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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

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SUMMARY

Recent studies have demonstrated the presence and the regulatory function of several neurotransmitters in the immune system. In the present study, we examined the presence of acetylcholine receptors, using pharmacological and molecular biological assays, and their transmembrane control and functions, using a biochemical assay, in a cloned human leukemic helper T lymphoma cell line, Jurkat. Several muscarinic agonists, such as acetylcholine, carbachol, muscarine, and oxotremorine-M (Oxo-M), at 100 μM caused a transient elevation of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), in contrast to the tonic elevation of $[Ca^{2+}]_i$ induced by 10 μg/ml phytohemagglutinin (PHA). It appeared that the elevation induced by Oxo-M, the most potent $[Ca^{2+}]_i$ elevator, was more effectively inhibited by *p*-fluorohexahydrosiladifenidol hydrochloride (*p*-F-HHSiD) and 4-diphenylacetoxy-*N*-methylpiperidine methiodine than by pirenzepine and 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*] [1,4]benzodiazepine-6-one (AF-DX 116), suggesting that a pharmacological M3 subtype of muscarinic receptors is involved in the elevation of $[Ca^{2+}]_i$. Northern blot analysis showed that the m3 type of receptors are expressed in Jurkat cells. Scatchard analysis of [^{3}H]quinuclidinyl benzilate binding to intact cells indicated a K_d of 14.1 nM and a B_{max} of 45,370 binding sites/cell. [^{3}H]Quinuclidinyl benzilate binding to cell membranes was also inhibited by *p*-F-HHSiD rather than by pirenzepine and AF-DX 116. Oxo-M induced formation of inositol trisphosphate, and 5′-*O*-(2-thio)diphosphate inhibited the formation. Cholera toxin treatment inhibited the PHA-induced $[Ca^{2+}]_i$ rise but did not affect the Oxo-M-induced rise. Neither pertussis nor botulinus (type C) toxin affected the rise induced by Oxo-M or PHA. Thus, bacterial toxin-insensitive GTP-binding proteins seem to be involved in the Oxo-M-induced increase in $[Ca^{2+}]_i$. Treatment with 12-*O*-tetradecanoylphorbol 13-acetate abolished the Oxo-M-induced $[Ca^{2+}]_i$ rise but did not affect that induced by PHA. m_3 Muscarinic receptors thus appear to cause Ca^{2+} mobilization from intracellular stores via bacteria toxin-insensitive GTP-binding proteins, phospholipase C activation, and inositol trisphosphate formation in Jurkat cells. Protein kinase C seems to negatively modulate the m_3 receptor system.

Recently, several studies suggested that the nervous system controls immune functions (1, 2). As morphological evidence, electron microscopic studies demonstrate that the vagus nerves exist in lymphoid tissues such as spleen and thymus and that nerve terminals form synaptic contacts with lymphocytes (3). By methods of radioligand binding, lymphocytes have been shown to possess various receptors for neurotransmitters and neuropeptides, such as the β-adrenoceptor (4), muscarinic ACh receptor (5, 6), 5-hydroxytryptamine$_2$ receptor (7), and type B cholecystokinin receptor (8), and a muscarinic receptor or several receptors mediate changes in the levels of cGMP in lymphocytes (9). In contrast, it is known that the system of β-adrenoceptor-G_S-adenylate cyclase in lymphocytes involves inhibitory regulation of IL-2 production and T cell proliferation (10). Although β-adrenergic receptor systems have been extensively investigated, detailed mechanisms of the muscarinic ACh

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ABBREVIATIONS: ACh, acetylcholine; IL-2, interleukin-2; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; G protein, GTP-binding protein; AF-DX 116, 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one; *p*-F-HHSiD, *p*-fluorohexahydrosiladifenidol hydrochloride; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodine; TCR/CD3, T cell antigen receptor/CD3 complex; McN-A343, (4-hydroxy-2-butynyl)-1-trimethylammonium *m*-chlorocarbanilate chloride; PHA, phytohemagglutinin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Gpp(NH)p, guanylylimidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; GDPβS, guanosine 5′-*O*-(2-thio)diphosphate; QNB, quinuclidinyl benzilate; IMDM, Iscove's modified Dulbecco's medium; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; CHO, Chinese hamster ovary; AM, acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Oxo-M, oxotremorine-M; InsP, inositol phosphate; PKC, protein kinase C; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate.

receptor system have never been elucidated, with respect to the intracellular signaling after activation of muscarinic receptors in lymphocytes and the immune functions of the receptor system.

Pharmacologically distinguishable forms of the muscarinic ACh receptor occur in different tissues and have been classified into M_1, M_2, and M_3 subtypes on the basis of the selectivity of novel antagonists, i.e., pirenzepine, AF-DX 116, *p*-F-HHSiD, and 4-DAMP (11, 12). Using molecular cloning techniques and sequencing analysis of cDNA, the genes corresponding to m1–m5 muscarinic receptor subtypes were identified, using the nomenclature of Bonner *et al.* (13). However, it is unknown which subtypes of muscarinic receptors may be expressed in lymphocytes.

The human leukemic T lymphocyte cell line Jurkat was first established by Gillis and Watson (14) as an IL-2-producing cell line. It has been shown that Jurkat cells possess the adenylate cyclase system involving A_2 adenosine receptors (15), prostaglandin E_2 and $F_{2\alpha}$ receptors (16), and cross-talk between the adenylate cyclase system, Ca^{2+} mobilization, and PKC (17, 18). In this cell line, stimulation of the TCR/CD3 complex is followed by a rapid rise in Ins(1,4,5)P_3 formation and in $[Ca^{2+}]_i$ (19). The finding that the TCR/CD3 complex-mediated increase in $InsP_3$ and $[Ca^{2+}]_i$ is inhibited by cholera toxin (20, 21) and GDP (22) suggests the possible involvement of G proteins in the TCR/CD3 complex-induced response (23, 24). Little has been elucidated in lymphocytes as to whether muscarinic receptors change $InsP_3$ and $[Ca^{2+}]_i$ by the same or different mechanisms of antigen-induced elevation (of $InsP_3$ and $[Ca^{2+}]_i$). In the present study, the presence of muscarinic receptors and the transmembrane signaling mechanism of receptor-mediated response were investigated in Jurkat cells.

Experimental Procedures

Materials. Fura-2/AM and synthetic Ins(1,4,5)P_3 were purchased from Dohjin (Kumamoto, Japan). Pirenzepine and AF-DX 116 (Nippon Boehringer Ingelheim) and pertussis toxin (islet-activating protein) (Kaken Pharmaceutical Co.) were generous gifts. Muscarine chloride, ACh chloride, nicotine, atropine sulfate, digitonin, heparin, and botulinus toxin (type C) (Wako); McN-A343, oxotremorine, Oxo-M, and (*RS*)-(±)-QNB (Research Biochemical Inc.); carbachol, PHA, TPA, ATP, and cholera toxin (Sigma); Gpp(NH)p, GTPγS, and GDPβS (Boehringer Mannheim); and leupeptin (Peptide Institute) were used. [^{3}H]QNB (1.6 TBq/mmol), *myo*-[2-^{3}H]inositol (473.6 GBq/mmol), and cDNA probes for human m1–m5 muscarinic receptors were from DuPont/NEN. The 3′-terminal labeling kit and [α-^{32}P]dideoxy-ATP were from Amersham. AG-1X8 was from Bio-Rad.

Cell culture and assay of the number of cells. The human leukemic T cell line Jurkat (clone JP111) was maintained in IMDM supplemented with 10% CO_2/90% air at 37°. The medium was supplied at 1:5 (v/v) every 48 hr. The cells were counted with a Coulter counter (model ZM; Coulter Electronics).

