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SIGNAL TRANSDUCTION OF MUSCARINIC ACETHLCHOLINE RECEPTORS

by

KEDAN LIN

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

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Degree Conferred: .....

To my parents

Jingxiang Cao

&

Lixing Lin

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Many people contributed towards the completion of this project. First of all I would like to thank my advisors Drs. Wolfgang Sadée and Jelveh Lameh for their generous support and guidance throughout my study and research.

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Signal Transduction of Muscarinic
Acetylcholine Receptors
Kedan Lin
August 2000

ABSTRACT

Muscarinic acetylcholine receptors (mAChRs) are G protein coupled receptors (GPCRs) responsive to the neurotransmitter acetylcholine and are involved in a broad range of biological functions. This work describes signal transduction and regulation of muscarinic receptors.

Receptor activity is under tight regulation for adaptation of the cells to their environment. One such regulatory response is receptor desensitization. Under repeated exposure to agonist, mAChR subtype 1 receptor (m1 mAChR) underwent rapid desensitization. Our results suggested a role for G protein-coupled receptor kinases in regulating desensitization, although other kinases could also be involved.

One of the emerging themes in receptor signaling is the interconnection between GPCR pathway and growth factor mediated pathways that activate nuclear transcription. To delineate the pathways of mAChRs responsible for activating the nuclear transcription factor serum response factor (SRF), genes encoding m1-3 mAChR were co-expressed with a reporter gene driven by SRF. Only m1 mAChR activated SRF through a pathway involving the small GTPase RhoA, independent of Gαi/o and Gαq. Transfection of constitutively active Gα13 strongly induced SRF activation, and activation of SRF by m1 mAChR was shown to involve calmodulin and the phosphatase calcineurin.

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To separate Gq coupling and nuclear signaling of SRF by muscarinic receptors, we tested a panel of receptor mutants and chimeras with the focus on the third intracellular (i3) loops. A m3 mAChR chimera with the i3 loop from m1 stimulated SRF activation; however m1 mAChR mutant with much of the i3 loop deleted could still activate SRF. This suggests that domains other than i3 can contribute to SRF activation of muscarinic receptors.

This thesis also includes the development of rapid screening assays, exploiting cell membrane and nuclear signaling of GPCRs. We developed a method to assay intracellular calcium ions in cultured cells on 96-well plates using a fluorometer. This assay can serve as a rapid screening method for active compounds at many different GPCRs. In addition, a reporter gene assay integrating cell surface receptor and nuclear signaling to screen for novel immunosuppressant compounds was developed. This assay was applied to the analysis of FK506 in patient blood samples and the data obtained was similar to a conventional method.

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INTRODUCTION

A. Muscarinic Acetylcholine Receptors and Cholinergic Effects

Neurotransmitters can produce a wide range of direct effects on target cells. Among known endogenous neurotransmitters, acetylcholine (ACh) was the first identified mediating the negative chronotropic response of cardiac muscarinic receptors. ACh is expressed at cholinergic synapses and neuroeffector junctions in the central and peripheral nervous systems. The cholinergic system is one of the most important modulatory neurotransmitter systems in the brain and controls activities related to conscious awareness (van der Zee & Luiten, 1999). In degenerative brain diseases, such as Alzheimer's disease, there is a correlation between loss of explicit memory and hypoactivity of cholinergic projections to the hippocampus and cortex (Perry *et al.*, 1999).

The actions of acetylcholine are mediated through nicotinic and muscarinic cholinergic receptors, which transduce signals via distinct mechanisms. Nicotinic receptors are ligand-gated ion channels, whose activation causes a rapid increase in cation permeability. Muscarinic receptors, on the other hand, belong to the G protein-coupled receptor (GPCR) superfamily, the largest family of integral membrane receptors. These receptors are seven transmembrane-spanning proteins that respond to a wide variety of stimuli including light, odor, taste, hormones and neurotransmitters (Bockaert & Pin, 1999). The main focus of my thesis work was on muscarinic receptors and their regulation.

Muscarinic receptors are found primarily on the autonomic effector cells that are innervated by postganglionic parasympathetic nerves. The cloning of the cDNAs that encode muscarinic receptors has so far identified five distinct gene products (Bonner *et al.*, 1987). They have been designated as M1 through M5 based on pharmacological specificity. This corresponds with the designation of m1 through m5 revealed by molecular cloning. m1 AChRs are found in ganglia and various secretory glands, m2 mAChRs predominate in the myocardium and in smooth muscle; and m3 and m4 are located in smooth muscle and secretory glands. All five subtypes are found in the CNS (Perry *et al.*, 1999). All of the muscarinic receptor subtypes interact with members of a group of guanine nucleotide-binding regulatory proteins (G proteins) that regulate a variety of effector proteins within cells (Wess, 1993).

B. G protein Coupled Receptors (GPCRs) and G proteins

Activation of GPCRs, which couple to second messenger production via multiple heterotrimeric G proteins, can result in 'direct' effects on a range of signaling proteins, including phospholipase C (PLC), adenylate cyclase (AC) and ion channels (Neer, 1995). This activation ultimately leads to changes in the cellular concentration of second messengers such as cAMP, Ca^{2+}, inositol phosphates (IP), or diacylglycerol (DAG).

G proteins, consisting of α, β and γ subunits, relay signals received by the cell-surface receptors to intracellular effectors. The onset/offset of this signaling event is controlled by the cycle of GTP binding to the α subunit (Bourne, 1997).

The replacement of GDP with GTP in the active site causes the dissociation of the α subunit from βγ heterodimer as well as the receptor. Both the GTP bound α subunit and the free βγ heterodimer can regulate the activity of their respective downstream effectors. The hydrolysis of bound GTP, either by the intrinsic GTPase activity of the α subunit or by the interaction with other regulators of G-protein signaling (RGS), returns the α subunit to a GDP-bound state (Figure 1). The α subunit can then reassociate with the βγ subunits and terminate signal transmission. Activation GPCR causes dissociation of G protein subunits following the binding of GTP to Gα; this can subsequently result in activation of distinct Gα and Gβγ sensitive isoforms of adenylate cyclase, PLC or other phospholipases.

G protein α subunits can be divided into four families: Gs, Gi (Gi, Go and transducin), Gq (Gq, G11, G14 and G16), and G12 (G12 and G13) (Gilman, 1987; Simon *et al.*, 1991). The interaction between GPCRs and the large number of different G protein subunits is highly diverse, as one GPCR is capable of interacting with more than one G protein to result in activation of a variety of targets. Thus, GPCRs can be useful targets for the design of therapeutic agents. However, research and development using G protein coupled receptors as drug targets has encountered great difficulty. The main obstacles in these studies have been the expression of a number of receptors in the same cells and the lack of subtype specific ligands. One way to tackle this

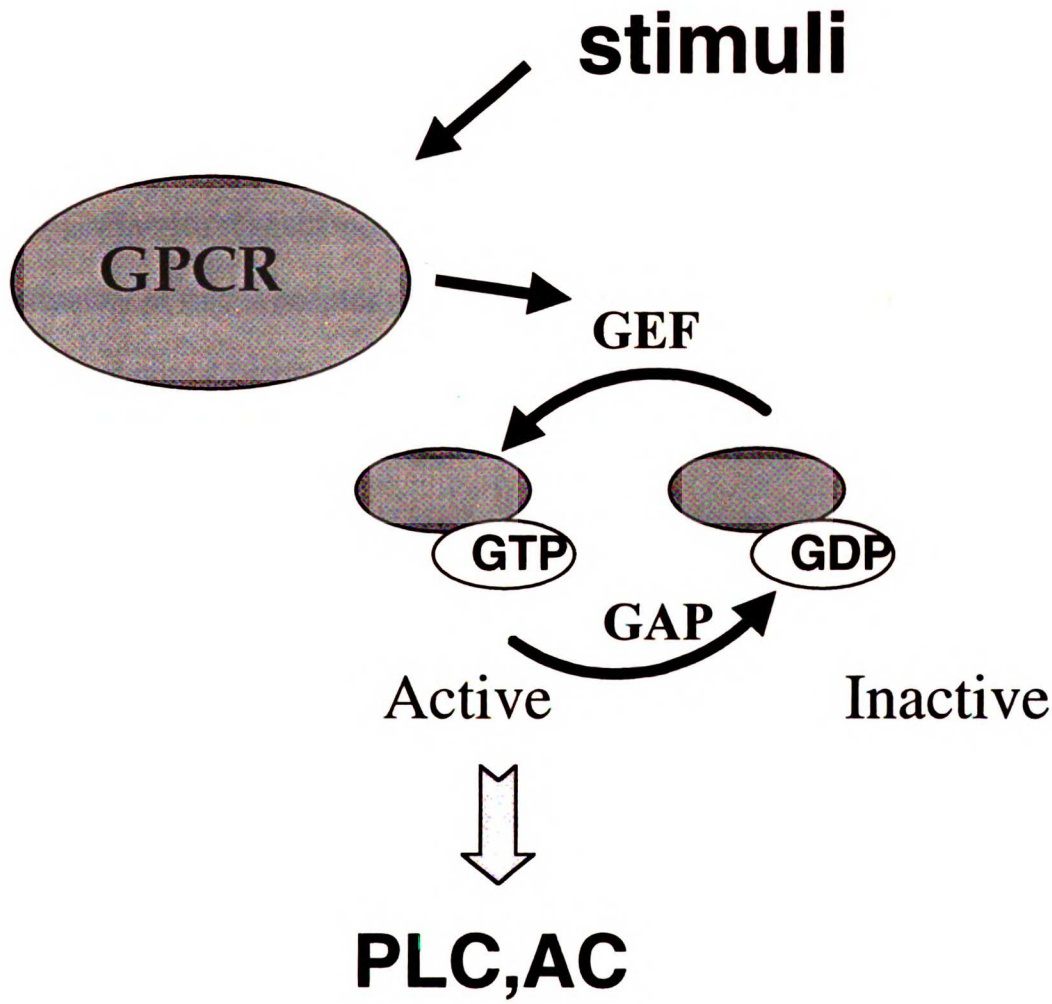


Figure 1. Activation of G protein coupled receptors. Binding of a G-protein-coupled receptor by its transmitter enables the receptor to interact with the G-protein. This interaction prompts GTP instead of GDP to bind to the G-protein which leads to dissociation of the α, β, and γ complex. The freed α subunit then diffuses through the membrane and activates its effectors or target proteins.

problem is to understand the specific pathways mediated by individual receptor subtypes and to develop subtype specific functional assays to screen lead compounds.

Chapter 2 and 3 describe studies on transcriptional regulation of m1-3 mAChRs with respect to G protein coupling pathways. The differential coupling capacity of each receptor provides us with a tool for designing specific drugs.

Agonist binding to G protein-coupled receptors results in ligand-induced receptor conformation changes. These conformational changes in turn result in dissociation of G protein-subunits from their partners leading to activation of the intracellular signaling pathways. In addition to signaling events, activated receptors undergo regulatory processes leading to adaptation of the cells to their environment. One such regulatory response is receptor desensitization, which results in a diminution in receptor responsiveness.

C. Receptor Desensitization and its Regulation

Receptor desensitization plays an important physiological role by curbing overstimulation of GPCR signaling cascades (Bünemann *et al.*, 1999; Ferguson *et al.*, 1998). It also provides a mechanism by which GPCR-mediated signals from multiple inputs can be orchestrated into meaningful information. The failure of a receptor to desensitize has been associated with diseases such as retinal degeneration, precocious puberty and thyroid tumors. Conversely, up-regulation of proteins associated with GPCR desensitization has also been

associated with diseases such as hypertension, congestive heart failure and drug dependence and tolerance (Iiri *et al.*, 1997; Iiri *et al.*, 1998).

Receptor desensitization is observed by a decrease in second messenger production, either ablating the maximum response, and/or increasing the EC50 of the agonist. Receptor desensitization can be the consequence of receptor phosphorylation, arrestin binding, sequestration and/ or down-regulation (Zhang *et al.*, 1997). GPCR phosphorylation is initiated within seconds to minutes of receptor activation and is mediated by both second messenger-dependent protein kinases and receptor-specific G protein-coupled receptor kinases (GRKs) (Benovic *et al.*, 1986). Desensitization in response to GRK-mediated phosphorylation involves the binding of arrestin proteins that serve to uncouple the receptor from its G protein. GPCR sequestration is also believed to play a critical role in GPCR resensitization.

A clear understanding of the interaction among the processes of receptor activation, desensitization and resensitization as well as the regulatory pathways of each of these processes, is critical to the development of novel drug targets.

In Chapter 1, m1 mAChR was used as a model to study the process of receptor desensitization. In this work, the role of phosphorylation in the regulation of receptor desensitization was investigated. Furthermore, a panel of receptor mutants was employed to identify the correlation among receptor domains, internalization and G protein coupling in receptor desensitization.

D. Nuclear signaling of G protein Coupled Receptors / Crosstalk with Growth Factor Mediated Pathways

Ligands that interact with G protein coupled receptors induce acute signaling functions. However, these ligands can also induce long term effects such as inducing gene expression and promoting cell growth (Marinissen *et al.*, 1999). These genetic and proliferative effects can be induced by activation of receptors that signal through heterotrimeric G-proteins of the Gq family, pertussis toxin-sensitive /Go proteins, Gs, or G12/G13. Numerous growth-promoting G protein-coupled receptors also activate the low molecular weight G-protein Ras and stimulate mitogen-activated protein kinase, possibly through interaction with low molecular weight G-proteins of the Rho family (Post & Brown, 1996). The mechanisms by which heterotrimeric G-proteins couple to these kinase cascades remain to be elucidated. It is likely that interactions between multiple signaling pathways are required for G protein-coupled receptors to propagate signals to the nucleus.

