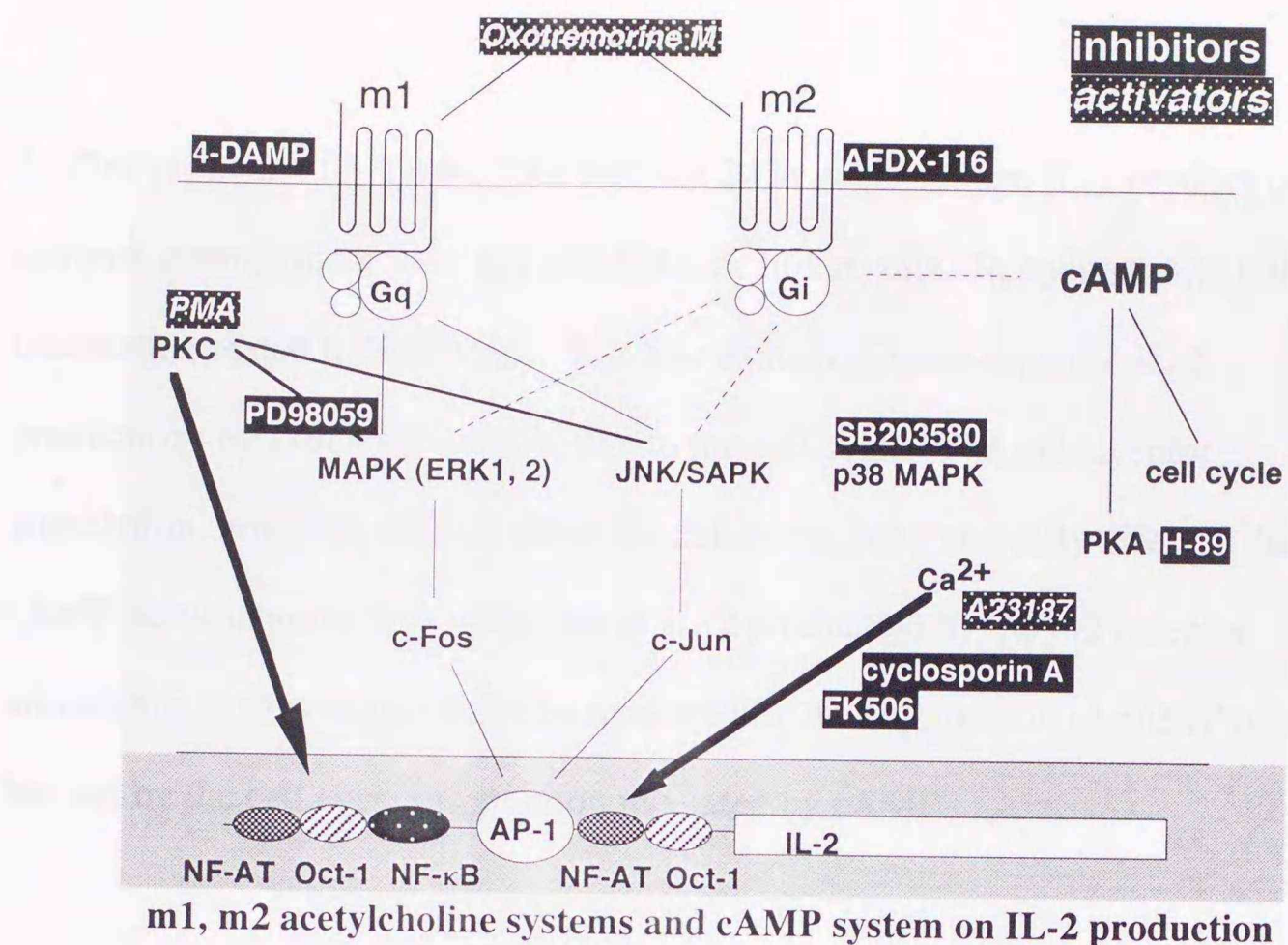


Fig. 3.9. Effects of m2 receptor stimulation on cAMP accumulation in Jurkat

cells Cells were treated 1 μ M 4-DAMP for 20 min alone (lane 1) or pretreated 1 μ M 4-DAMP for 20 min then treated 100 μ M Oxo-M for 30 min (lane 2). Cells were treated 1 μ M of 4-DAMP for 20 min then treated 1 nM PMA and 500 nM A23187 for 30 min (lane 3) or pretreated 1 μ M of 4-DAMP for 20 min then treated 100 μ M Oxo-M and 1 nM PMA and 500 nM A23187 for 30 min (lane 4). Cells were treated 1 μ M 4-DAMP for 20 min and treated 100 μ M Oxo-M for 18 hr, then added 1 nM PMA and 500 nM A23187 for 30 min (lane 5). The amount of cAMP was assessed as described in the "Experimental procedures". Each point is the mean $\pm$ SE of three assays. The activity of 4-DAMP was the same value as vehicle and estimated as 100%.