Measurement of $[Ca^{2+}]_i$. The cells were washed and resuspended in IMDM. Loading of cells with fura-2 was carried out with a 10-min incubation with 3.0 μM fura-2/AM at 37° in a water bath. The loaded cells were diluted 1:10 with IMDM and incubated further for 30 min at 37°. They were then washed by centrifugation at 200 × *g* for 3 min at room temperature. An aliquot of 1–5 × 10^6 cells was used for the autofluorescence measurement. The cell suspension in Tyrode-HEPES buffer (137 mM NaCl, 3.3 mM KH_2PO_4, 0.7 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM dextrose, 20 mM HEPES, pH 7.4) was transferred to a fluorometer cuvette housed in a thermostatted holder. Fluorescence readings were taken with a fluorescent spectrophotometer (F-2000; Hitachi) at excitation and emission wavelengths of 340/380 nm and 510 nm, respectively. After each measurement, the change in fura-2 fluorescence intensity was calibrated as $[Ca^{2+}]_i$ by the two-wavelength method described by Grynkiewicz *et al.* (25). The K_d of the fura-2/Ca^{2+} interaction was taken to be 224 nM.

Treatment with cholera toxin, pertussis toxin, botulinus toxin, digitonin, and TPA. To examine the involvement of G proteins in the Oxo-M-induced rise of $[Ca^{2+}]_i$, cells in logarithmic growth phase were pretreated with 500 ng/ml cholera toxin for 1 hr, 100 ng/ml pertussis toxin for 20 hr, or 4 μg/ml botulinus toxin (type C) for 20 hr, in the culture medium. After being washed twice with Tyrode-HEPES buffer, cells were resuspended in Tyrode-HEPES buffer (pH 7.4) with or without 1 mM $CaCl_2$ or 2 mM EGTA and the effects of Oxo-M and PHA on $[Ca^{2+}]_i$ were examined.

Permeabilized cells were prepared by incubation for 5 min with 15 μM digitonin in Tyrode-HEPES buffer (pH 7.4) containing 0.1% bovine serum albumin, 1 mM ATP, 5 μM leupeptin, 2 μM phenylmethylsulfonyl fluoride, and 0.1 mM EGTA and further incubation for 30 min after the addition of 10 volumes of Tyrode-HEPES buffer containing the same reagents without digitonin. The reaction mixture was centrifuged to obtain permeabilized cells, which were resuspended in Tyrode-HEPES buffer.

The cells were incubated in Tyrode-HEPES buffer (pH 7.4) with 100 nM TPA for 1–10 min or 20 hr at 37°. In the case of H-7 treatment,

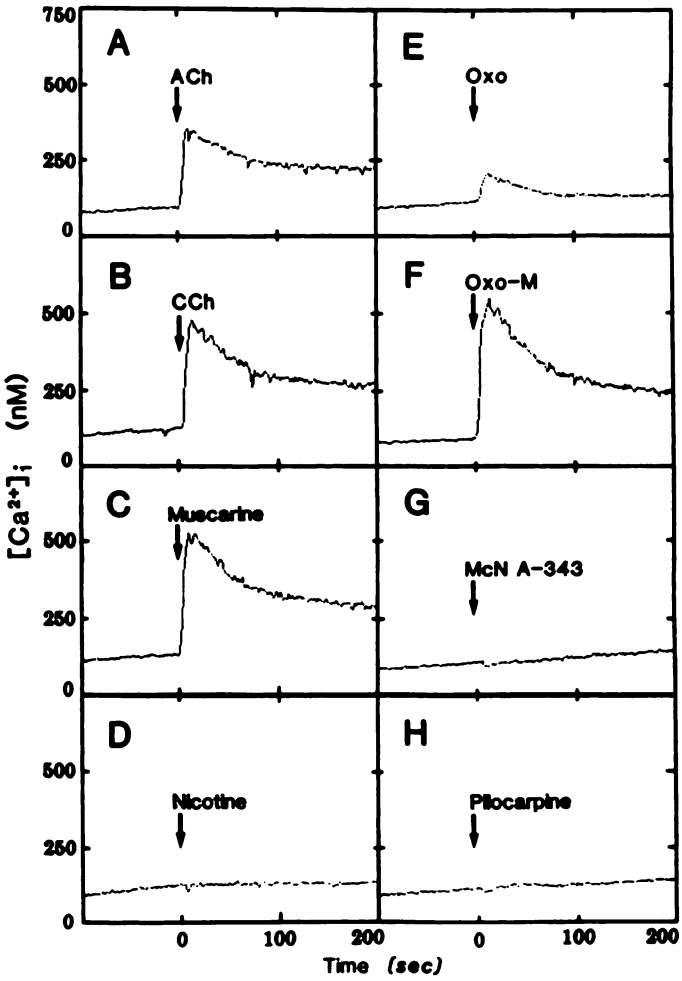

Fig. 1. Effects of cholinergic agonists on $[Ca^{2+}]_i$ rise in Jurkat cells. Time course of $[Ca^{2+}]_i$ rise was examined after addition of ACh (A), carbachol (*CCh*) (B), muscarine (C), nicotine (D), oxotremorine (*Oxo*) (E), Oxo-M (F), McN-A343 (G), or pilocarpine (H), at a final concentration of 100 μM, in Tyrode-HEPES buffer (pH 7.4). Jurkat cells were preloaded with 3.0 μM fura-2/AM for 10 min and then washed. From fluorescence intensity of fura-2, $[Ca^{2+}]_i$ was determined by the method of Grynkiewicz *et al.* (25), using a Hitachi F-2000 spectrophotomer.

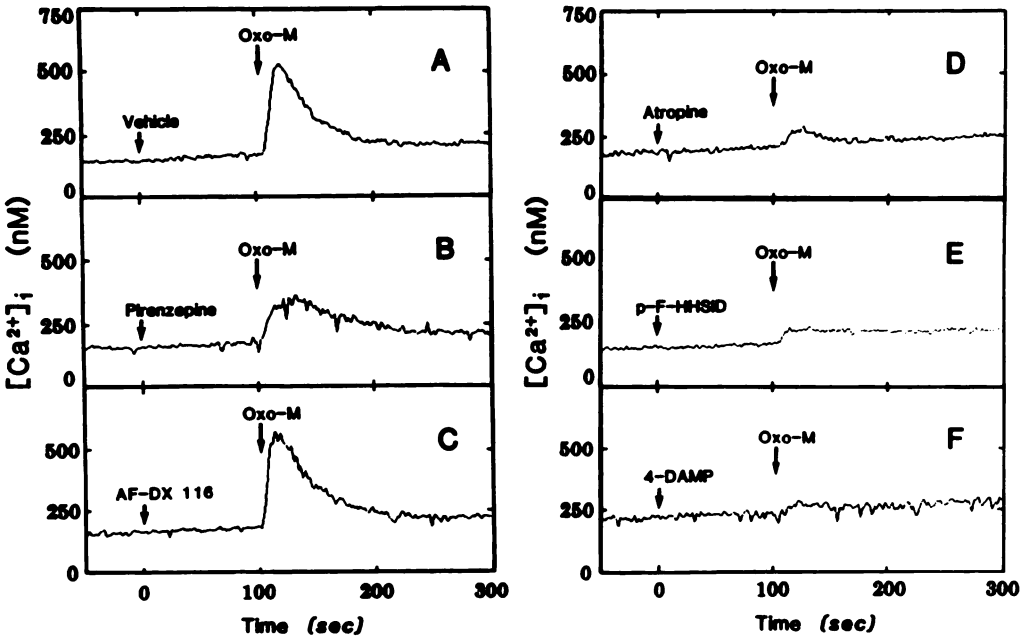

Fig. 2. Influences of pirenzepine, AF-DX 116, atropine, *p*-F-HHSiD and 4-DAMP on Oxo-M-induced $[Ca^{2+}]_i$ rise in Jurkat cells. After pretreatment with vehicle (A), 1 μM pirenzepine (B), 1 μM AF-DX 116 (C), 1 μM atropine (D), 1 μM *p*-F-HHSiD (E), or 1 μM 4-DAMP (F) for 100 sec, the effects of 1 mM Oxo-M were examined.

cells were preincubated with H-7 (100 μM) for 2 min at 37° before TPA addition.