In chapter 2 and 3, the components involved in m1-m3 mAChR nuclear activation pathways were studied. In addition, the role of receptor internalization in activating *c-fos*, as a measure of nuclear signaling of the receptor, was investigated.

E. Drug Discovery and Receptor Screening

The present-day drug targets address only about 500 molecular targets. 45% of these targets are cell membrane receptors, largely GPCRs. In

comparison, enzymes only account for 28% of all current drug targets (Drews, 1999). With the finishing of human genome project in 2000, another ~3000 GPCR will be identified (Kolakowski, 1994; Probst *et al.*, 1992). The rapid expansion in identification of disease-related genes and compound collections demands more efficient screening assays (Debouck & Goodfellow, 1999).

Therapeutic development has been directed at identification of multiple receptor subtypes and their specific direct actions to develop agonists and antagonists with fewer side-effects. In chapter 4, the detection of receptor mediated calcium influx using fluorescent dye is described. A large percentage of G protein coupled receptors can elevate intracellular calcium levels either by releasing Ca^{2+} from intracellular stores via the PLC pathway or by indirect/direct G protein activation of calcium channels.

In chapter 5, another functional receptor assay is described. This assay integrates the membrane GPCR with the nuclear signaling of NFAT (nuclear factor of activated T cells). In this assay, the firefly luciferase reporter genes functionally coupled to NFAT pathway were used to screen for immunosuppressant compounds. The availability of several tools makes development of functional assays for large scale screening possible.

The development of these techniques not only can be used to functionally characterize these receptors and their signaling pathways, but also can be exploited for the development of high-throughput drug-screening systems, the expression cloning of new receptors and identification of endogenous ligands for orphan receptors.

CHAPTER 1

DESENSITIZATION OF MUSCARINIC ACETYLCHOLINE m1 RECEPTORS

I. SUMMARY

Agonist binding to heterotrimeric G protein-coupled receptors triggers several events that result from ligand-induced changes in receptor conformation, from the initial dissociation of G protein complex to a rapid decrease in the maximum response, and/or an increase in the EC50 of the agonist. This adaptive change serves to diminish the response of cells to further stimulation. Activation of m1 mAChR increases the inositol phosphates (IP) production and intracellular Ca^{2+} concentration through activation of phospholipase C (PLC). PLC pathway was thought to be more resistant to desensitization compared to cyclic AMP pathway. Whether m1 mAChR receptors undergo desensitization and how this process is regulated has been subject of controversy. It has been hypothesized that phosphorylation of GPCRs by receptor-specific kinases is a critical event in receptor regulation, especially desensitization and internalization.

To address whether m1 receptors desensitize in a recombinant expression system, we measured both G protein coupling and second messenger production under repeated exposure to m1 agonist carbachol (CCh). m1 mAChR underwent desensitization after 5 min pretreatment with CCh.

To test the hypothesis that muscarinic receptor phosphorylation by G protein-coupled receptor kinases (GRKs) plays a role in its regulation, and to distinguish the contribution of individual members of the GRK family, we studied the desensitization effect in HEK 293 cells co-transfected with GRK2 through GRK6. Among them, GRK2 exhibited the strongest desensitization effect on m1 mAChR. To further test the effect of phosphorylation by G protein receptor kinase 2 (GRK2 or βARK), we either accentuated or ablated phosphorylation by overexpression GRK2 or a dominant-negative GRK2 mutant (K220W or DN-GRK2) and observed the changes in receptor regulation. GRK2 strongly inhibited second messenger production measured by Inositol (1,4,5) trisphosphates (IP_3) production, while DN-GRK2 also attenuated IP_3 production, although to a lesser extent. These results suggest a role for GRKs in regulating desensitization. However, GRKS may not be the only kinases involved in this process.

To identify the regions on m1 AChR receptor that interact with GRKs, we tested a panel of mutants with Ser/Thr deletion in intracellular loop 3 (i3) and -COOH terminal. Mutants with i3 loop mutations were indistinguishable from wild-type, while a portion of the COOH terminus of muscarinic receptor was shown to enhance sensitivity of receptor to phosphorylation.

II. INTRODUCTION

Agonist binding to heterotrimeric G protein-coupled receptors triggers several events as a consequence conformation change of the receptor. First, dissociation of heterotrimeric G protein subunits from each other leads to activation of the intracellular signaling pathways. Second, there is a rapid desensitization of the receptors. The production of intracellular second messengers decreases in a second exposure to the agonist. This is observed by a decrease in the maximum response, and/or an increase in the EC50 of the agonist (Bünemann & Hosey, 1999).

Receptor desensitization can be a homologous or heterologous event. In homologous desensitization only the activated receptors desensitize, and the desensitization is strictly agonist-dependent. In heterologous desensitization the activation of one type of receptor can result in the desensitization of other, unrelated receptors, particularly receptor types that activate common signaling pathways. The molecular events underlying desensitization of GPCRs have not been entirely elucidated (Zhang *et al.*, 1997).

Phosphorylation of the GPCRs has been implicated in the process of uncoupling in both homologous and heterologous desensitization. Two types of receptor phosphorylation mechanisms have been identified. Phosphorylation by second messenger activated protein kinases, such as PKA and PKC, has been demonstrated to play a role in heterologous desensitization of G protein coupled receptors (Ferguson *et al.*, 1998). For homologous desensitization, which is the focus of our study, a distinct family of "receptor

specific" protein kinases known as G protein coupled receptor kinases (GRKs) has been proposed (Benovic *et al.*, 1986).

G protein coupled receptor kinases phosphorylate only agonist-occupied members of the GPCR superfamily; inactivated receptors are not phosphorylated at significant rates by these kinases. After the receptors phosphorylation, proteins known as arrestins bind to the receptors and "arrest" receptor-G protein coupling by disrupting receptor/G protein interactions (Attramadal *et al.*, 1992).

The GRK protein family of Ser/Thr kinases consists of six members which can be subclassified according to both sequence homology and functional similarity: rhodopsin kinase (GRK1), β-adrenergic receptor kinase 1 (βARK1 or GRK2) and βARK2 (GRK3), the GRK4-6 subfamily (Premont *et al.*, 1995). GRKs phosphorylate GPCRs at serine and threonine residues residing within the carboxyl-terminal tail (e.g., β2AR) or third intracellular loop (e.g., m2 muscarinic acetylcholine receptor, m2 mAChR) (Hosey *et al.*, 1999). The GRKs share a central "kinase" domain with high homology and variability in the N-and C-terminal domains. Several differences in substrate specificity and mechanisms of regulation have been revealed (Premont *et al.*, 1995; Premont *et al.*, 1994; Stoffel *et al.*, 1994; Zhang *et al.*, 1997).

GRK2 was first isolated as a kinase that phosphorylates the β2-adrenergic receptor in an agonist-dependent manner *in vitro*, and so named β-adrenergic receptor kinase (βARK) (Benovic *et al.*, 1986). However, the

substrate specificity of " βARK" is much broader than β-adrenergic receptor. *In vitro*, βARK phosphorylates the m2 mAChR, β2-adrenergic receptors and substance P receptors (Aramori *et al.*, 1997; Hall *et al.*, 1999; Hosey *et al.*, 1999). However, its role in human muscarinic receptor hm1 mAChR regulation is not clear. To determine if agonist-induced phosphorylation is of general importance in the regulation of m1 mAChR, we tested whether the receptors behave differently in the absence of GRK. There are no specific GRK inhibitors. For *in vitro* phosphorylation assay using peptide pseudo-substrates, the low affinity interactions with the GRKs has limited their usage. A dominant negative mutant of GRK was shown to prevent desensitization of adrenergic receptors in a bronchial epithelial cell line (Diviani *et al.*, 1996) and attenuate adenosine A2 receptor desensitization (Mundell *et al.*, 1997). This mutant K220W was mutated at kinase catalytic center with normal substrate binding activity (Tsuga *et al.*, 1994). To address whether phosphorylation by βARK enhances receptor desensitization, we analyzed HEK 293 cells transfected with expression vector encoding the hm1 receptor and vector encoding a βARK or a dominant-negative βARK mutant (K220W or DN-GRK2).

Internalization of G protein-coupled receptors rapidly follows agonist binding and has initially been proposed to be involved in receptor desensitization. However, recent studies have shown that internalization of the receptor-ligand complex and homologous desensitization were independent processes (Maestes *et al.*, 1999; Olli-Lahdesmaki *et al.*, 1999). To demonstrate

that receptors desensitization and internalization are two events that can proceed independently of each other in m1 mAChR expression system, the role of β-arrestin, that facilitates internalization of GRK phosphorylated receptors, in desensitization of m1 mAChR was studied.

Regions within the receptor responsible for the specificity of G protein coupling have been defined for some GPCRs, primarily by mutagenesis and receptor subtype chimera studies. The determinants in the GPCRs that allow the receptors to be GRK substrates are not well defined. In this study, we examined the role of various receptor domains in modulating agonist-induced desensitization.

III. EXPERIMENTAL PROCEDURES

A. Materials

[^{3}H]-NMS (specific activity 85 Ci/mmol) and [^{3}H]-QNB (specific activity 47 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Carbachol was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

B. Transient and Stable Expression of hm1 Receptor

The gene encoding the human muscarinic cholinergic subtype 1 receptor (hm1) was obtained from a human placental genomic library as previously described (Maeda *et al.,* 1990). The sequence of a selected clone was verified and the plasmid was co-transfected with indicated plasmids or with pRSV^{neo}

(for stable transfection) into human embryonic kidney (HEK 293) cells by the calcium phosphate precipitation method. Clonal cell lines expressing hm1 sites were selected in DMEM/H-16/F-12 with 10% fetal calf serum, 1% penicillin/streptomycin, and 400 mg/ml G418 in 5% CO_2 and maintained in this medium with 200 μg/ml G418. A clone expressing >3 X 10^5 sites/cell as determined by [^{3}H]-NMS binding was selected for further experiments.

C. Construction of Mutants

Construction of m1 mAChR mutants were previously described (Moro *et al.*, 1993; Moro *et al.*, 1993). d232-358 is a mutant with intracellular third loop deletion from residue 232 to 358, K447 is a mutant with truncated COOH terminal from residue 447.

D. Receptor Binding Assay

Cells were seeded onto 12-well dishes, allowed to attach overnight, and then incubated for 90 minutes at 12°C with 2 nM [^{3}H]-QNB in PBS to measure total sites or with 2 nM [^{3}H]-NMS in PBS to measure surface sites. Non-specific binding was determined in the presence of 10 μM atropine. After labeling, cells were placed on ice, harvested with PBS, and filtered (S&S #32 glass fiber filter), followed by three rinses with ice-cold PBS. The radioactivity on the filters was determined by scintillation counting. Percent binding values were compared between carbachol-treated and untreated cells. Data for the time courses

presented are the averages of triplicate measurements from a representative experiment (repeated 2-3 times), and the error bars represent standard deviation.

E. Desensitization of m1 mAChR in G protein Coupling

Membranes from the cells stably expressing wild-type or mutant m1 AChRs were prepared as previous described (Burford & Nahorski, 1996). Briefly, cells were grown to confluence and harvested using 10 mM Hepes, 500 μM EDTA and 150 mM NaCl, pH 7.4. Cells were homogenized using a Dounce homogenizer and centrifuged at 500 rpm for 10 minutes. The supernatant was centrifuged at 12,000g for 30 minutes and the pellet resuspended in ice-cold buffer consisting of 10 mM HEPES, 10 mM MgCl2, and 100 mM NaCl (pH 7.4) at a final concentration of 100 μg/ml. Incubations were conducted in a final assay volume of 1 ml at 30°C for 60 min in the presence of 10 μM GDP and ~ 70 pM [^{35}S] GTPγS (1202 Ci/mmol) with or without CCh. Solubilized G proteins were then immunoprecipitated by incubation with G protein specific anti-serum and protein G-Sepharose beads at 4°C for 2 h. After extensive washing, the radioactivity on the beads was determined by scintillation counting.

F. Desensitization of Carbachol-Induced Inositol Phosphate Release

Cells stably expressing wild-type or mutant m1 mAChRs were assayed

for carbachol-stimulated phosphoinositide hydrolysis. Cells were plated onto 6-well culture dishes and allowed to reach ~80% confluency. Cells were labeled with [^{3}H]-myoinositol (0.2 mM) at 37°C for 24-48 hours. After inositol labeling, cells were incubated in incomplete medium with or without CCh (1 mM) for 5 min or indicated times. Pretreated cells were washed three times with PBS and then rechallenged with 1 mM carbachol at 37°C for the indicated time (0-5 minutes). Reactions were stopped by removal of medium and addition of methanol to each well. Cells were scraped and inositol phosphates recovered by methanol/chloroform extraction. Inositol (1,4,5) trisphosphate was isolated as previously described (Arden *et al.*, 1992; Maeda *et al.*, 1990). Results are expressed as fold increase in eluted inositol phosphates over basal level.

Desensitization was assessed by comparing the dose-response curves of total IP production as a function of increasing CCh concentrations for control cells and cells pretreated for indicated times with 1 mM CCh at 37°C.

G. *In vitro* Phosphorylation Using Rhodopsin as Substrate

Phosphorylation assays were carried out by Dr. Matthias Kassack (unpublished results). Briefly, the pEF-GRK2 expression construct (gift from T Haga, University of Tokyo, Japan) was stably transfected into HEK 293 cells. The culture medium was supplemented with 10 mM sodium butyrate for 16 h before the experiment. The cells were scrapped into 600 µl of ice-cold buffer (25 mM pH 7.5 Tris-HCl, 5 mM EDTA, 10 mM $Mg(OAC)_2$, 5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatine, 10 µg/ml leupeptine, and 10

μg/ml benzamidine), homogenized with a Dounce homogenizer. The samples were then centrifuged at 300,000g at 4°C for 30 min, and the supernatant recovered.