General conclusions



1. Muscarinic receptor m1 and m2 mRNAs were expressed in hPBL and pretreatment of these receptors with Oxo-M enhanced PHA-induced IL-2 production. Oxo-M-induced enhancement of IL-2 production may not be due to [Ca²⁺]_i elevation. Stimulatory effects of Oxo-M on IL-2 production could be due to the intracellular signaling mechanism.

2. a) Jurkat cells expressed m1 and m2 receptor mRNAs.

Stimulation of m1 receptors enhanced PMA/A23187-induced IL-2 production and IL-2 promoter activity. AP-1 could be involved in this enhancement and both MAPK and JNK also seem to be involved.

b) Pretreatment of m2 receptors for 18 hr decreased PMA/A23187-induced IL-2 production and promoter activity. AP-1 binding activity and MAPK and JNK activities were decreased by m2 receptor stimulation compared to those in the control cells. m2 receptors stimulation with simultaneous PMA/A23187 treatment decreased AP-1 and NF-κB binding activities.

3. Pretreatment with 1 mM DBcAMP for 24 hr enhanced the IL-2 production, in contrast, pretreatment for 6 hr inhibited it in Jurkat cells. In addition, the DBcAMP treatment arrested the cell cycle. The mechanism of these biphasic IL-2 productions by DBcAMP may be due to the cell cycle. The m2 receptor stimulation, however, did not affect the cell cycle, but transiently affected the cAMP accumulation. The inhibition of IL-2 production by the m2 receptor stimulation is thus suggested to be mediated by the suppression of cAMP pathways, but not by the cell cycle progression mediated by cAMP.

References

1. Bonner, T. I., A. C. Young, M. R. Brann, and N. J. Buckley. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1**: 403-410 (1988).
2. Fujino, H., Y. Kitamura, T. Yada, T. Uehara, and Y. Nomura. Stimulatory roles of muscarinic acetylcholine receptors on T cell antigen receptor/CD3 complex-mediated interleukin-2 production in human peripheral blood lymphocytes. *Mol. Pharmacol.* **51**: 1007-1014 (1997).
3. Rabey, J. M., L. Shaenkman, and G. M. Gilad. Cholinergic muscarinic binding by human lymphocytes: changes with aging, antagonist treatment, and senile dementia of the Alzheimer type. *Ann. Neurol.* **20**: 628-631 (1986).
4. Arnaiz-Villena, A., M. Timo, C. Rodriguez-Gallego, M. Perez-Blas, A. Corell, J. M. Martin-Villa, and J. R. Regueiro. Human T-cell activation deficiencies. *Immunol. Today* **13**: 259-265 (1992).
5. Bulloch, K. Neuroanatomy of lymphoid tissue: a review, in *Neural Modulation of Immunity* (Guillemin et al., eds). Raven Press, New York, 111-141 (1985).
6. Kammer, G. M. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. *Immunol. Today* **9**: 222-229 (1988).
7. Strom, B. T., A. M. Lane, and K. George. The parallel, time-dependent, bimodal change in lymphocyte cholinergic binding activity and cholinergic influence upon lymphocyte-mediated cytotoxicity after lymphocyte activation. *J. Immunol.* **127**: 705-710 (1981).
8. Morecki, S., S. L. Topalian, W. W. Myers, D. Okrongly, T. B. Okarma, and S. A. Rosenberg. Separation and growth of human CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes and peripheral blood mononuclear cells by direct positive