Isolation of RNA and Northern blot analysis. Total cellular RNA was isolated by guanidium isothiocyanate/CsCl methods (26). Analysis of m1–m5 muscarinic receptor mRNA was carried out by the formaldehyde/agarose gel method, using ^{32}P-labeled probes. The 3′ ends of 48- or 49-base cDNA oligonucleotides of human m1–m5 muscarinic receptor sequence (m1, nucleotides 4-51, 721-768, and 811-858; m2, nucleotides 3-51, 681-728, and 781-828; m3, nucleotides 4-51, 721-768, and 791-838; m4, nucleotides 4-51, 841-888, and 921-968; m5, nucleotides 4-51, 671-718, and 741-788) were ^{32}P-labeled with [α-^{32}P] dideoxy-ATP, with an Amersham 3′-end labeling kit, and were hybridized. The filters obtained were exposed to Kodak X-Omat film at −80° for 2–7 days.

[^{3}H]QNB binding assay. Binding studies for quantitating muscarinic receptors were performed with intact cells (2×10^6 cells/tube) by using the muscarinic antagonist [^{3}H]QNB (0.5–30 nM). Nonspecific binding was determined in the presence of 10 μM atropine. Incubation was performed at 37° for 90 min in IMDM. In the experiment using Jurkat cell membranes (0.5–1 mg of protein/ml), membranes were incubated at 30° for 150 min with [^{3}H]QNB (0.4–4 nM) in 50 mM Tris·HCl buffer (pH 7.4) containing 100 mM NaCl. After incubation, the bound radioactivity was determined by filtration of the reaction mixture through a glass filter (Whatman GF/C) under reduced pressure. The filter was washed three times with 7.5 ml of Tyrode-HEPES buffer (for intact cells) or 50 mM Tris·HCl buffer (for membranes). The bound radioactivity on the filters was determined by liquid scintillation counting. A linear regression Scatchard plot (27) was estimated by the least squares method. Displacement curves of muscarinic antagonists were computer-analyzed by a nonlinear least squares curve-fitting procedure.

Assay of $InsP_3$ formation. The cells were incubated with *myo*-[^{3}H]inositol (4 μCi/ml) for 48 hr in IMDM and resuspended in Tyrode-HEPES buffer (pH 7.4) containing 0.1% bovine serum albumin and 10 mM LiCl. After preincubation for 10 min, cells were incubated for 10 min with test drugs at 37°. The reaction was terminated by the addition of HCl (final concentration, 0.1 N). After centrifugation (2000 × *g* for 5 min), the supernatant was applied to an AG-1X8 column to separate InsPs or $InsP_3$, as described by Berridge *et al.* (28), and radioactivity in $InsP_3$ was counted.

Results

Presence of muscarinic receptors and effects on $[Ca^{2+}]_i$ rise in Jurkat cells. Fig. 1 shows the effects of several cholinergic agonists at 100 μM on $[Ca^{2+}]_i$ in Jurkat cells. Although nicotine had no effect at all, ACh, carbachol, and muscarine induced a transient rise followed by a gradual decrease from the peak within several minutes, suggesting the presence of muscarinic ACh receptors in Jurkat cells and the involvement of the receptors in the $[Ca^{2+}]_i$ rise. Oxo-M, an agonist of M_1/M_3 muscarinic receptors, caused the greatest rise. McN-A343 (a selective M_1 agonist) (29), pilocarpine (potent M_1 agonist) (29), and oxotremorine (a potent M_2 agonist) (30) had no or only slight effect. Furthermore, to characterize the subtypes of muscarinic receptors mediating $[Ca^{2+}]_i$ elevation, the inhibitory effects of muscarinic antagonists (at 1 μM), i.e., atropine (nonselective antagonist), pirenzepine ($M_1 > M_3 \geq M_2$) (11, 31), AF-DX 116 ($M_2 \geq M_1 > M_3$) (11, 12), 4-DAMP ($M_1 \geq M_3 > M_2$) (11, 12), and *p*-F-HHSiD ($M_3 > M_1 > M_2$) (31), on Oxo-M-induced $[Ca^{2+}]_i$ rise were compared. As shown in Figs. 2 and 3A, the order of inhibitory potency of muscarinic antagonists (at 1 μM) for the Oxo-M-induced $[Ca^{2+}]_i$ rise was 4-DAMP > *p*-F-HHSiD ≥ atropine > pirenzepine > AF-DX 116. In addition, IC_{50} values for effects on the 100 μM Oxo-M-induced $[Ca^{2+}]_i$ rise were 13.2 nM [(±)-QNB], 62.6 nM (*p*-F-HHSiD), 1281 nM (pirenzepine), and >10,000 nM (AF-DX 116) (Fig. 3B). These results indicate that the pharmacologically defined M_3 subtype of muscarinic receptors exists on the Jurkat cell surface and mediates $[Ca^{2+}]_i$ rise.

To clarify which subtypes of muscarinic receptor mRNA are expressed in Jurkat cells, Northern blot analysis was carried out on total RNA extracted from Jurkat cells in a logarithmic growth phase, using ^{32}P-labeled probes of human m1–m5 muscarinic receptor cDNA. Fig. 4 shows that only the m3 subtype of mACh receptor mRNA was expressed. This result agrees with the pharmacological findings, suggesting that the M_3