Rhodopsin phosphorylation was carried out using urea-treated bovine rod outer segments obtained as described (Bennett & Sitaramayya, 1988). The assay (50 μl final volume) contained 5 μM rhodopsin, 20 mM Tris-HCl (pH7.5), 2 mM EDTA, 6 mM $MgCl_2$, 100 μM ^{32}P-ATP (1000-1500 cpm/pmol) and 1 μg of HEK 293 cells cytosolic proteins. The samples were incubated in the dark or in room light at 25°C for 20 min. The reaction was quenched by adding 12.5 μl of SDS sample buffer, and electrophoresed through a 10% homogeneous SDS polyacrylamide slab gel. The gel was fixed, stained with Coomassie blue, vacuum-dried and exposed at -70 °C for 16-48 h for autoradiography.

H. Receptor Internalization

Measurements of muscarinic receptor internalization were performed in intact cells as previously described (Maeda *et al.*, 1990). Briefly, the cells were replated onto 12-well culture dishes and allowed to attach overnight. After incubated with or without 1 mM carbachol for up to 2 h, cells were placed on ice and washed three times with ice-cold phosphate-buffered saline to remove residual carbachol. Cell surface receptor and total receptors were assayed by incubating with 2 nM [^{3}H] NMS or [^{3}H] QNB in PBS at 12°C for 90 min respectively. At the end of incubation, the cells were placed on ice, removed from the wells, and filtered quickly on glass-fiber filters (SS#32), followed by

three rapid rinses with PBS. Three replicate independent samples were assayed for each treatment condition.

IV. RESULTS

A. G Protein Coupling and IP_3 Production of hm1 mAChR in HEK 293 cells

Using a stable HEK 293 cell line expressing wild-type hm1 mAChR, we studied at m1 mAChR activation at both G protein coupling level and second messenger production level. Particularly time course of activation by different concentrations of the muscarinic receptor agonist carbachol (CCh) was investigated. Using G protein specific antibody to immunoprecipitate individual Gα proteins, we measured m1 coupling to Gαs, Gαq, Gαi and Gαo proteins. CCh stimulated a 7 fold increase in Gq coupling, 2-3 fold increase in Gi coupling, and had negligible effects on the levels of Gs or Gi coupling (Figure 1.1). This is consistent with the notion that m1 is a Gq coupled receptor that stimulates inositol phosphate production.

To assess the onset of G protein coupling and IP_3 production, we compared the time course of m1 mAChR activation using both GTPγS binding and PI turnover assays. HEK 293 cells stably expressing hm1 mAChR were stimulated with 1 mM CCh, G protein coupling and second messenger production were then studied during a 60 min interval. Figures 1.2 A & B show that both G protein coupling and IP_3 production peaked at 5 min and reached a plateau after 10 min exposure to 1 mM CCh.

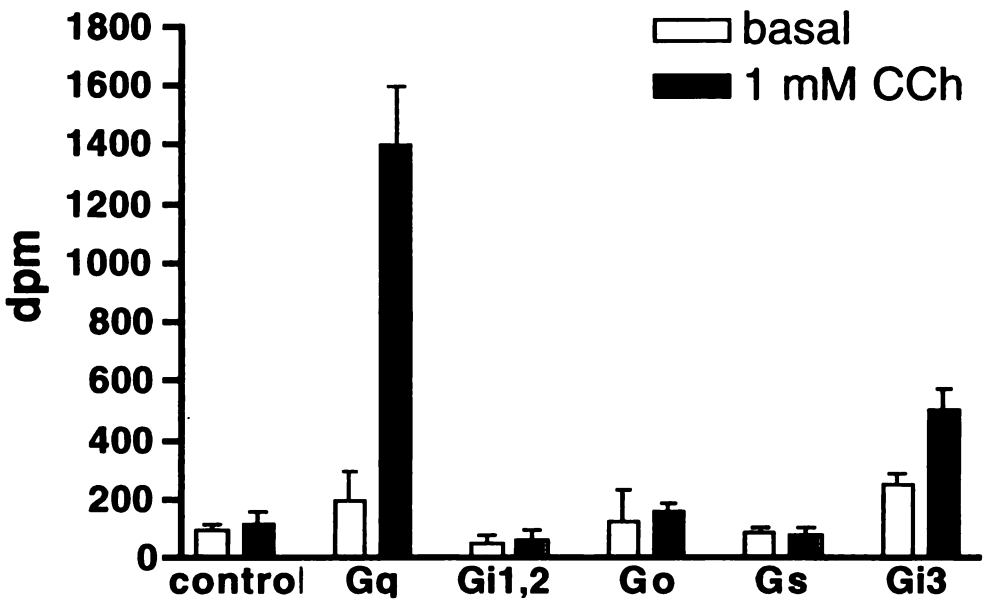


Figure 1.1. Carbachol-induced G protein activation by m1 mAChR as measured by [^{35}S] GTPγS binding assay. Membrane from untransfected and transfected HEK 293 m1 mAChR cells were prepared as decribed in Experiment Procedures, [^{35}S]GTPγS binding was determined with 1 mM CCh. Data are mean ± SD from four to six experiments.

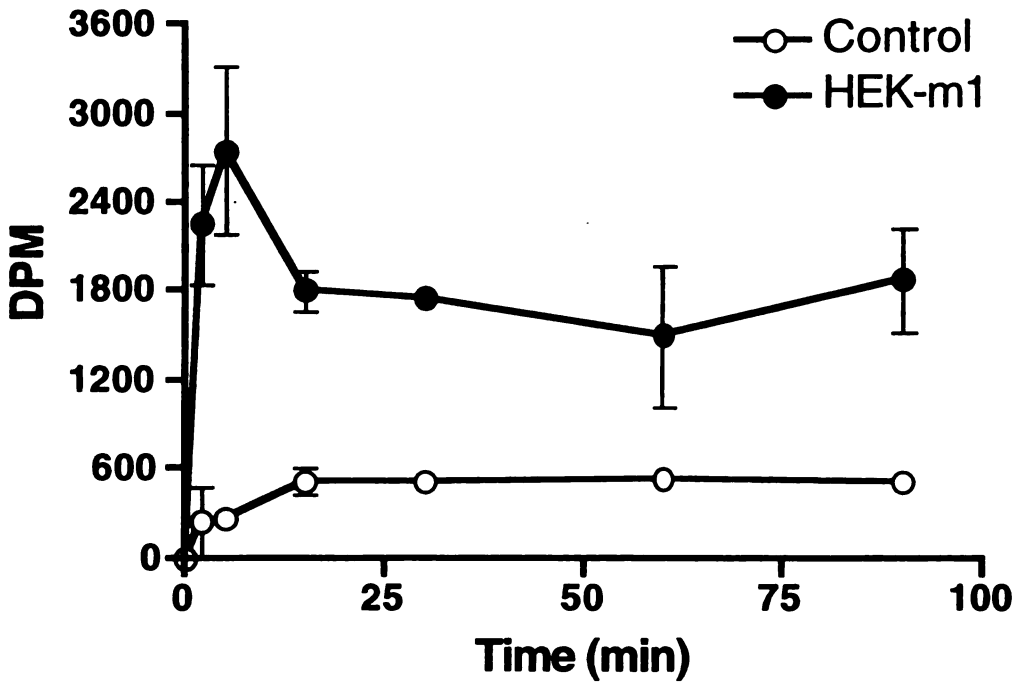


Figure 1.2A. Time course of CCh induced G protein coupling in m1 mAChR transfected HEK293 cells. Membranes from transfected cells were treated with 1 mM CCh for the indicated times, and Gq specific [^{35}S]GTPγS binding was determined. Data are mean ± SD from four to six experiments.

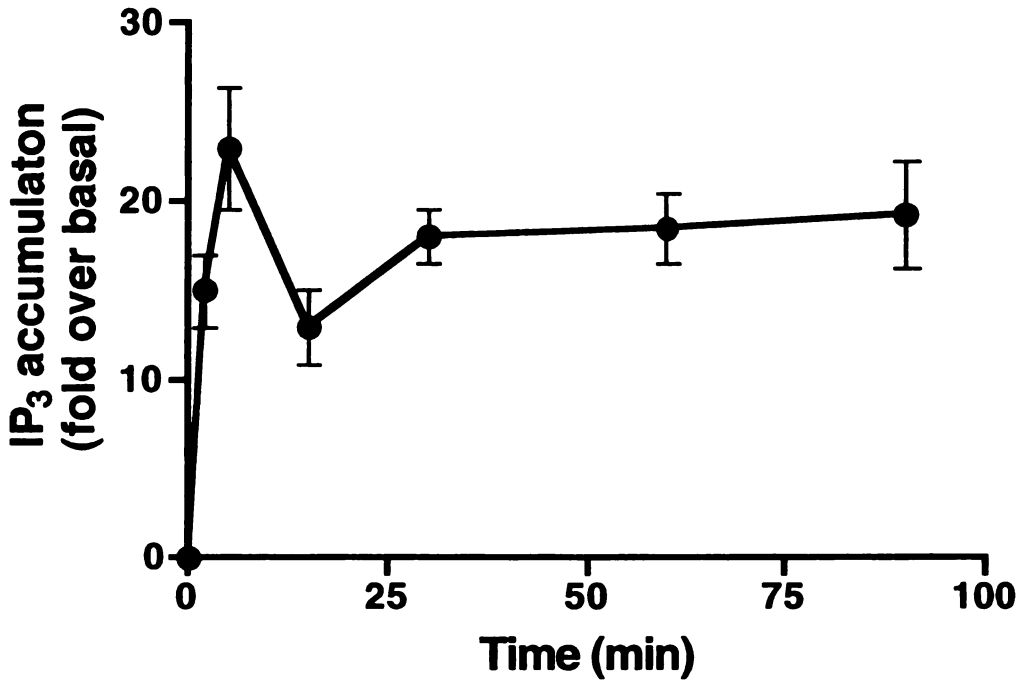


Figure 1.2B. Time course of CCh induced IP_3 production. Cells were treated with 1 mM CCh for the indicated times, and IP_3 production was measured as described in Experimental Procedures. Data are mean ± SD from four to six experiments.

B. Desensitization of m1 mAChR After 5 min Agonist Pretreatment

To determine whether carbachol-stimulated desensitization occurs at G protein coupling level, stably transfected HEK 293-hm1 cells were pretreated with 1 mM CCh for 5 min prior to a membrane preparation and subsequent restimulation with 1 mM CCh. Compared to medium stimulated control, 5 min pretreatment led to a 30% decrease in Emax, and the depression lasted for 60 min (Figure 1.3). Similar results were obtained for IP_3 production (Data not shown).

C. Role of Kinases in m1 mAChR Desensitization

To investigate the potential role of kinases and phosphorylation process in hm1 desensitization, we used transiently transfected system expressing both m1 mAChR and G protein coupled receptor kinases (GRKs) to characterize the changes in second messenger production. Among the five GRKs tested, the inhibition of IP turnover ranged from high to low GRK2> GRK3> GRK4, GRK5 and GRK6 (Figure 1.4). The kinase activity of these GRKs were shown to be similar using *in vitro* Rhodopsin phosphorylation assay by Dr. Mattias Kassack (Data not shown). We further characterized the function of GRK2 in regulating m1 mAChR desensitization.

D. Effects of GRK2 and DN-GRK2

There are several ways to study receptor phosphorylation, either through *in vivo* phosphorylation on the receptor itself or inhibition of kinase activity.

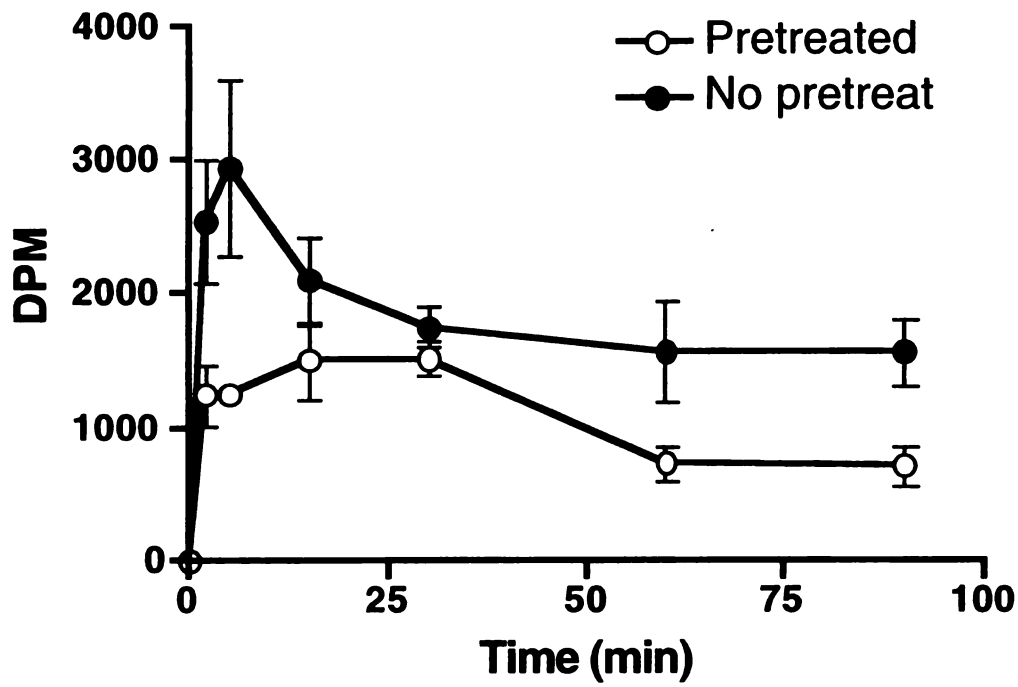


Figure 1.3. Desensitization of m1 mAChR measured by G protein coupling. Cells stably expressing m1 mAChR were pretreated with 1 mM CCh or medium for 5 min, washed with PBS three times. Membranes from both treatments were treated with 1 mM CCh for the indicated times, and Gq specific [^{35}S]GTPγS binding was determined. Data are mean ± SD from four to six experiments.