- panning on covalently attached monoclonal antibody-coated flasks. *J. Biol. Response Modif.* **9**: 463-474 (1990).
9. Grynkiewicz, G., M. Poenie, and Y. R. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**: 3440-3450 (1985).
 10. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159 (1987).
 11. Baumgartner, M., K. J. Wei, and R. S. Aronstam. Retinoic acid-induced differentiation of a human neuroblastoma cell line alters muscarinic receptor expression. *Dev. Brain Res.* **72**: 305-308 (1993).
 12. Sussman, D. J., and G. Milman. Short-term, high-efficiency expression of transfected DNA. *Mol. Cell. Biol.* **4**: 1641-1643 (1984).
 13. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475-1489 (1983).
 14. Westin, G., T. Gerster, M. M. Muller, G. Schaffner, and W. Schaffner. OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts. *Nucleic Acids Res.* **15**: 6787-6798 (1987).
 15. Northrop, J. P., S. H. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* **369**: 497-502 (1994).
 16. Chen, R.-H., and J. Blenis. Identification of *Xenopus* S6 protein kinase homologs (pp90^{sk}) in somatic cells; phosphorylation and activation during initiation of cell proliferation. *Mol. Cell. Biol.* **10**: 3204-3215 (1990).
 17. Rao, P. N., and R. T. Johnson. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* **225**: 159-164 (1970).

18. Hadden, J. W., and R. G. Coffey. Early biochemical events in T-lymphocyte activation by mitogens, in *Immunopharmacology Reviews: Volume 1* (J. W. Hadden and A. Szentivanyi, eds.). Plenum Press, New York, 273-376 (1990).
19. Abraham, R. T., L. M. Karnitz, J. P. Secrist, and P. J. Leibson. Signal transduction through the T-cell antigen receptor. *Trends Biochem. Sci.* **17**: 434-438 (1992).
20. Downward, J., J. Graves, and D. Cantrell. The regulation and function of p21^{ras} in T cells. *Immunol. Today* **13**: 89-92 (1992).
21. Schreiber, S. L. Chemistry and biology of the immunophilines and their immunosuppressive ligands. *Science* **251**: 283-287 (1991).
22. Schreiber, S. L., and G. R. Crabtree. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13**: 136-142 (1992).
23. Clipstone, N. A., and G. R. Crabtree. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**: 695-697 (1992).
24. Kitamura, Y., T. Arima, Y. Kitayama, and Y. Nomura. Regulation of $[Ca^{2+}]_i$ rise activated by doxepin-sensitive H₁-Histamine receptors in Jurkat cells, cloned human T lymphocytes. *Gen. Pharmac.* **27**: 285-291 (1996).
25. Kaneda, T., Y. Kitamura, and Y. Nomura. Presence of m3 subtype muscarinic acetylcholine receptors and receptor-mediated increases in the cytoplasmic concentration of Ca^{2+} in Jurkat, a human leukemic helper T lymphocyte line. *Mol. Pharmacol.* **43**: 356-364 (1993).
26. Jhon, D.-Y., H.-H. Lee, D. Park, C.-W. Lee, K.-H. Lee, O. J. Yoo, and S. G. Rhee. Cloning, sequencing, purification, and Gq-dependent activation of phospholipase C- β 3. *J. Biol. Chem.* **268**: 6654-6661 (1993).
27. Asaoka, Y., S. Nakamura, K. Yoshida, and Y. Nishizuka. Protein kinase C, calcium and phospholipid degradation. *Trends Biochem. Sci.* **17**: 414-417 (1992).

28. Novak, T. J., and E. V. Rothenberg. cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells. *Proc. Natl. Acad. Sci. USA* **87**: 9353-9357 (1990).
29. Tamir, A., and N. Isakov. Cyclic AMP inhibits phosphatidylinositol-coupled and -uncoupled mitogenic signals in T lymphocytes. *J. Immunol.* **152**: 3391-3399 (1994).
30. Tsuruta, L., H. J. Lee, E. S. Masuda, N. K. Nakagawa, N. Arai, K. Arai, and T. Yokota. Cyclic AMP inhibits expression of the IL-2 gene through the nuclear factor of activated T cells (NF-AT) site, and transfection of NF-AT cDNAs abrogates the sensitivity of EL-4 cells to cyclic AMP. *J. Immunol.* **154**: 5255-5264 (1995).
31. Taniguchi, T., and Y. Minami. The IL-2/IL-2 receptor system: a current overview. *Cell* **73**: 5-8 (1993).
32. Durand, D. B., M. R. Bush, J. G. Morgan, A. Weiss, and G. R. Crabtree. A 275 bp fragment at the 5' end of the interleukin 2 gene enhances expression from the T cell antigen receptor. *J. Exp. Med.* **165**: 395-407 (1987).
33. Fujita, T., H. Shibuya, T. Ohashi, K. Yamanishi, and T. Taniguchi. Regulation of human IL-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**: 401-405 (1986).
34. Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. *EMBO J.* **8**: 465-473 (1989).
35. Durand, D. B., J. P. Shaw, M. R. Bush, R. E. Replogle, R. Belageje, and G. R. Crabtree. Characterization of antigen receptor response elements within the interleukin 2 enhancer. *Mol. Cell. Biol.* **8**: 1715-1724 (1988).
36. Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J. M. Musacchio, G. D.