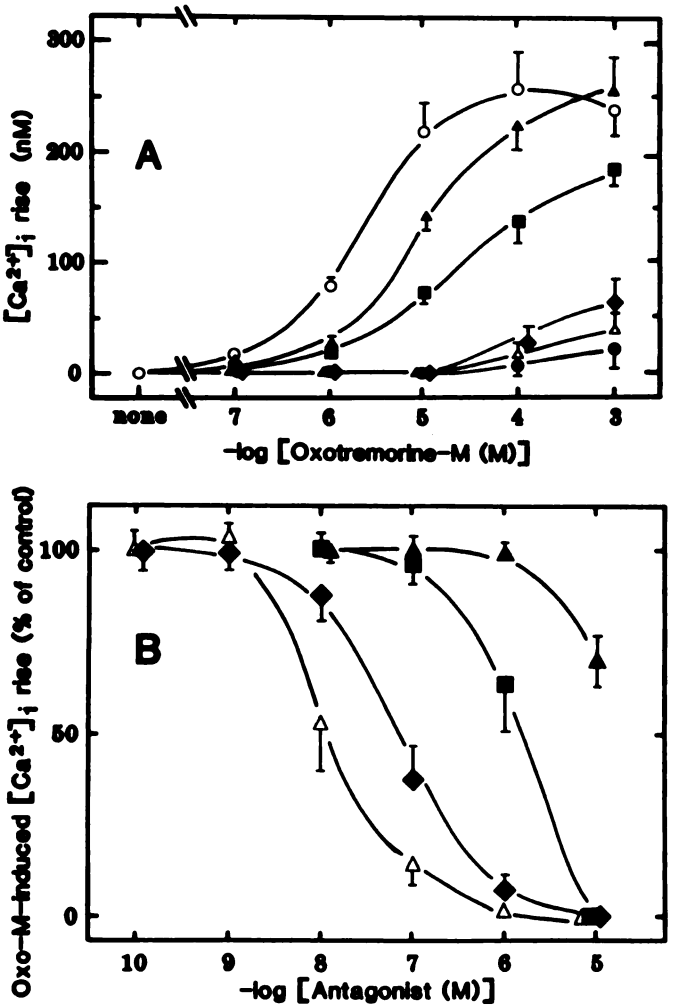

Fig. 3. Inhibitory effects of muscarinic antagonists on Oxo-M-induced $[Ca^{2+}]_i$ rise in Jurkat cells. A, After pretreatment with vehicle (○), 1 μM pirenzepine (■), 1 μM AF-DX 116 (▲), 1 μM atropine (△), 1 μM *p*-F-HHSiD (◆), or 1 μM 4-DAMP (●) for 100 sec, the effects of Oxo-M (100 nM to 1 mM) were examined in Tyrode-HEPES buffer. Each *point* shows the mean ± standard error of three to five independent experiments. B, After pretreatment with (±)-QNB (△), *p*-F-HHSiD (◆), pirenzepine (■), or AF-DX 116 (▲) at various concentrations (0.1 nM to 10 μM) for 100 sec, the effects of 100 μM Oxo-M were examined in Tyrode-HEPES buffer. Each *point* shows the mean ± standard error of triplicate determinations. IC_{50} values of the antagonists were 13.2 nM (QNB), 63.6 nM (*p*-F-HHSiD), 1281 nM (pirenzepine), and >10,000 nM (AD-DX 116). One hundred percent activity (100 μM Oxo-M-induced $[Ca^{2+}]_i$ rise) was 263 ± 17 nM.

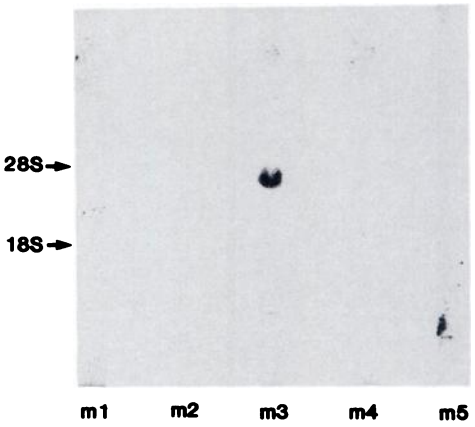

Fig. 4. Northern blot analysis of m1–m5 subtypes of muscarinic receptor mRNA extracted from Jurkat cells. Total RNA extracted by the method of Glisin *et al.* (26) was subjected to electrophoresis and then blotted to nitrocellulose membranes. Hybridization with probes of ^{32}P-labeled cDNA for m1–m5 human muscarinic receptors was carried out at 42° for 18 hr. Only the m3 subtype ACh receptor probe was hybridized. The positions of 28 S and 18 S rRNA are indicated.

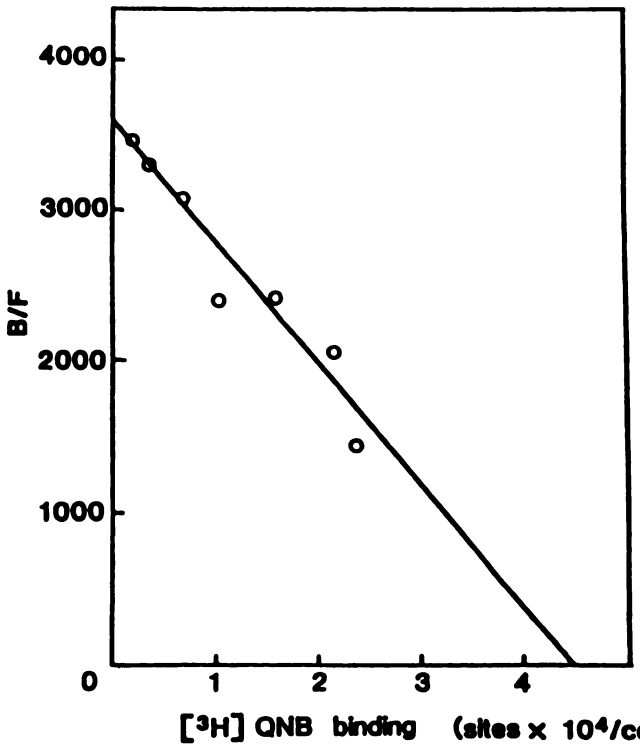

Fig. 5. Scatchard analysis of [^{3}H]QNB binding in intact Jurkat cells. Intact Jurkat cells were incubated with [^{3}H]QNB (0.5–30 nM) at 37° for 90 min. The K_d and B_{max} values were determined to be 14.1 ± 2.3 nM and 45,370 ± 5,018 sites/cell respectively, by linear regression. Each *point* shows the mean of four independent experiments.

subtype of muscarinic receptors is involved in $[Ca^{2+}]_i$ rise in Jurkat cells.

We then examined the kinetic properties of [^{3}H]QNB binding to muscarinic receptors in intact Jurkat cells. Scatchard analysis indicated the presence of [^{3}H]QNB binding sites with a K_d of 14.1 ± 2.3 nM and a B_{max} of 45,370 ± 5,018 binding sites/cell (Fig. 5). Using membranes of Jurkat cells, K_d and B_{max} for [^{3}H]QNB binding were 0.47 ± 0.12 nM and 10.6 ± 1.7 fmol/mg of protein, respectively (Fig. 6A). In addition, IC_{50} values for effects on [^{3}H]QNB binding to the membranes were 16.0 nM (*p*-F-HHSiD), 811 nM (pirenzepine), and >10,000 nM (AF-DX 116) (Fig. 6B). These inhibitory potencies agree with results on the Oxo-M-induced $[Ca^{2+}]_i$ rise (Fig. 3B).

Ca^{2+} mobilization and $InsP_3$ formation stimulated by Oxo-M. Fig. 7 shows the time course of $[Ca^{2+}]_i$ elevation induced by 100 μM Oxo-M and 10 μg/ml PHA in the absence or presence of 2 mM EGTA in the medium. The tonic effects of PHA on $[Ca^{2+}]_i$ rise were almost suppressed by eliminating external Ca^{2+} but the effect of Oxo-M was partially inhibited. Ca^{2+} influx through membrane Ca^{2+} channels and Ca^{2+} release from intracellular storage pools appear to be the main reasons for the $[Ca^{2+}]_i$ rise in response to PHA and Oxo-M, respectively.

Because Oxo-M may elevate $[Ca^{2+}]_i$ mainly by intracellular Ca^{2+} mobilization, we examined whether InsP formation is stimulated by Oxo-M. Oxo-M at 1–100 μM caused InsP formation in a concentration-dependent manner (Fig. 8). Thus, the Oxo-M-evoked $[Ca^{2+}]_i$ rise may possibly be mediated by