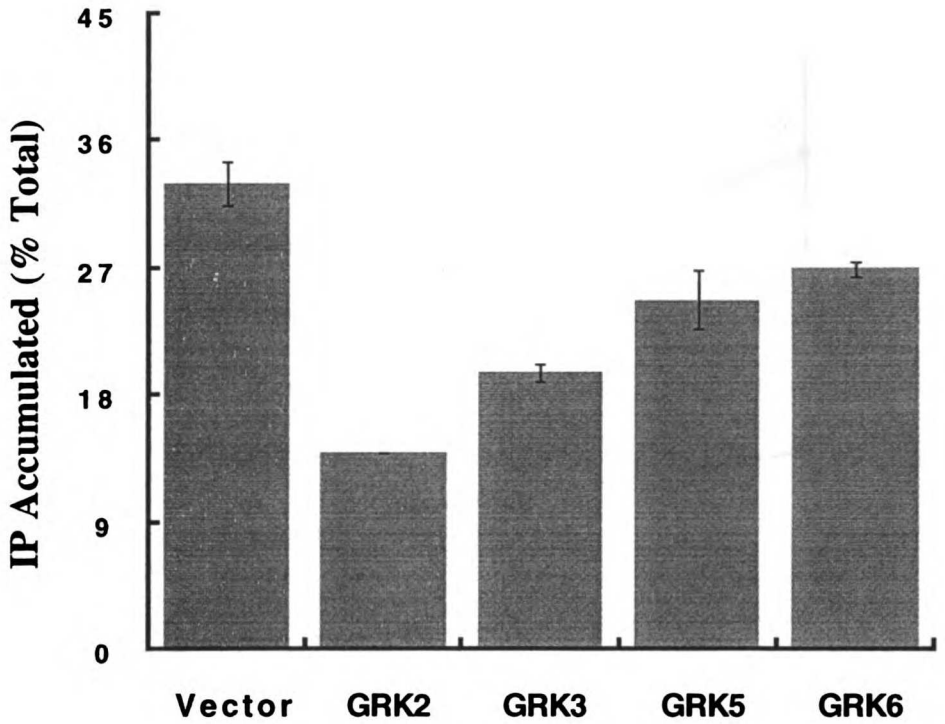


Figure 1.4. Effects of GRKs on m1 mAChR activation. Cells were transiently transfected with m1 and GRKs plasmid, IP3 production was determined 48 h after transfection as described in Experimental Procedures. Results are expressed as the fold increase in eluted IP_3 over basal (untreated). Data are mean ± SD from four to six experiments.

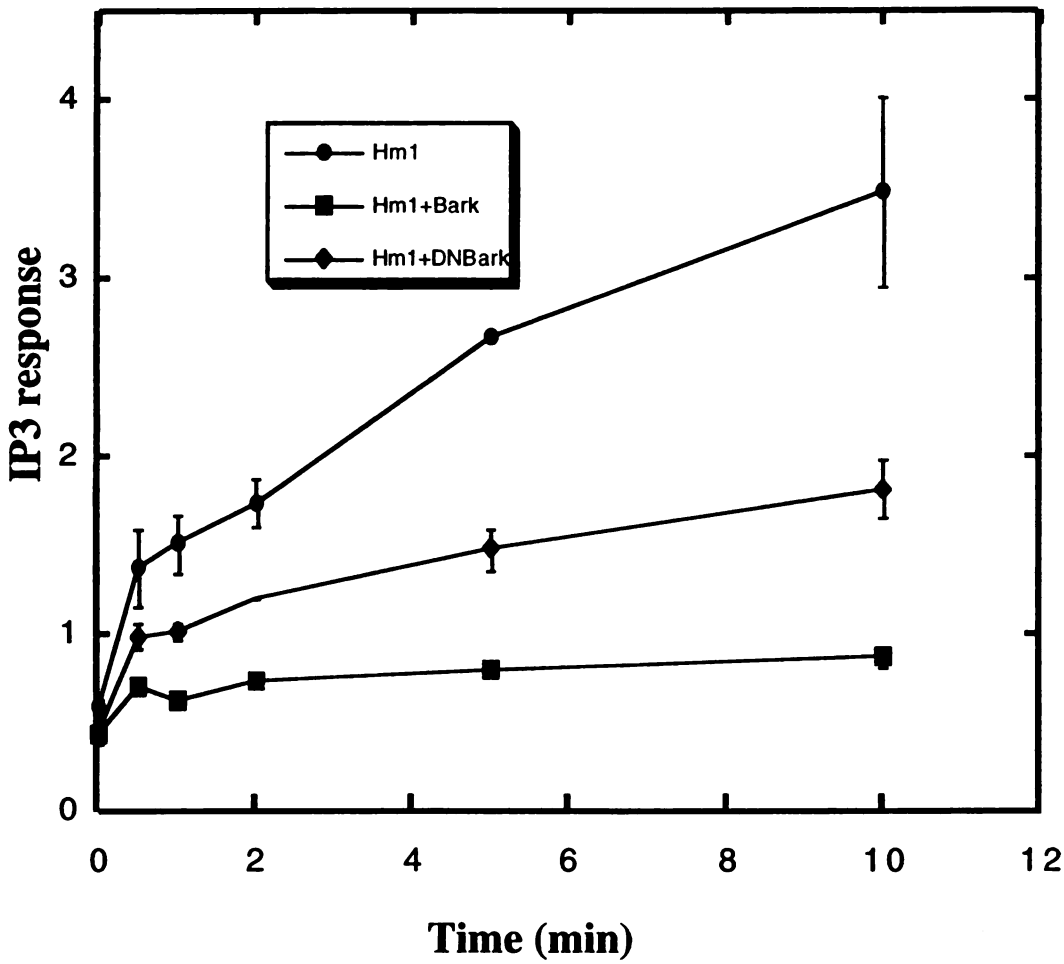


Figure 1.5. Time course of CCh induced IP_3 production in GRK2 and DN-GRK2 overexpression cell lines. HEK 293 cells were transfected with m1 mAChR DNA in pSG5 together with empty vector, 0.5 μg of pEF-GRK2 or DN-GRK2. Total amount of DNA was kept constant. Cells were stimulated at the indicated times, and IP_3 production was determined as described in Experimental Procedures.

Since there is no selective inhibitors for GRKs, we chose to express the dominant negative kinase to suppress the kinase activity. Dominant negative GRK2 (DN-GRK2) was constructed with a single amino acid substitution at the kinase catalytic domain (K220W). This mutant kinase was able to block the binding of active kinase to m2 mAChR and suppress the receptor phosphorylation (Tsuga *et al.*, 1998). When purified from HEK 293 cells over-expressing DN-GRK2, DN-GRK2 was able to block the rhodopsin phosphorylation by GRK2 in *in vitro* assay (Data not shown). Figure 1.5 shows the time course of CCh stimulated IP_3 production with overexpression of either GRK2 and DN-GRK2. Desensitization of m1 mAChR by GRK2 could be measured by a decrease in the Vmax for IP_3 production by about 70%, and to a lesser extent (~20%) by DN-GRK2.

E. Effect of Altered Ser/Thr Sites on Receptor Desensitization

The consensus sequences for GRKs phosphorylation have not been definitively determined. Previous studied suggested that phosphorylation sites for other GPCRs are located in both the third intracellular loop and carboxyl terminus (Bouvier *et al.*, 1988; Inglese *et al.*, 1993). To identify the possible phosphorylation sites of hm1, we evaluated desensitization of mutants with serine or threonine deletions. The first mutant d232-358 was constructed with deletion in i3 loop from residue 232 to 358, which includes most of serine/threonine residues in this region. K447 is a mutant with C- terminal truncated at residue 447. Other COOH terminal ser/thr residues are embedded

in membrane based on receptor structure prediction (Baldwin, 1993) and presumably would not be accessible to kinases. These mutants were transiently cotransfected with GRK2 or DN-GRK2 in HEK 293 cells, and their IP production after exposure to CCh was assessed as described above. Both mutants stimulated ~10 fold increase over basal levels (untransfected cells), which indicates normal coupling to G proteins as measured by IP production (data not shown). Figure 1.6 shows the comparison between mutant and wildtype. d232-358 exhibited similar pattern as wildtype with GRK2 or with DN-GRK2. In contrast, carboxyl terminal truncation mutant K447 was sensitive to both GRK2 and DN-GRK2.

F. Effect of Internalization on m1 mAChR Desensitization

The adaptor protein β-arrestin has been shown to cause desensitization of GPCRs by precluding further interactions of the GPCRs and G proteins. To examine the role of enhanced internalization on m1 mAChR desensitization, β-arrestin was co-expressed with m1 and GRK2 or DN-GRK2 respectively. The coexpression of β-arrestin alone did not significantly increase the extent of desensitization of wild type receptor. We also did not observe any synergistic effect with the combination of GRK2 and β-arrestin (Figure 1.7). This is also consistent with the observation that m1 mAChR mutants with internalization defects exhibited identical desensitization profile as wild-type (Data not shown).

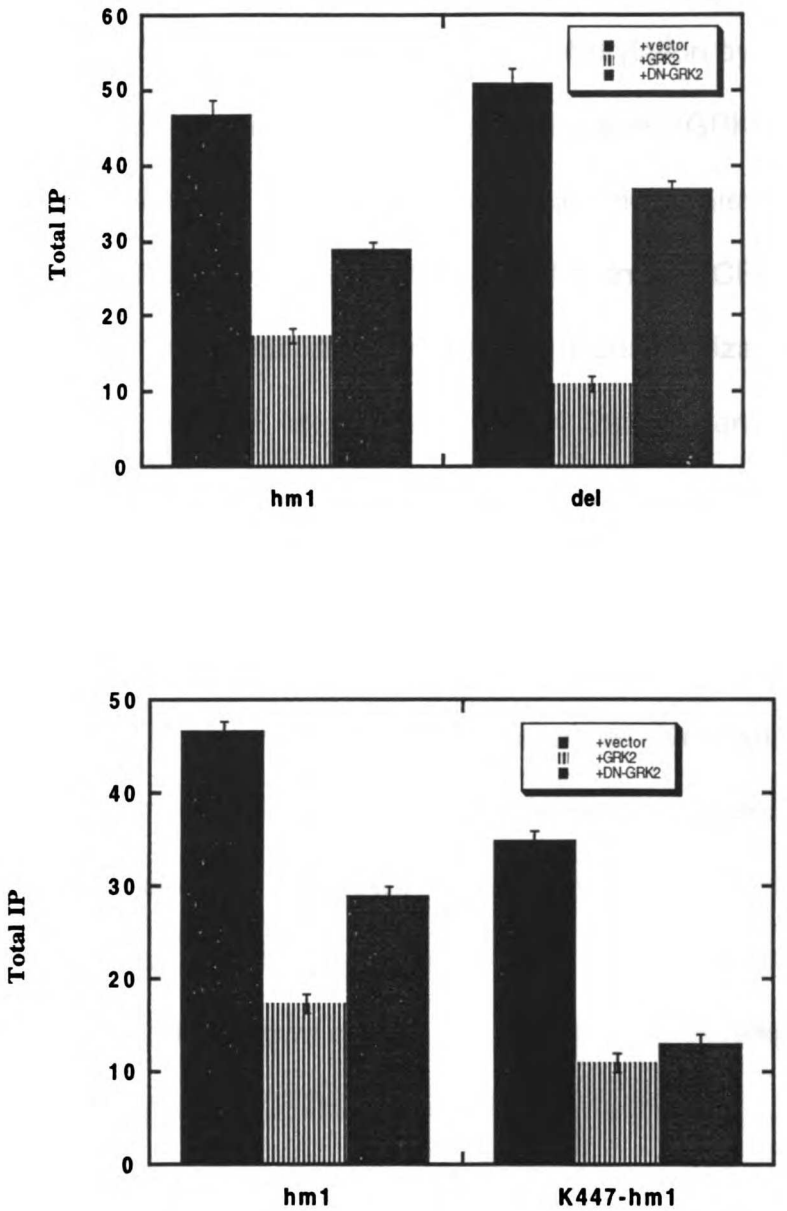


Figure 1.6. Effect of Mutants with Altered Ser/Thr Sites on Receptor Desensitization. HEK 293 cells were transfected with d282-385 mAChR or K447 DNA in pSG5 together with empty vector, 0.5 μg of pEF-GRK2 or DN-GRK2. Total amount of DNA was kept constant. Cells were stimulated at the indicated times, and IP_3 production determined as described in Experimental Procedures.

V. DISCUSSION

This study focused on the effect of phosphorylation by kinases, especially receptor-specific G protein-coupled receptor kinases (GRKs), on regulation of receptor desensitization. While the molecular mechanisms involved in the phosphorylation-dependent desensitization of many GPCRs have been fairly well delineated, the mechanism of m1 mAChR desensitization has been less forthcoming. m1 mAChR expressed in HEK 293 desensitized after 5 min pretreatment with 1 mM CCh as measured by GTPγS assay. We employed both stable and transient transfected cell line systems to study the GRK2 effect on the G protein coupling. Our results showed that overexpressing GRK2 caused desensitization. Surprisingly, over-expression of dominant negative GRK2 mutant also attenuated the m1 activity. In comparison, protein kinase A and C inhibitors had no effect on m1 mAChR desensitization (data not shown). One hypothesis is that DN-GRK2 binds to the phosphorylation sites of the endogenous GRK2, but allows access of other kinases to different sites thereby desensitizing the receptor. This suggests that GRK2 might not be the major kinase involved in phosphorylation of hm1, neither were the other members of GRK family. However, phosphorylation by GRKs, if not totally required, may nevertheless participate in the desensitization process. Phosphorylation of the receptor by GRK2 appeared to be responsible for part of the observed homologous desensitization.