- Plowman, B. Ruby, and J. Schlessinger. Protein tyrosine kinase PYK2 involved in Ca^{2+} -induced regulation of ion channel and MAP kinase functions. *Nature* **376**: 737-745 (1995).
37. van Biesen, T., B. E. Hawes, D. K. Luttrell, K. M. Krueger, K. Touhara, E. Porfiri, M. Sakaue, L. M. Luttrell and R. J. Lefkowitz. Receptor-tyrosine-kinase- and Gbg-mediated MAP kinase activation by a common signalling pathway. *Nature* **376**: 781-784 (1995).
 38. Mattingly, R. R., and I. G. Macara. Phosphorylation-dependent activation of the Ras-GRF/CDC25^{Mm} exchange factor by muscarinic receptors and G-protein $\beta\gamma$ subunits. *Nature* **382**: 268-272 (1996).
 39. Winitz, S., M. Russell, N.-X. Qian, A. Gardner, L. Dwyer, and G. L. Johnson. Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.* **268**: 19196-19199 (1993).
 40. Crabtree, G. R. Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**: 355-361 (1989).
 41. Uehara, Y., and H. Fukuzawa. Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* **201**: 370-379 (1991).
 42. Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J. Biol. Chem.* **270**: 27489-27494 (1995).
 43. Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young, and J. C. Lee. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**: 229-233 (1995).
 44. Flanagan, W. M., B. Corthesy, R. J. Barm, and G. R. Crabtree. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A.

- Nature* **352**: 803-807 (1991).
45. Liu, J., J. D. Farmer, W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complex. *Cell* **66**: 807-815 (1991).
 46. Henney, C. S., L. M. Lichtenstein, E. Gillespie, and R. T. Rolley. In vivo suppression of the immune response to alloantigen by cholera enterotoxin. *J. Clin. Invest.* **52**: 2853-2857 (1973).
 47. Leung, K. H., and E. Mihich. Prostaglandin modulation of development of cell-mediated immunity in culture. *Nature* **288**: 597-600 (1980).
 48. Chouaib, S., K. Welte, R. Mertelsmann, and B. Dupont. Prostaglandin E2 acts at two distinct pathways of T lymphocyte activation: inhibition of IL-2 production and down-regulation of transferring receptor expression. *J. Immunol.* **135**: 1172-1179 (1985).
 49. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. Suppression of human T cell mitogenesis by prostaglandin: existence of a prostaglandin-producing suppressor cell. *J. Exp. Med.* **146**: 1719-1734 (1977).
 50. Gilbert, K. M., and M. K. Hoffman. cAMP is an essential signal in the induction of antibody production by B cells but inhibits helper function of T cells. *J. Immunol.* **135**: 2084-2089 (1985).
 51. DosReis, G. A., A. F. Nobrega, and R. P. de Carvalho. Purinergic modulation of T lymphocyte activation: differential susceptibility of distinct activation steps and correlation with intracellular 3', 5'-cyclic adenosine monophosphate accumulation. *Cell. Immunol.* **101**: 213-231 (1986).
 52. Lerner, A., B. Jacobson, and R. Miller. Cyclic AMP concentrations modulate both calcium flux and hydrolysis of phosphatidylinositol phosphates in mouse T lymphocytes. *J. Immunol.* **140**: 936-940 (1988).
 53. Bismuth, G., I. Theodorou, H. Gouy, S. Le Gouvello, A. Bernard, and P. Debre.