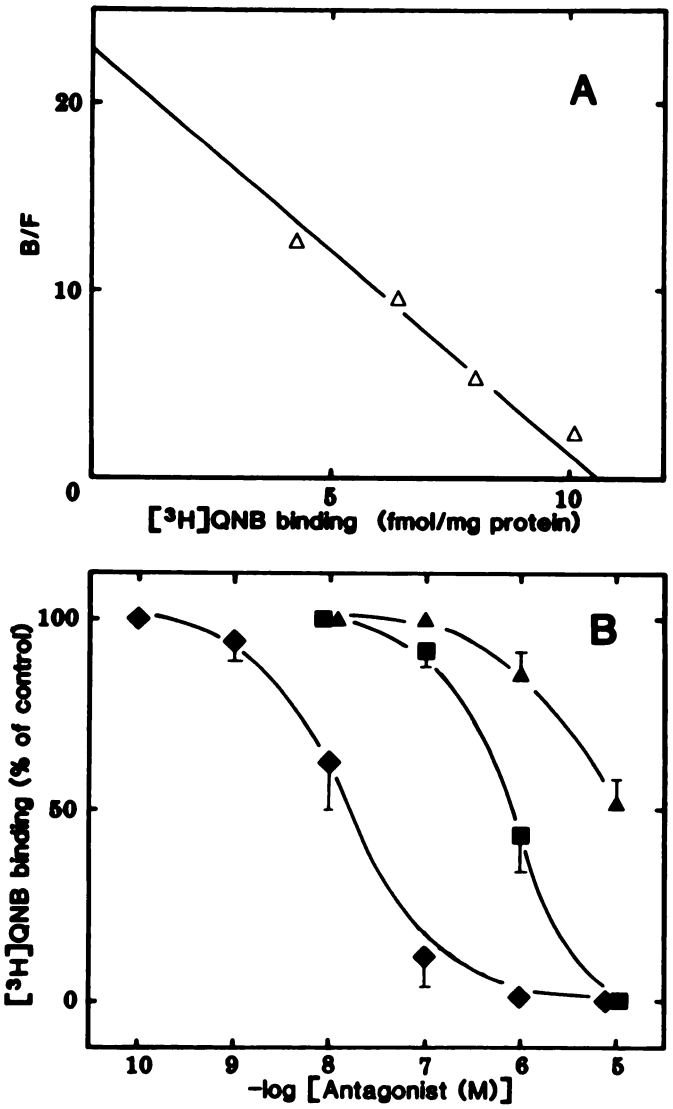

Fig. 6. Scatchard analysis and competition of muscarinic antagonists for [^{3}H]QNB binding in the membranes of Jurkat cells. A, Jurkat cell membranes (0.5–1 mg of protein/ml) were incubated with [^{3}H]QNB (0.4–4 nM) at 30° for 150 min. The K_d and B_{max} values were determined to be 0.47 ± 0.12 nM and 10.6 ± 1.7 fmol/mg of protein respectively, by Scatchard analysis. Each *point* shows the mean of four independent experiments. B, Competition curves of *p*-F-HHSiD (◆), pirenzepine (■), and AF-DX 116 (▲) for 1.57 nM [^{3}H]QNB binding to the membranes (0.7 mg of protein/ml). Each *point* shows the mean ± standard error of triplicate determinations. IC_{50} values of the antagonists were 16.0 nM (*p*-F-HHSiD), 811 nM (pirenzepine), and 11,230 nM (AF-DX 116).

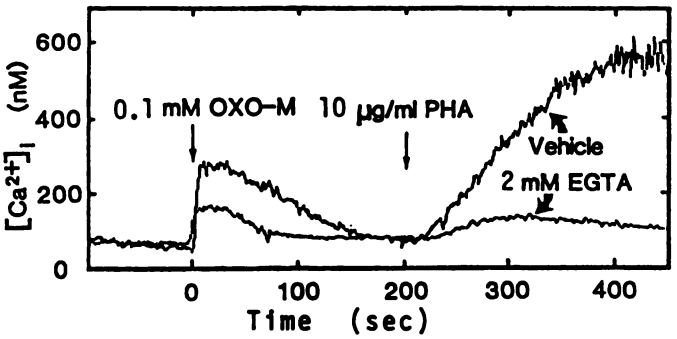

Fig. 7. Effects of extracellular Ca^{2+} on Oxo-M- and PHA-induced $[Ca^{2+}]_i$ rise. In the presence of 1 mM Ca^{2+} (*Vehicle*) or 2 mM EGTA, 100 μM Oxo-M was injected at 0 sec, and then 10 μg/ml PHA was injected at 200 sec. Extracellular Ca^{2+}-free conditions partially inhibited the $[Ca^{2+}]_i$ rise induced by 0.1 mM Oxo-M and markedly inhibited the $[Ca^{2+}]_i$ rise induced by 10 μg/ml PHA.

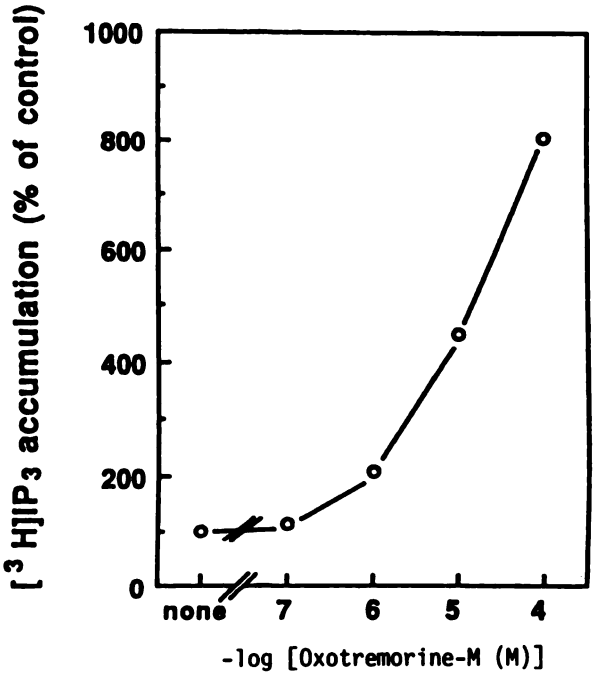

Fig. 8. Effects of Oxo-M on $InsP_3$ accumulation in Jurkat cells. Jurkat cells were preloaded with *myo*-[^{3}H]inositol for 48 hr and then washed. Oxo-M at concentrations of 0.1–100 μM was incubated for 10 min at 37° in the presence of 10 mM LiCl. [^{3}H]$InsP_3$ (IP_3) produced was separated by AG-1X8 column chromatography, as described by Berridge *et al.* (28). Each *point* shows the mean of three or four independent experiments.

$InsP_3$. To confirm the involvement of $Ins(1,4,5)P_3$ in Ca^{2+} liberation from storage pools induced by receptor activation, we examined whether synthetic $Ins(1,4,5)P_3$ (32) causes elevation of $[Ca^{2+}]_i$ and whether heparin [an antagonist for the $Ins(1,4,5)P_3$ receptor] (33) inhibits the effect of $Ins(1,4,5)P_3$ in cells permeabilized by digitonin. Synthetic $Ins(1,4,5)P_3$ (20–100 μM) was found to cause a transient $[Ca^{2+}]_i$ rise. Heparin (100 μg/ml) completely suppressed the effect of $Ins(1,4,5)P_3$ (Fig. 9).

Involvement of G proteins in Oxo-M-induced $[Ca^{2+}]_i$ rise. Fig. 10 shows the effects of cholera toxin, pertussis toxin, and botulinus toxin (type C) on the 100 μM Oxo-M- or 10 μg/ml PHA-induced $[Ca^{2+}]_i$ rise. Pretreatment with 500 ng/ml cholera toxin for 1 hr abolished the PHA-induced rise but not the effect of Oxo-M to induce an increase. Neither pertussis toxin (100 ng/ml, 20 hr nor botulinus toxin (type C) (4 μg/ml, 20 hr affected Oxo-M- and PHA-evoked increases in $[Ca^{2+}]_i$. To analyze whether G proteins are involved in the Oxo-M- and PHA-induced $[Ca^{2+}]_i$ rise, the effects of Gpp(NH)p on $[Ca^{2+}]_i$ rise were then examined in Jurkat cells permeabilized by pretreatment with digitonin. Gpp(NH)p at 20–100 μM caused transient elevation of $[Ca^{2+}]_i$ (Fig. 11). GTPγS at 0.1–100 μM enhanced the formation of InsPs in the presence and absence of 100 μM Oxo-M in digitonin-permeabilized cells (Fig. 12). GDPβS inhibited the stimulatory effect of 50 μM GTPγS on the formation of InsPs in the presence and absence of 100 μM Oxo-M, suggesting the possible involvement of G proteins in the Oxo-M-induced $[Ca^{2+}]_i$ rise.