The above results are consistent with the observation made by Budd et al. (Budd *et al.*, 2000). They have proposed a new family of receptor specific

kinases- muscarinic receptor kinases responsible for desensitization of muscarinic receptors. They have suggested an alternative route to receptor phosphorylation by casein kinase (Budd *et al.*, 2000).

To further explore the correlation between the phosphorylation sites and receptor desensitization, we studied mutants with phosphorylation site deletions. The major sites phosphorylated by GRK2 were reported in the third intracellular loop (Haga *et al.*, 1996). Measured by IP_3 production, intracellular third loop deletion mutant d282-358 showed similar pattern when co-expressed with GRK2 or DN-GRK2 from wildtype. This suggests that GRK2 may not be the major kinase involved, even though GRK2 have been shown to phosphorylate m1 mAChR *in vitro* (Haga *et al.*, 1996). In contrast, carboxyl terminus deletion K447 showed sensitivity to both GRK2 and DN-GRK2. These results suggest that C-terminal region might be the major phosphorylation sites for other kinases, such as muscarinic receptor specific kinases (Tobin *et al.*, 1997).

A complex series of events participate in the 'turn-off' of GPCRs in both physiological and pharmacological settings. Internalization of receptor-ligand complexes is an essential aspect of the function of G protein-coupled receptors. Recent studies have shown the paradoxical relationship between receptor internalization and desensitization. Receptor desensitization could be suppressed by removing the GRK phosphorylation sites of the β2-adrenergic receptor without impairing receptor internalization (Strader *et al.*, 1987). Furthermore, mutants unable to internalize were still capable of desensitization (Barak *et al.*, 1994; Yu *et al.*, 1993). However, m2 muscarinic acetylcholine

receptor internalization can be facilitated by overexpression of GRK2 and expression of a dominant negative mutant of this kinase decreases the rate of receptor internalization (Tsuga *et al.*, 1998). Furthermore, endocytosis of an internalization-resistant mutant of the α_2-adrenergic receptor could be induced by overexpression of GRK2, which phosphorylated the receptor (Ferguson *et al.*, 1995). Our study with over-expression of β-arrestin suggested that desensitization and internalization may be controlled by different molecular mechanisms in m1 mAChR regulation.

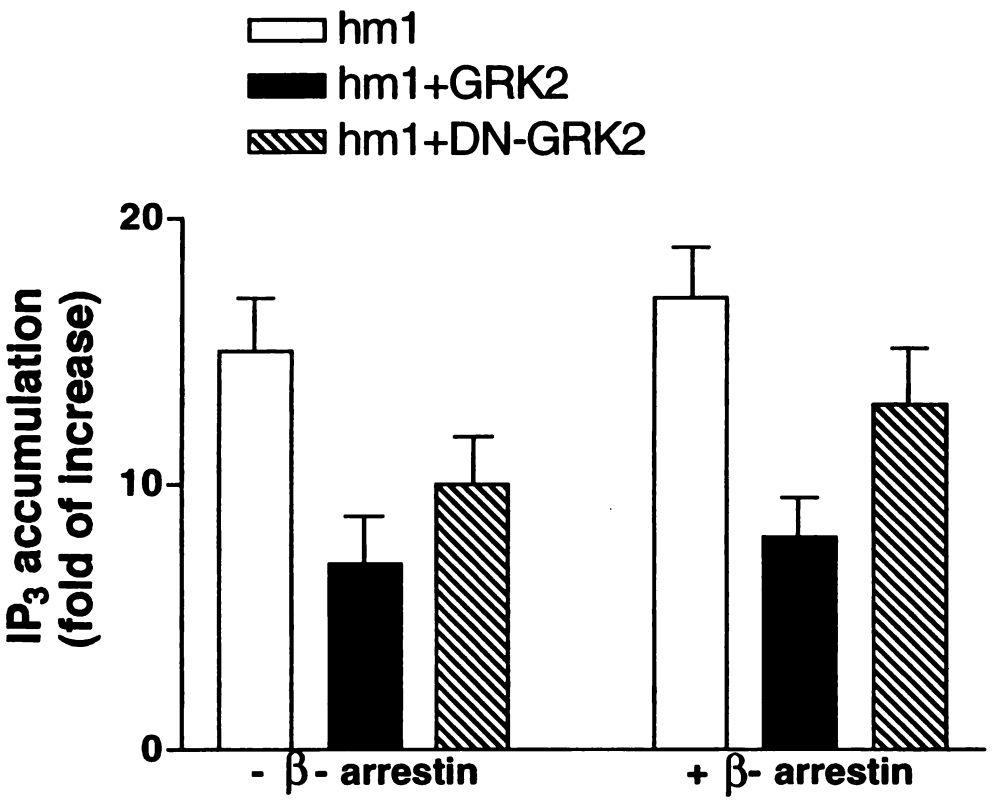


Figure 1.7. Effect of β-arrestin overexpression on receptor desensitization. HEK 293 cells were transfected with m1 mAChR, β-arrestin and pEF-GRK2 or DN-GRK2. Total amount of DNA was kept constant. Cells were stimulated at the indicated times, and IP_3 production was determined as described in Experimental Procedures.

CHAPTER 2

HUMAN m1 MUSCARINIC ACETYLCHOLINE RECEPTOR ACTIVATES SRF-MEDIATED GENE TRANSCRIPTION THROUGH Gq INDEPENDENT PATHWAYS

I. SUMMARY

How GPCRs stimulate nuclear transcription factors is still poorly understood. To delineate the pathways of mAChRs responsible for activating the nuclear transcription factor, serum response factor (SRF), genes encoding m1~3 mAChR were co-expressed with a reporter gene driven by SRF in lymphocyte Jurkat T cells. We show that only m1 mAChR activated SRF through a pathway involving the small GTPase RhoA. This was shown to be independent of Gαi/o and Gαq. Transfection of constitutively active Gα13 strongly induced SRF activation, but additional m1 mAChR stimulation further activated SRF. This finding raised the possibility that m1 mAChR might activate SRF through multiple independent pathways. One such pathway from m1 mAChR to SRF was shown to involve calmodulin (CaM) and the phosphatase calcineurin (PP2B). Whereas CaM and calcineurin are known to regulate activation of NFAT (nuclear factor of activated T cells), this is a novel pathway for SRF activation. The complete pathway remains to be elucidated.

II. INTRODUCTION

GTP-binding proteins (G proteins) are recognized as central elements in many types of membrane-mediated cell signaling, coupling extracellular stimuli to intracellular effector responses (Gilman, 1987). Two distinct G protein superfamilies, monomeric and heterotrimeric, have been identified. Historically, they have been thought to regulate distinct classes of cellular function. The monomeric, small molecular weight G proteins (Ras and Ras like) mediate slow signals that control cellular events such as mitogenesis and differentiation, largely through regulating gene transcription. They are activated by growth factors via membrane tyrosine kinases and target a variety of downstream effectors, which, in turn, activate transcription factors (Janknecht & Hunter, 1996). Such pathways are critical for development and differentiation. In contrast to monomeric G proteins, the heterotrimers (consisting of α and $\beta\gamma$ subunits) are generally considered to mediate faster signals controlling acute cellular responses such as synaptic transmission or sensory reception that do not require alterations in gene transcription. They are activated by a myriad of transmitters and sensory stimuli. Their downstream target effectors, such as cyclases, phospholipases, and ion channels, are also distinct from those activated by monomeric G proteins (Figure 2.1).

Increasing evidence suggests that pathways mediated by two families of G proteins are intertwined and GPCRs interact with growth factor activated pathways (Marinissen *et al.*, 1999). The interaction occurs through the major components that participate in growth factor signaling. Growth factor signaling

is mediated, in large, by several groups of sequentially activated protein kinases, collectively known as the mitogen-activated protein kinase (MAPK) cascades (Seger & Krebs, 1995). These pathways also involve recruitment of adaptor molecules and activation of small GTPases such as Ras, Rap, Rac and CDC42 that transmit the signal further to other pathways. Many of the components that participate in growth factor signaling (small GTPases, MAPKs) are also activated by ligands stimulating G-protein coupled receptors (GPCRs). For example, Gi/o coupled receptors, such as m2 muscarinic receptors and mu-opioid receptors, exert their mitogenic effects through MAPK pathways (Schulz & Hollt, 1998). However, much less is known about the mechanisms underlying GPCRs activation of growth related transcription.

Memory consolidation involves changes in gene expression, especially an increase in transcription of immediate early genes (IEGs), such as *c-fos* and *c-jun*. It has been reported that muscarinic receptors mediate induction of *c-fos* expression in rat brain, glial cells and other neuronal cells (Chou *et al.*, 1992; Cohen *et al.*, 1996; Ding *et al.*, 1998; Larsson *et al.*, 1994; Tsiokas & Watson, 1995). However, the mechanism of nuclear signaling pathway of muscarinic receptors remains unclear.

The focus of the present work was on the transcriptional regulation of IEGs mediated by muscarinic receptors. IEGs can be induced in the absence of new protein synthesis, and they regulate the expression of a variety of proteins involved in mitogenesis and neuronal differentiation (Burstein *et al.*, 1998; Hill *et al.*, 1993).

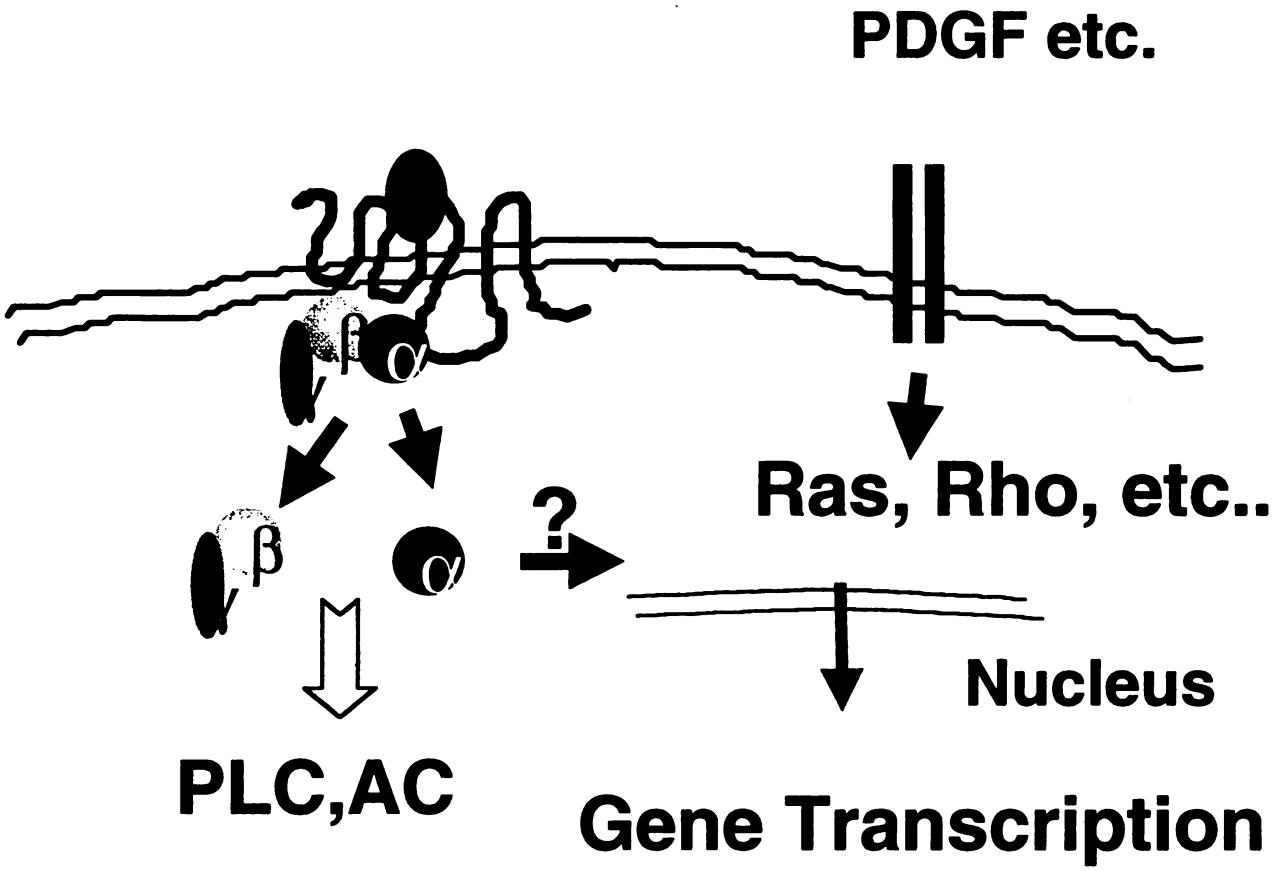


Figure 2.1. Signal transduction pathways involving growth factors, tyrosine kinase receptors and G protein coupled receptors.

Serum response factor (SRF) is a key regulator of IEG transcription. It is a ubiquitous transcription factor that mediates serum and growth factor-induced transcriptional activation of the *c-fos* proto-oncogene. SRF plays a central role in the transcriptional response of mammalian cells to a variety of extracellular signals. The importance of SRF in growth factor-regulated transcription is suggested by the identification of SRF binding sites, serum response elements (SRE) within the regulatory region of many transiently expressed serum-inducible genes (Spencer & Misra, 1996).