- Cyclic AMP-mediated alteration of the CD2 activation process in human T lymphocytes. Preferential inhibition of the phosphoinositide cycle-related transduction pathway. *Eur. J. Immunol.* **18**: 1351-1357 (1988).
54. Granja, C., L.-L. Lin, E. J. Yunis, V. Relias, and J. D. Dasgupta. PLC γ 1, a possible mediator of T cell receptor function. *J. Biol. Chem.* **266**: 16277-16280 (1991).
55. Patel, M. D., L. E. Samelson, and R. D. Klausner. Multiple kinase and signal transduction: phosphorylation of the T cell antigen receptor complex. *J. Biol. Chem.* **262**: 5831-5838 (1987).
56. Klausner, R. D., J. J. O'Shea, H. Luong, P. Ross, J. A. Bluestone, and L. E. Samelson. T cell receptor tyrosine phosphorylation, variable coupling for different ligands. *J. Biol. Chem.* **262**: 12654-12659 (1987).
57. Johnson, K. W., B. H. Davis, and K. A. Smith. cAMP antagonizes IL-2-promoted T cell cycle progression at a discrete point in early G1. *Proc. Natl. Acad. Sci. USA* **85**: 6072-6076 (1988).
58. Isakov, N., and A. Altman. Tumor promoters in conjunction with calcium ionophores mimic antigenic stimulation by reactivation of alloantigen-primed T lymphocytes. *J. Immunol.* **135**: 3674-3680 (1985).
59. Takayama, H., G. Trenn, and M. V. Sitkovsky. Locus of inhibitory action of cAMP-dependent protein kinase in the antigen receptor-triggered cytotoxic T lymphocyte activation pathway. *J. Biol. Chem.* **263**: 2330-2336 (1988).
60. Link, D. S., M. A. Chan, and E. W. Gelfand. Increased cyclic adenosine monophosphate levels block progression but not initiation of human T cell proliferation. *J. Immunol.* **145**: 449-455 (1990).
61. Tamir, A., and N. Isakov. Increased intracellular cyclic AMP levels block PKC-mediated T cell activation by inhibition of c-jun transcription. *Immunol. Lett.* **27**: 95-99 (1991).

62. Oksenberg, D., J. R. Oksenberg, K. Sakai, S. J. Peroutka, and L. Steinman. Cyclic adenosine 3', 5'-monophosphate metabolism in activated T-cell clones. *Immunology* **67**: 484-488 (1989).
63. Dumont, J. E., J. C. Jauniaux, P. P. Roger. The cyclic AMP-mediated stimulation of cell proliferation. *TIBS* **14**: 67-71 (1989).
64. Hamilton, J. A. Glucocorticoids and prostaglandins inhibits the induction of macrophage DNA synthesis by macrophage growth factor and phorbol ester. *J. Cell. Physiol.* **115**: 67-74 (1983).
65. Rock, C. O., J. L. Cleveland, and S. Jackowski. Macrophage growth arrest by cyclic AMP defines a distinct checkpoint in the mid-G1 stage of the cell cycle and overrides constitutive *c-myc* expression. *Mol. Cell. Biol.* **12**: 2351-2358 (1992).
66. Vario, G., S. Argyriou, A. M. Bordum, G. Whitty, and J. A. Hamilton. Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP: lack of effect on early responses to colony stimulating factor-1. *J. Biol. Chem.* **265**: 2692-2701 (1990).
67. Kato, J., M. Matsuoka, K. Polyak, J. Massague, and C. J. Sherr. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27^{kip1}) of cyclin-dependent kinase 4 activation. *Cell* **79**: 487-496 (1994).
68. Smith, K. A. Interleukin 2: inception, impact and implications. *Science* **240**: 1169-1176 (1988).
69. Cantrell, D. A., and K. A. Smith. The interleukin-2 T-cell system: a new cell growth model. *Science* **224**: 1312-1316 (1984).
70. Crabtree, G. R. Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**: 355-361 (1989).
71. Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal

- human lymphocytes. *Proc. Natl. Acad. Sci. USA* **83**: 3982-3986 (1986).
72. Ullman, K. S., J. P. Northrop, C. L. Verweij, and G. R. Crabtree.
Transmission of signals from the T lymphocyte antigen receptor to the genes
responsible for cell proliferation and immune function: the missing link. *Annu.
Rev. Immunol.* **8**: 421-452 (1990).
73. Parker, E. M., K. Kameyama, T. Higashijima, and E. M. Ross.
Reconstitutively active G protein-coupled receptors purified from baculovirus-
infected insect cells. *J. Biol. Chem.* **266**: 519-527 (1991).
74. Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz. The GTP-binding
protein, Go, regulates neuronal calcium channels. *Nature* **325**: 445-447
(1987).
75. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu.
Rev. Biochem.* **56**: 615-649 (1987).
76. Tang, W.-J., and A. G. Gilman. Adenylyl cyclases. *Cell* **70**: 869-872
(1992).
77. Camps, M., A. Carozzi, P. Schnabel, A. Scheer, P. J. Parker, and P. Gierschik.
Isozyme-selective stimulation of phospholipase C- β 2 by G protein $\beta\gamma$ -subunits.
Nature **360**: 684-686 (1992).