Effects of TPA on Oxo-M- and PHA-elevated $[Ca^{2+}]_i$. To confirm the possible involvement of PKC in the $[Ca^{2+}]_i$ rise

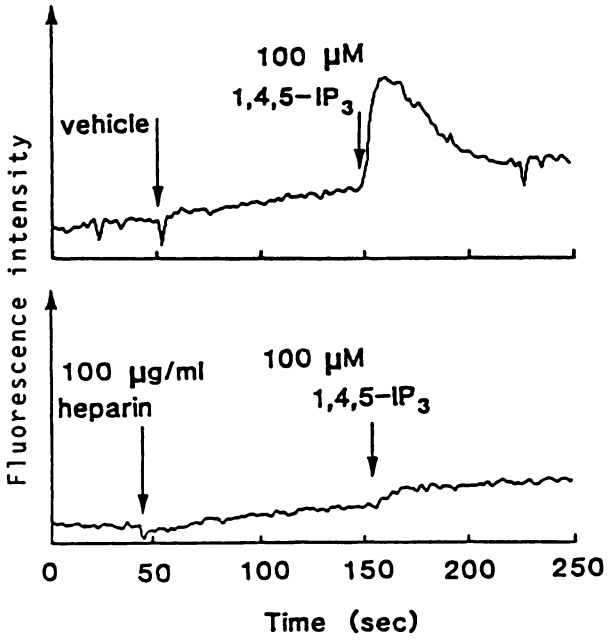

Fig. 9. Effects of heparin on synthetic Ins(1,4,5)P_3-induced increase of $[Ca^{2+}]_i$ in digitonin-permeabilized Jurkat cells. Digitonin-permeabilized cells were prepared by treatment with 15 μM digitonin (30 min), as described in Experimental Procedures. After pretreatment with vehicle or 100 μg/ml heparin for 100 sec, 100 μM synthetic Ins(1,4,5)P_3 (*1,4,5,-IP3*) was injected.

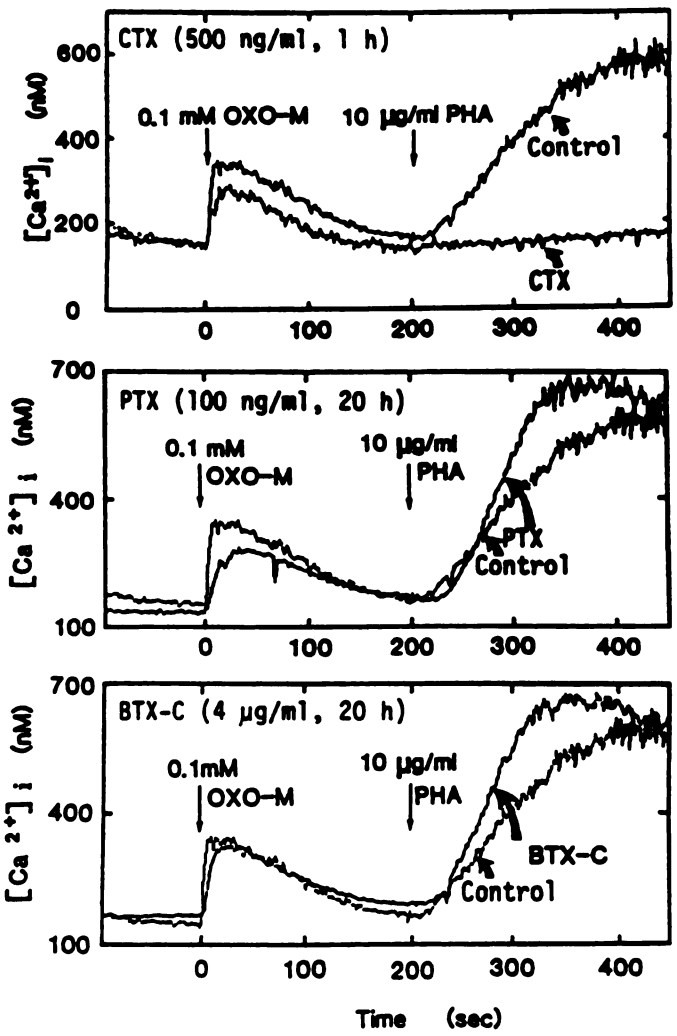

Fig. 10. Effects of bacterial toxins (cholera, pertussis, and botulinus toxin) on Oxo-M- and PHA-induced $[Ca^{2+}]_i$ rise in Jurkat cells. Jurkat cells were treated with cholera toxin (*CTX*) (500 ng/ml, 1 hr), pertussis toxin (*PTX*) (100 ng/ml, 20 hr), botulinus toxin type C (*BTX-C*) 4 μg/ml, 20 hr), or vehicle. After washing twice with Tyrode-HEPES buffer, 100 μM Oxo-M was injected at 0 sec, and then 10 μg/ml PHA was injected at 200 sec.

induced by Oxo-M and PHA, we examined the effects of 100 nM TPA for 20 hr on the Oxo-M- and PHA-induced $[Ca^{2+}]_i$ rise. As shown in Fig. 13, treatment with TPA abolished the Oxo-M (100 μM)-induced rise. However, TPA inhibited only the initial phase of the 10 μg/ml PHA-induced $[Ca^{2+}]_i$ rise and did not affect the maximal level of the Oxo-M-induced $[Ca^{2+}]_i$ rise. Inhibition by TPA was dependent on incubation time, and 3–5 min was required for complete inhibition (Fig. 14). H-7 (100 μM) a potent inhibitor of PKC, almost completely suppressed the inhibition produced by 5-min TPA treatment. PKC thus appears likely involved in negative feedback regulation of the Oxo-M-induced $[Ca^{2+}]_i$ rise.

Discussion

Recently, genes for muscarinic receptor subtypes have been classified as m1–m5, based on results from cDNA cloning experiments (34, 35). In addition, several muscarinic antagonists have been used to determine affinity profiles for five cloned human muscarinic receptors (m1–m5) stably expressed in CHO cells. In brief, the profile for pirenzepine is m1 > m4 ≥ m5 ≥ m3 ≥ m2, for AF-DX 250 [the (+)-enantiomer of AF-DX 116] is m2 ≥ m4 ≥ m1 ≥ m3 > m5, for *p*-F-HHSiD is m3 ≥ m1 ≥ m4 > m5 ≥ m2, and for 4-DAMP is m3 = m1 ≥ m5 = m4 > m2 (36). It is known that T lymphocytes possess muscarinic receptors (5), which stimulate cGMP formation (9). The results presented here demonstrate the presence of functional muscarinic ACh receptors mediating $[Ca^{2+}]_i$ rise. In addition, the Oxo-M-induced rise was inhibited by 4-DAMP ≥ atropine ≥ *p*-F-HHSiD ≫ pirenzepine > AF-DX 116 in Jurkat cells. Thus, the receptor on the Jurkat cell surface is of the m_3 subtype of muscarinic receptor. McN-A-343 and pilocarpine stimulate phosphoinositide hydrolysis is m1-expressing CHO cells more than in m3-expressing CHO cells (29). In the present study, McN-A-343 and pilocarpine did not cause a $[Ca^{2+}]_i$ rise in Jurkat cells. In contrast, McN-A343 caused a $[Ca^{2+}]_i$ rise in NGPM1-8 cells, i.e., NG108-15 cells in which porcine m1 receptor genes are expressed (NG108-15 cells express the m4 gene natively), but did not in porcine m3-expressing cells (NGPM3-332) (37), although muscarine and Oxo-M caused an increase in both NGPM1-8 and NGPM3-332 cells.[1] These characteristics also support the idea that the muscarinic receptor in Jurkat cells is of the m3 subtype. Furthermore, the result of Northern blot analysis clearly shows expression of the m3 subtype in Jurkat cells. It has been reported that the m3 subtype gene is expressed in several human cell lines, e.g., H4 (neuroglioma), TE671 (medulloblastoma), A172 (glioblastoma), SK-N-SH (neuroblastoma), and 1321N1 (astrocytoma), but the m1 subtype gene is not expressed in these cell lines (38). In addition, the distribution of m3-expressing tissues in swine is cerebrum, lacrimal gland, parotid gland, small and large intestines, urinary bladder, submaxillary gland, and pancreas (35, 39). These observations suggested that the m3 subtype gene is

[1] Y. Kitamura, R. Imaizumi, M. Tohda, H. Higashida, and Y. Nomura, unpublished observations.