SRE presents in many immediate-early gene promoters, and is activated by both growth factor stimulation and oncogene products such as Src, Ras and Raf. Growth factors activate cellular mitogen-activated protein kinases (MAP kinases) via a conserved kinase pathway under the control of Ras and Raf proteins, suggesting that the MAP kinase pathway is involved in SRE activation (Treisman, 1994).

Several lines of evidence support the hypothesis that SRF activation is mediated through small GTPase RhoA and several GTP binding proteins. Rho is known to mediate the assembly of integrin-containing focal adhesions and actin stress fibers (Treisman *et al.*, 1998). Recently several groups have shown that heterotrimeric G proteins can also mediate SRE activation through RhoA. However, the identity and specificity of G protein(s) involved in this process is controversial (Gutkind, 1998).

In this study, lymphocyte Jurkat T cells were transfected with genes encoding m1-3 mAChR to study the signaling pathway from receptor to SRF

using luciferase reporter assay. In this system, m1 mAChR activated SRF mediated gene transcription through Rho in a Gi/o and Gq independent manner. Constitutively active Gα13 activated Rho pathway independent of receptor activation.

G13 mediated pathway was not affected by calmodulin and phosphatase calcineurin inhibitors, while m1 mAChR was. These findings raise the possibility of the existence of multiple independent pathways dictating muscarinic receptor nuclear signaling pathways.

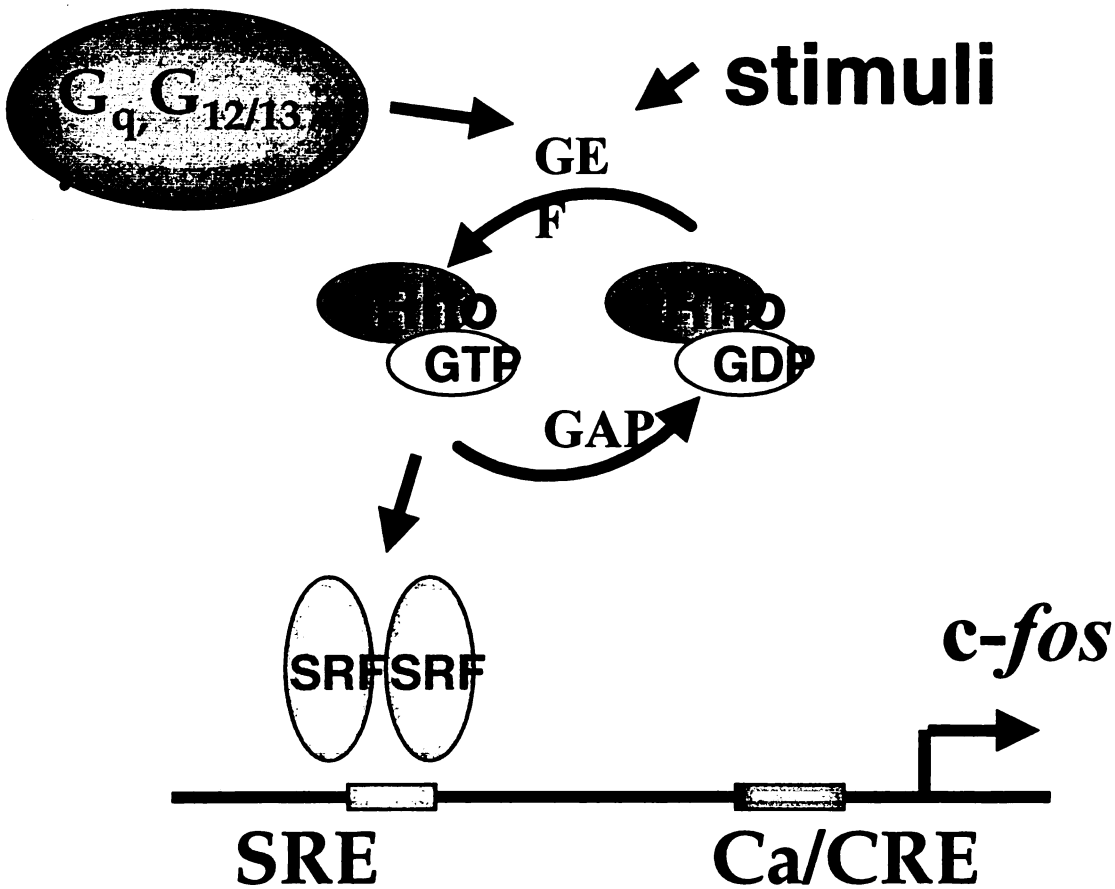


Figure 2.2 Heterotrimeric and monomeric G proteins activate *c-fos* pathway.

III. EXPERIMENTAL PROCEDURES

A. Cell Culture and Transfection

Jurkat Leukemic T cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 50 units/ml penicillin and 50 μg/ml streptomycin in 5% CO_2 incubator at 37°C. Transfections were performed using LipofectAMINE Plus (Life Technologies, Inc.) according to manufacturer's instructions. Jurkat T cells ($1x10^7$) were cotransfected with 0.2 μg SRE.L-Luciferase plasmid and 2 μg of either pSG5a vector, or muscarinic receptor cDNA containing plasmid. The transfection was stopped after 5 h by adding medium containing 0.5% serum.

B. DNA Constructs

Gαq and Gα12 protein constructs (pCDNAI) and Gα13 constructs (pCEV29) were kindly provided by Drs. Ethan Burstein and Mark Brann (Acadia Pharmaceuticals, San Diego). Rho constructs were gifts from Dr. Marc Symons (The Picower Institute for Medical Research). SRE.L-Luciferase reporter plasmid (Buhl *et al.*, 1995) and C. botulinum C3 ADP-ribotransferase (C3) were kindly provided by Dr. Songzhu An (UCSF).

The genes encoding the human muscarinic cholinergic subtypes receptors (m1-3) in pSG5 vector were obtained from a human placental genomic library as previously described (Maeda *et al.*, 1990). m3/m1 chimera was made as described (Moro *et al.*, 1993).

C. Reporter Gene Assay

Eighteen hours after transfection, cells were replated in 96 well plates and stimulated with medium or reagents at 37 °C for 4 h as indicated. Cells were then lysed by Reporter Lysis Buffer (Promega, Madison, WI), and luciferase activities measured by luminometer (EG& G).

The Dual-Luciferase® Reporter (DLR) Assay System (Promega, Madison, WI) was used to normalize the transfection efficiency. pRL-CMV Vector containing constitutive expression of Renilla luciferase was co-transfected with SRE.L reporter genes and dual luciferase activities were measured according to the protocol.

D. Receptor Binding Assay

The levels of m1-3 mAChR expressed by the cells were determined by measuring the binding of [^{3}H] NMS on the surface of live cells as previously described (Lameh *et al.*, 1992). Briefly, transfected cells were incubated with 1.5-2.0 nM [^{3}H]-N-methyl scopolamine (NMS) in PBS at 12°C for 90 min. After labeling, cells were placed on ice, filtered and rinsed with ice-cold PBS three times (S&S #32 glass fiber filter). The radioactivity on the filters was determined by scintillation counting.

E. Intracellular Ca^{2+} Measurement

Measurement of intracellular calcium release is described in Chapter 4. Briefly, 18 hr after transfection, cells were washed with Kreb-HEPES buffer and

loaded with 3 μM Oregon Green 488 fluorescent dye at room temperature for 30 min. After loading, cells were washed with Kreb-HEPES buffer with 0.5% BSA three times and diluted to approximately $2x10^6$ cells/ml and distributed evenly (ca. $3x10^5$ cells/well) into an opaque, white 96-well plate (Corning Costar, Cambridge, MA). Buffer alone or buffer containing test compounds was injected sequentially into separate wells and the fluorescence intensity monitored at 1 s intervals using an excitation wavelength of 485 nm and emissions of 538 nm.

F. Effect of PTX on m2 mAChR mediated Adenylate Cyclase Inhibition

As control for PTX pretreatment in m1 mAChR mediated SRF activation, Jurkat cells transfected with m1 mAChR was pretreated with 300 nM PTX and cAMP inhibition was measured using cAMP kit (Amersham).

G. Data Analysis

Statistical analyses were performed using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA).

IV. RESULTS

A. Activation of SRF by Muscarinic mAChR Receptors

We tested the ability of transfected m1-m3 mAChRs to regulate serum response factor mediated gene transcription in Jurkat cells. SRF-mediated gene transcription was evaluated by the activity of luciferase as reporter enzyme. The DNA construct used here, SRE.L, is a derivative of c-Fos serum response element (SRE) and is only activated by serum response factor (SRF) but not tertiary complex (Hill *et al.*, 1994). So the production of luciferase under control of SRE.L depends mainly on SRF activation. The plasmid expressing different muscarinic receptors were cotransfected with the SRE.L-Luciferase reporter gene into Jurkat cells. Treatment with muscarinic receptor agonist carbachol induced SRF activation only in m1 transfected cells (Figure 2.3). Although all three transfected cell lines expressed comparable levels of receptor on the surface as measured by membrane impermeable antagonist NMS (Figure 2.4). SRF activation induced by m1 mAChR was antagonized with 10 μM atropine, indicating that activation was a receptor-mediated process.

SRF mediated gene transcription was activated in m1 mAChR transfected cells within 1 h, and reached a maximum after 4 h carbachol treatment (Figure 2.5). Thus, 4 hrs of treatment was used in all the follow-up experiments. SRF was activated in a dose-dependent manner, with carbachol EC50 at 10 μM, which is comparable to the EC50 measured in IP_3 production and Ca^{2+} release by m1 (Figure 2.6).

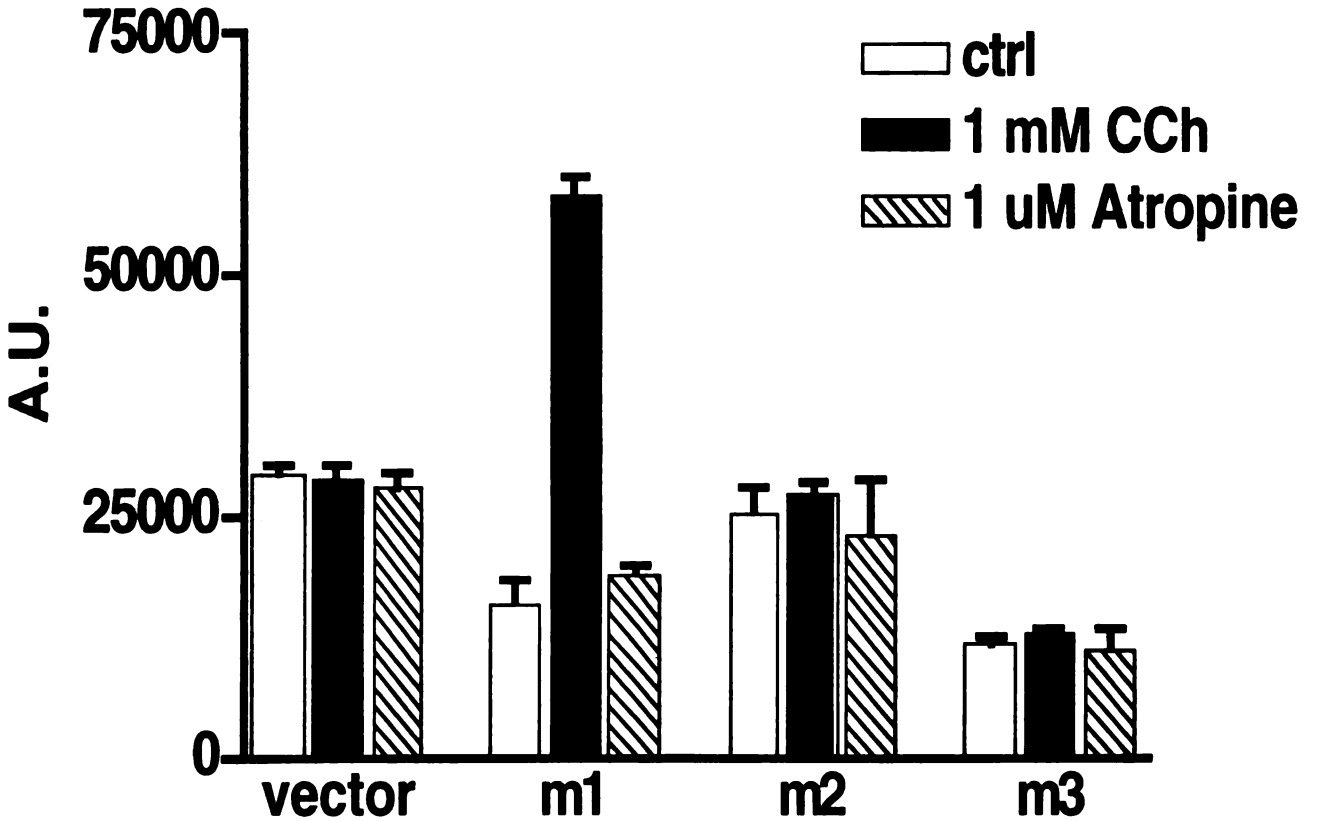


Figure 2.3. Differential regulation of SRF-mediated gene transcription by muscarinic receptor subtypes in Jurkat cells. Jurkat cells were cotransfected with 0.2 μg of SRE.L-luciferase reporter plasmid, and 2 μg pSG5 vector, m1, m2 or m3 receptor cDNA. 18 h later, aliquots of $1x10^5$ cells in 0.15 ml were transferred to 96-well plates and treated with media control, 100 μM carbachol(CCh) or CCh with 1 μM atropine as indicated. Cells were lysed 4 h later and luciferase activity was measured. Experiments were done at least twice and each point is the average of triplicate. Error bars represent S.D.