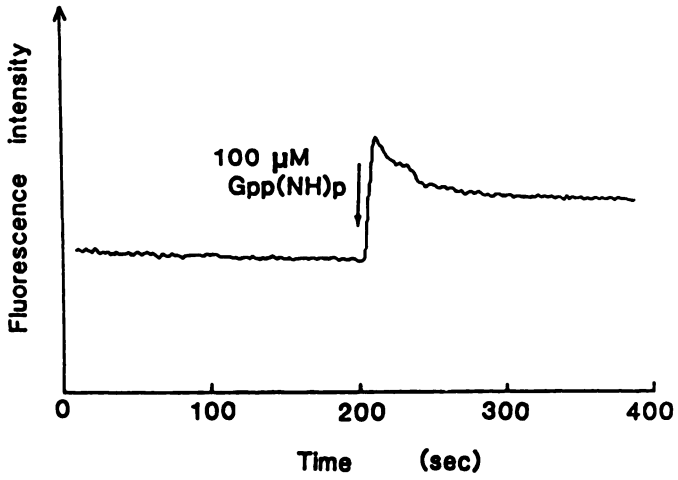

Fig. 11. Effects of Gpp(NH)p on intracellular Ca^{2+} accumulation in digitonin-permeabilized Jurkat cells. Permeabilized cells were prepared by treatment with 15 μM digitonin (30 min) in Tyrode-HEPES buffer, as described for Fig. 9. After preincubation, 100 μM Gpp(NH)p was injected at 200 sec.

expressed in peripheral secretory tissues and brain, corresponding to the present results.

Binding parameters of [^{3}H]QNB in Jurkat cells are K_d = 14.1 nM and B_{max} = 45,370 binding sites/cell using intact cells and K_d = 0.47 nM and B_{max} = 10.6 fmol/mg of protein using cell membranes. Although the K_d value of [^{3}H]QNB binding is approximately 100–1000 times the value (15–173 pM) in intact human embryonic kidney cells into which m3 receptor cDNA (termed HM4) has been transfected (35, 40), this value is similar to the value (3–60 nM) in intact human peripheral blood lymphocytes (6, 41). In addition, 13.2 nM, which is the IC_{50} for QNB effects on the Oxo-M-induced $[Ca^{2+}]_i$ rise (Fig. 3B), agrees with the K_d value (14.1 nM) for [^{3}H]QNB binding to intact cells (Fig. 5). A low affinity of K_d value in the intact cells may be due to a difference of microenvironment surrounding the receptors on the surface of human lymphocytes. Recently, an interesting finding was reported, that B_{max} values for [^{3}H]QNB binding to peripheral blood lymphocytes from patients with Alzheimer's disease, in which dysfunction of the cholinergic system in the central nervous system is observed, are markedly decreased, in comparison with the values in normal volunteers, without changes in the K_d values (42). The immune system may be modulated by the central nervous system through the cholinergic system.

The Oxo-M-induced $[Ca^{2+}]_i$ rise was reduced approximately 50% in the presence of extracellular 2 mM EGTA. Thus, not only Ca^{2+} release from intracellular storage pools but also Ca^{2+} influx are involved in Oxo-M-evoked $[Ca^{2+}]_i$ elevation. In contrast to the effects of Oxo-M, the potent and persistent effects of the mitogenic lectin PHA, a nonspecific activator of TCR/CD3, were almost abolished by extracellular EGTA. However, gradual slight elevation of $[Ca^{2+}]_i$ by PHA was observed in EGTA-containing medium, suggesting that PHA induces intracellular Ca^{2+} mobilization. Mouse monoclonal antibody against human CD3 (NU-T3) yielded results similar to those obtained with the PHA.[2] These observations are supported by the finding that PHA actually caused $InsP_3$ formation via TCR/CD3 (19, 20). The fact that intracellular Ca^{2+} mobilization may be related to the Oxo-M-induced rise in $[Ca^{2+}]_i$ is supported by the observation that $InsP_3$ formation is stimulated by Oxo-M in Jurkat cells. Cells permeabilized by digitonin treatment were responsive to Ins(1,4,5)P_3, in terms of $[Ca^{2+}]_i$ rise. The complete suppression by heparin of the $InsP_3$-induced $[Ca^{2+}]_i$ rise further supports the hypothesis that $[Ca^{2+}]_i$ elevated by Oxo-M may be due to Ca^{2+} mobilization by $InsP_3$. A recent report (43) suggests that cAMP is able to release Ca^{2+} from intracellular pools by a mechanism distinct from that involving the $InsP_3$-sensitive pool in Jurkat cells. However, it is unlikely that cAMP is involved in $[Ca^{2+}]_i$ elevation by Oxo-M, because Oxo-M had no effect on cAMP formation.[3] Recently, several interesting findings in Jurkat cells were reported, namely that (i) PHA and Ins(1,4,5)P_3 induced activation of Ca^{2+}-permeable channels, measured using the patch-clamp technique (44–46); (ii) activation of TCR/CD3 by anti-CD3 antibody (OKT3) induced an increase in Ins(1,3,4,5)P_4 within 10 min, which remained

[2] Y. Kitamura, Y. Kitayama, and Y. Nomura. Regulation of $[Ca^{2+}]_i$ rise activated by doxepin-sensitive H_1-histamine receptors in Jurkat cells. Manuscript in preparation.

[3] T. Kaneda and Y. Nomura, unpublished observations.

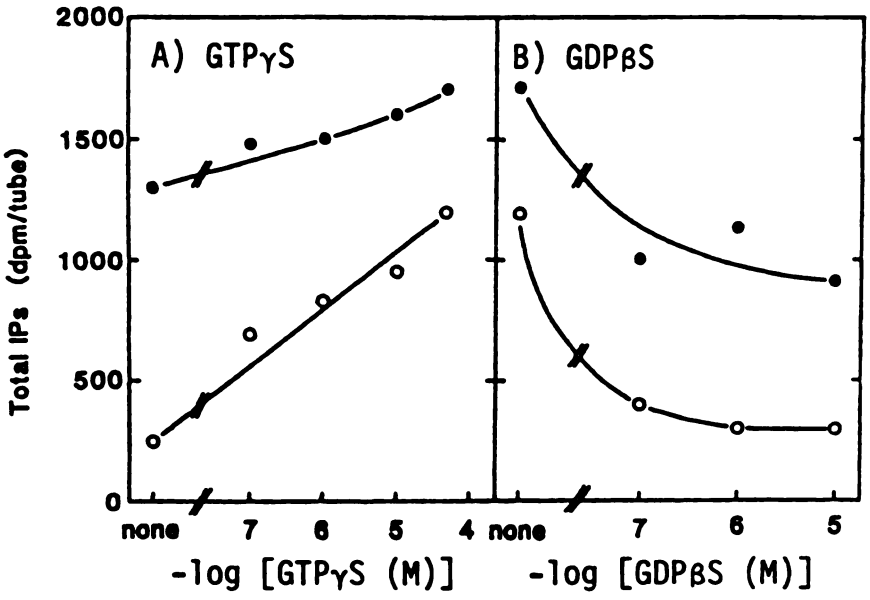

Fig. 12. Effects of GTPγS and GDPβS on Oxo-M-induced InsP accumulation in permeabilized Jurkat cells. A, Preparation of permeabilized cells was as described in Experimental Procedures. Cells were incubated with GTPγS (0–50 μM) in the presence (● or absence (○) of 100 μM Oxo-M for 15 min at 37°, in the presence of 10 mM LiCl. Accumulated [^{3}H]InsPs (*Total IPs*) were measured by the method of Berridge *et al.* (28). B, Cells were incubated with GTPγS (50 μM) and various concentration of GDPβS (0–10 μM) in the presence (●) or absence (○) of 100 μM Oxo-M for 15 min at 37° in the presence of 10 mM LiCl. Each *point* shows the means of three independent experiments.

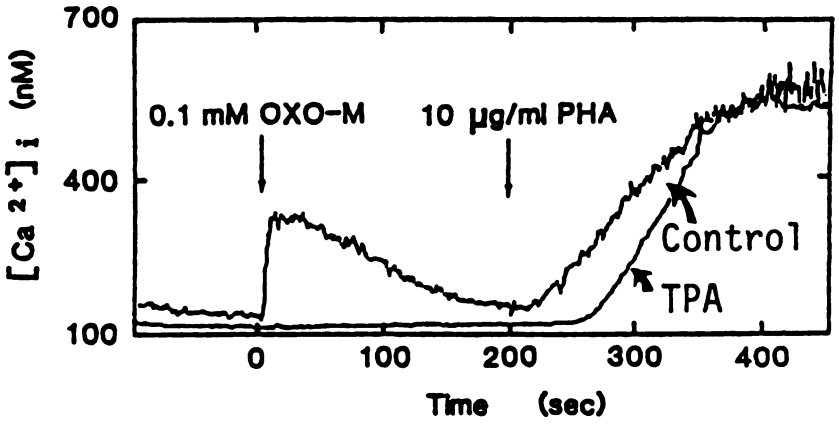

Fig. 13. Effects of TPA on Oxo-M- and PHA-induced $[Ca^{2+}]_i$ rise in Jurkat cells. Cells were pretreated with 100 nM TPA or vehicle (*Control*) for 20 hr at 37° and subsequently washed twice. Oxo-M (100 µM) was injected at 0 sec, and then 10 µg/ml PHA was injected at 200 sec.

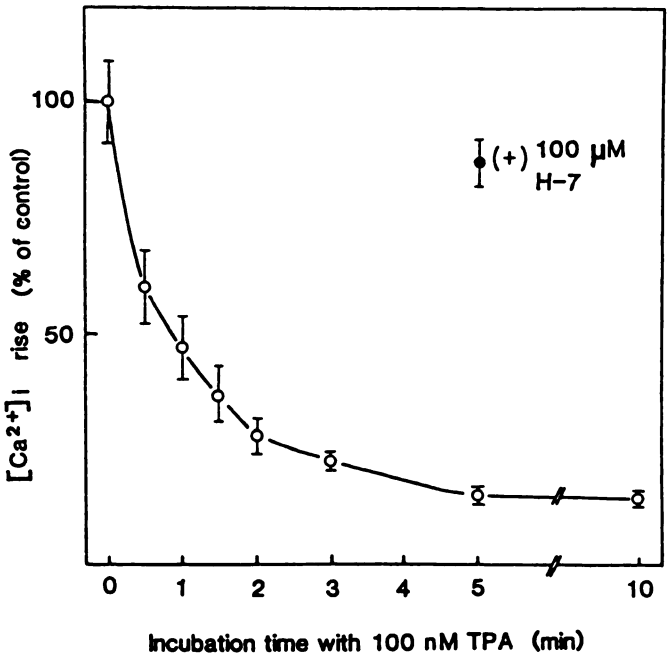

Fig. 14. Inhibitory effects of TPA on Oxo-M-induced $[Ca^{2+}]_i$ rise and effect of H-7 in Jurkat cells. Cells were pretreated with 100 nM TPA for 10 min at 37° for various times (○), as described for Fig. 13. For examination of the effect of H-7, cells were preincubated with 100 µM H-7 for 2 min and further incubated in the presence of 100 nM TPA for 5 min (●). The $[Ca^{2+}]_i$ rise induced by 100 µM Oxo-M was then measured. Each *point* shows the mean ± standard error of three to five independent experiments.

elevated for >30 min (47); and (iii) $Ins(1,3,4,5)P_4$ activates an endothelial Ca^{2+} channel (48).

The involvement of G proteins in the Oxo-M-induced $[Ca^{2+}]_i$ rise was also suggested, based on results from experiments using digitonin-permeabilized cells; (i) Gpp(NH)p transiently evoked a $[Ca^{2+}]_i$ rise and (ii) GTPγS enhanced Oxo-M-evoked InsP production, whereas GDPβS decreased it. As for the properties of the G proteins involved, little is known because three types of bacterial toxin, cholera, pertussis, and botulinus toxin (type C), failed to have any effect on the Oxo-M-induced $[Ca^{2+}]_i$ rise. The pertussis toxin-insensitive G protein G_q has been shown to activate the β1-type of phospholipase C (49, 50). Thus, m3 muscarinic receptors may possibly couple with G_q in Jurkat cells.

The finding that TPA abolished the Oxo-M-induced $[Ca^{2+}]_i$ rise indicates that PKC may modulate m3 receptor-mediated $[Ca^{2+}]_i$ rise. In addition, (i) injection of purified rat PKC also inhibited the Oxo-M-induced rise and H-7 reversed this inhibition and (ii) mastoparan, a direct activator of G proteins (51), stimulated a $[Ca^{2+}]_i$ rise and TPA inhibited the mastoparan-induced rise.[4] PKC may thus phosphorylate m3 receptors, G proteins, and/or phospholipase C in the pathways of signal transduction between the activation of m3 receptors and intracellular Ca^{2+} mobilization, because PKC phosphorylates at least mACh receptors purified from porcine cerebra and atria (52) and G_i (53). However, it is of interest that TPA inhibited only the initial rise of $[Ca^{2+}]_i$ induced by PHA and did not affect the maximal $[Ca^{2+}]_i$ persistently increased by PHA. The finding that PKC inhibits the initial $[Ca^{2+}]_i$ rise induced by a sequential pathway, such as that involving the TCR/CD3 complex, G proteins, and phospholipase C, but does not affect Ca^{2+} influx through Ca^{2+} channels in plasma membranes may be an explanation for this.

The functions of $[Ca^{2+}]_i$ elevations produced by each receptor, however, are not clear at present. Ca^{2+} may also affect the formation of cGMP and arachidonic acid by the activation of guanylate cyclase and phospholipase A_2, respectively. Oxo-M induces elevation of cGMP but not that of arachidonic acid in Jurkat cells.[3] The activation of muscarinic receptors induces cGMP formation (9) and mitosis of immune cells (54). The stimulatory effects of Oxo-M were also noted on (i) c-*fos* mRNA expression in Jurkat cells and (ii) PHA-induced DNA synthesis and IL-2 production in human peripheral blood lymphocytes (55). The functions of m3 receptors and particularly the interactions with TCR/CD3 and cross-talk of intracellular signaling mechanisms between m3 receptors and TCR/CD3 in T cells should be clarified in the future.

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