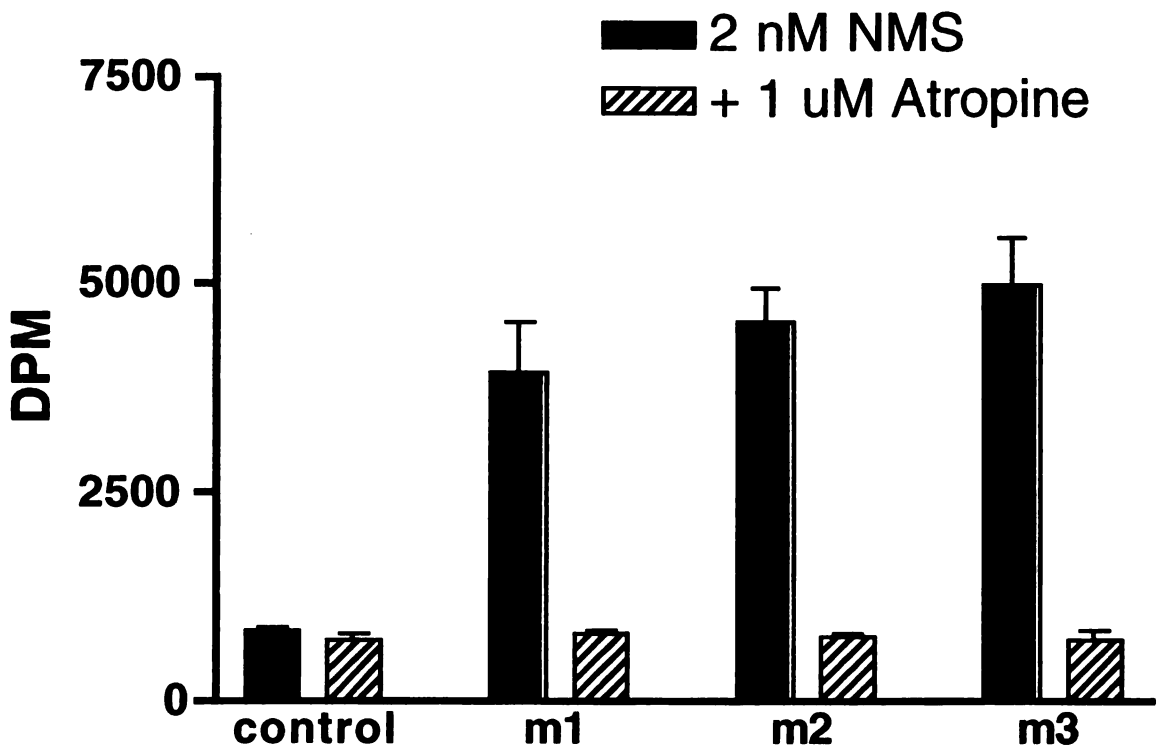


Figure 2.4. Muscarinic receptor expression level measured by ^{3}H-NMS. Using same pool of transfected cells as in Fig.2.3, cells were incubated with 2 nM ^{3}H-NMS with or without 1 μM atropine at room temperature for 45 min. Cells were then harvested in PBS, and radioactivity was quantified by liquid scintillation counting. Results were expressed as the total dpm and are representative of three independent experiments performed in triplicate.

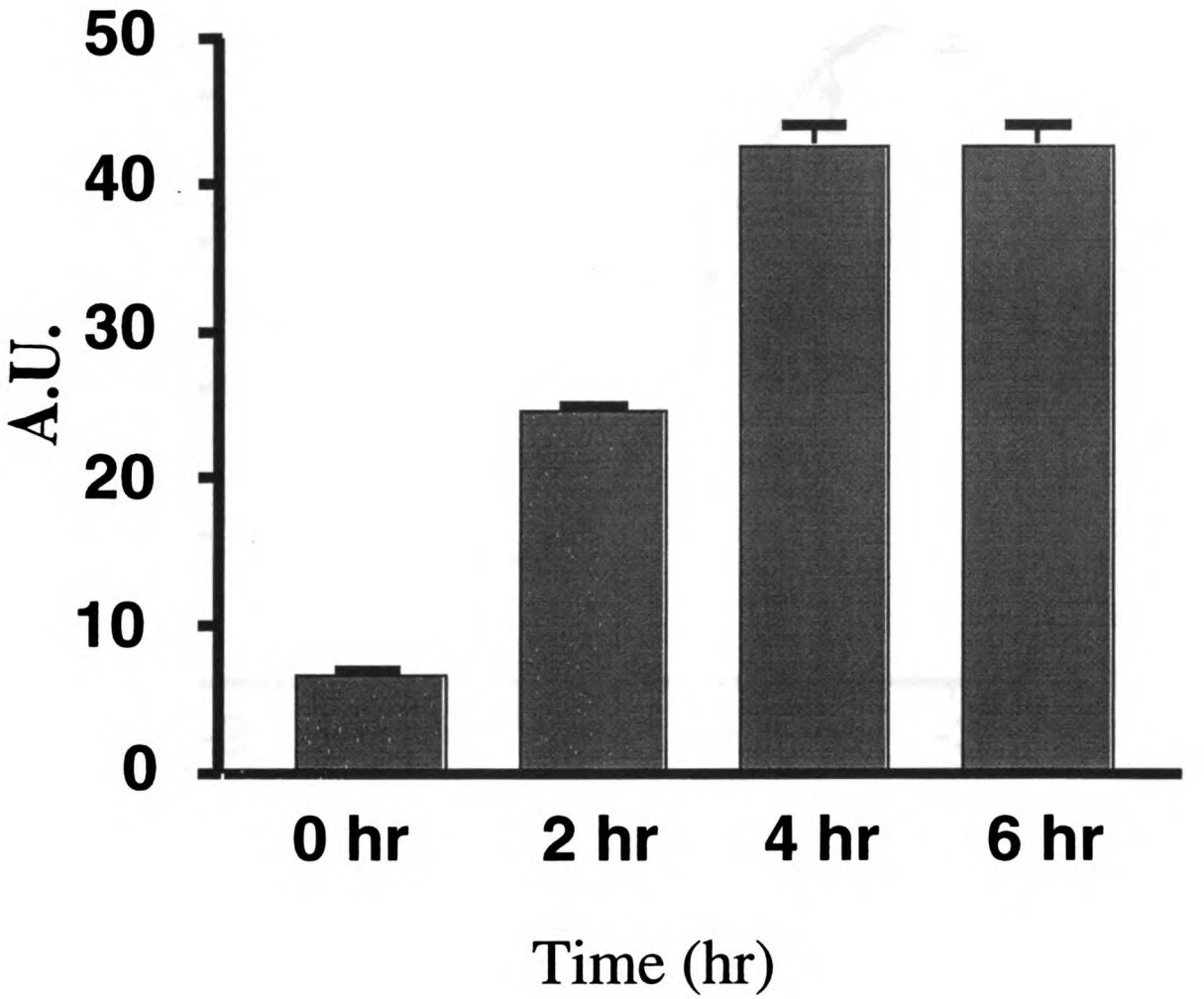


Figure 2.5. Time course of SRF activation by m1 mAChR. Cells transfected with m1 and SRF plasmids were stimulated with 100 μM CCh as described in Figure 2.4, a portion of cells were lysed and measured at indicated times . Experiments were carried out at least twice and each point is average of triplicates. Error bars represent S.D.

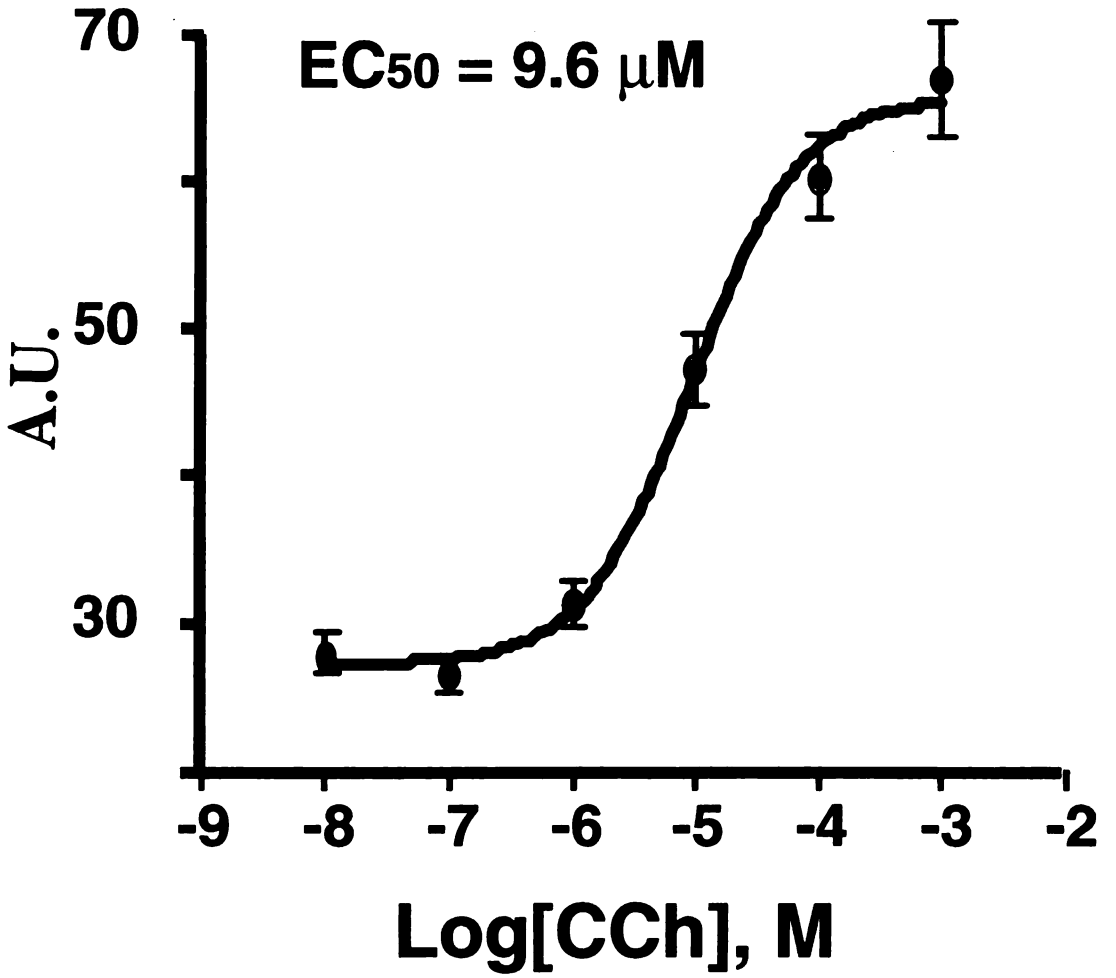


Figure 2.6. Concentration-dependent stimulation of SRF by m1 mAChR. Jurkat T-Ag cells were cotransfected with 2 μg of m1 receptor expressing plasmid and 0.2 μg of SRE.L-luciferase plasmid. 18 h later, cells were stimulated with increasing concentration of CCh. Cells were lysed 4 h later and luciferase activity measured. Experiments were done at least twice and each point is average of triplicates. Error bars represent S.D.

B. Involvement of Rho in SRF Activation by m1 mAChR

It has been reported that small GTPase such as RhoA and CDC42 are involved in SRF activation in various cellular systems. In the present study, two different approaches were utilized to confirm the role of small GTPases in SRF activation. First, the C3 toxin (Clostridium butulinum C3 transferase), which specifically inhibits RhoA by ADP-ribosylation (Quilliam *et al.*, 1989) was expressed along with m1 mAChR. Second, the dominant negative RhoA construct Rho N19A, which is deficient in binding to downstream effectors, was used. In the transfection system used, both C3 (Figure 2.7A) and dominant negative Rho (Figure 2.7B) blocked the SRF activation in a DNA dose-dependent manner.

C. Involvement of PTX Sensitive G protein in m1 mAChR Mediated SRF Activation

It has been shown that m1 mAChR is capable of activating multiple G proteins, including Gq/11 and Gi/o (Table 2.1). Gi/o coupled receptors have been shown to activate SRE through α subunits or MAPK pathway. To test whether Gi/o is involved in this process, cells transfected with m1 mAChR and SRF were pretreated with 300 nM pertussis toxin (PTX) for three hours, which completely suppressed Gi/o mediated adenylate cyclase inhibition in a parallel control experiments (Data not shown). PTX had no effect on SRF activation. (Figure 2.8). This result is consistent with the observation that Gi/o coupled m2 mAChR did not activate SRF (Figure 2.2).

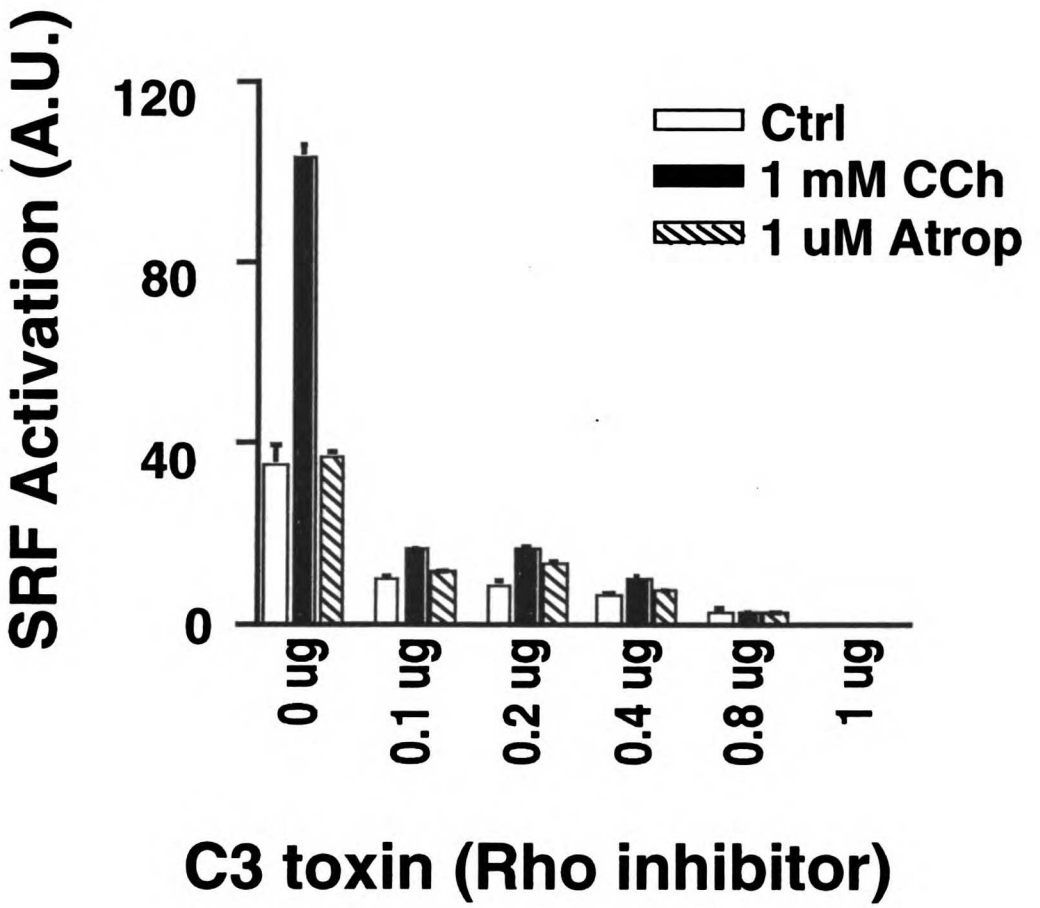


Figure 2.7 A. Rho is involved in SRF activation. Jurkat cells were cotransfected with 0.2 μg SRE.L-luciferase, 0.5 μg of m1 mAChR plasmid, and increasing concentration of C3 expression plasmid as indicated. 18 h later, cells were treated with 100 μM CCh and lysed for luciferase activity 4 h later. Results are representative of three independent experiments performed in triplicates. Error bars represent S.D.

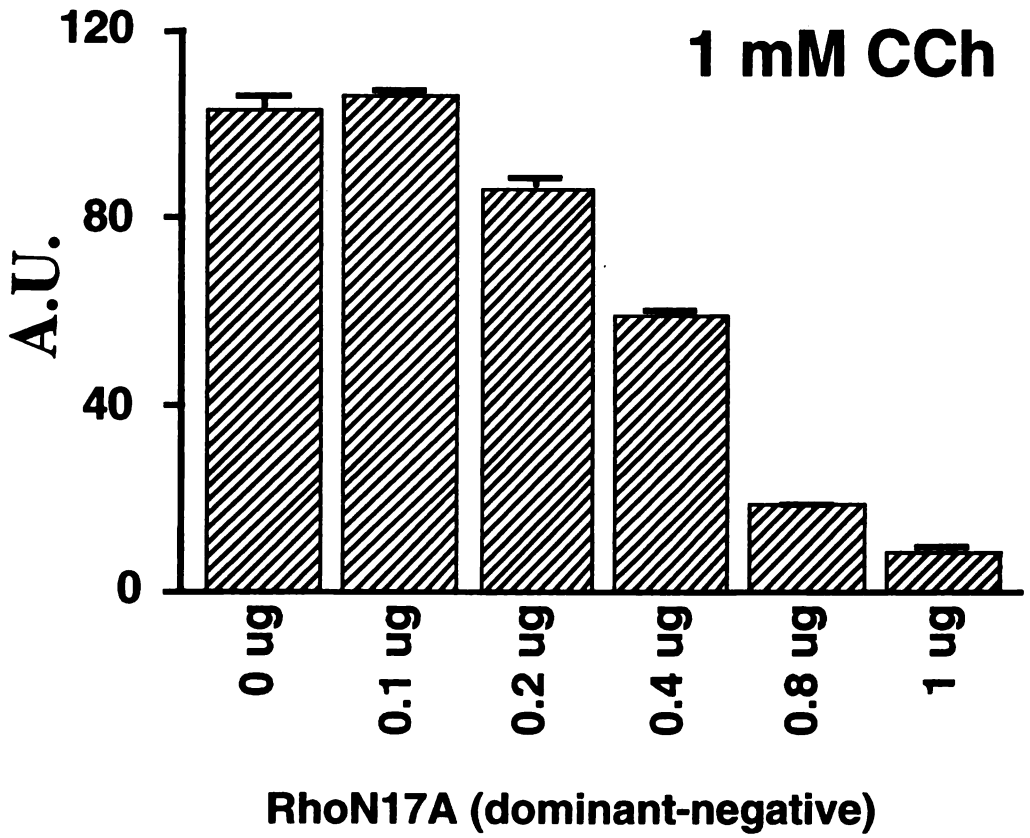


Figure 2.7 B. Rho is involved in SRF activation. Jurkat cells were cotransfected with 0.2 μg SRE.L-luciferase, 0.5 μg of m1 mAChR plasmid, and increasing concentration of RhoN19A expression plasmid as indicated. 18 h later, cells were treated with 100 μM CCh and lysed for luciferase activity 4 h later. Results are representative of three independent experiments performed in triplicate. Error bars represent S.D.

D. Role of Gq in SRF Activation by m1 mAChR

It has been demonstrated that m1 mAChR activates SRF through heterotrimeric G proteins, both Gq and G12/13 have been implicated in this process (Gutkind, 1998; Mao *et al.*, 1998). However, the close relative of the m1 mAChR receptor, Gq-coupled m3, failed to activate SRF in Jurkat cells. The inability of m3 mAChR to activate reporter gene transcription could have been due to the lack of functional expression. To rule out the possibility that m3 muscarinic receptors may inefficiently couple to Gq in Jurkat cells, the intracellular Ca^{2+} release by CCh was measured. Both receptors elicited Ca^{2+} mobilization (Figure 2.9 A & B) with similar intensity and duration, suggesting that both m1 and m3 mAChRs coupled to Gq/11 efficiently in this system. Furthermore, to rule out the possibility that the differences in cell surface receptor expression contributed to the discrepancy in SRF activation between m1 and m3 mAChR, receptor binding assays were carried out. The activation or inactivation of SRF by m1 and m3 mAChR respectively was independent of receptor density, since different levels of receptor expression did not alter the SRF activation pattern (data not shown).

Table 2.1. Activation of Specific G protein by Muscarinic receptor subtypes.

Receptors	G proteins	Effectors
m1/m3/m5	$G_{q/11}(G_{i/o}, G_{12/13}?)$	PLC-β↓
m2/m4	$G_{i/o}$ ($G_{12/13}?$)	AC

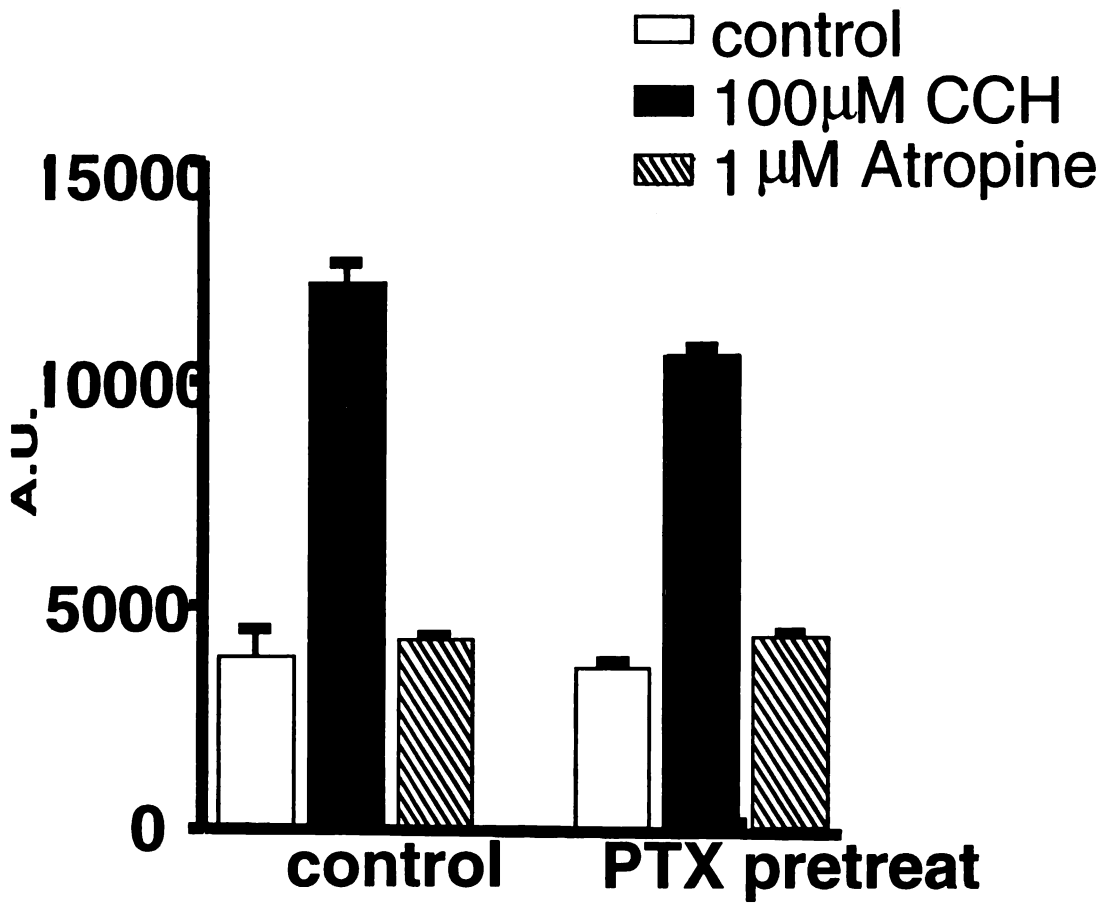


Figure 2.8. Effect of PTX on SRF activation by m1 mAChR. Jurkat cells were transfected as described in Figure 2.4. 300 ng/ml of PTX was added prior to agonist stimulation for 3 h. Luciferase activity was measured as previously described. Data is average of triplicate from a representative experiment. Error bars represent S.D.

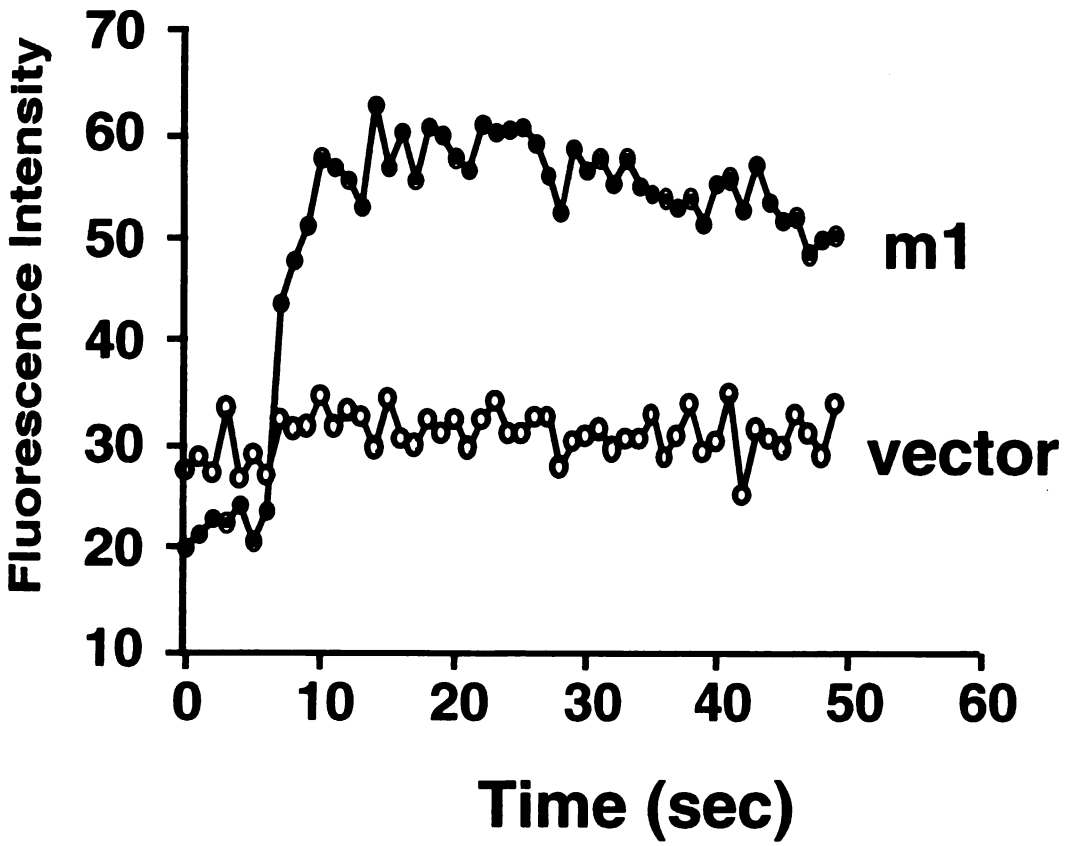


Figure 2.9 A. m1 mAChR activated calcium release in Jurkat cells. Jurkat cells were cotransfected with reporter gene and cDNA encoding m1, as described in Figure 2.3. Same pool of cells used for Luciferase assay were also used in Ca^{2+} assay. Cells were loaded with 3 μM Oregon Green 488 for 30 min in Krebs-HEPES buffer and rinsed with same buffer containing 0.5% BSA, 100 μM CCh was injected at time 0 se c and fluorescence intensity monitored by fluorometer.

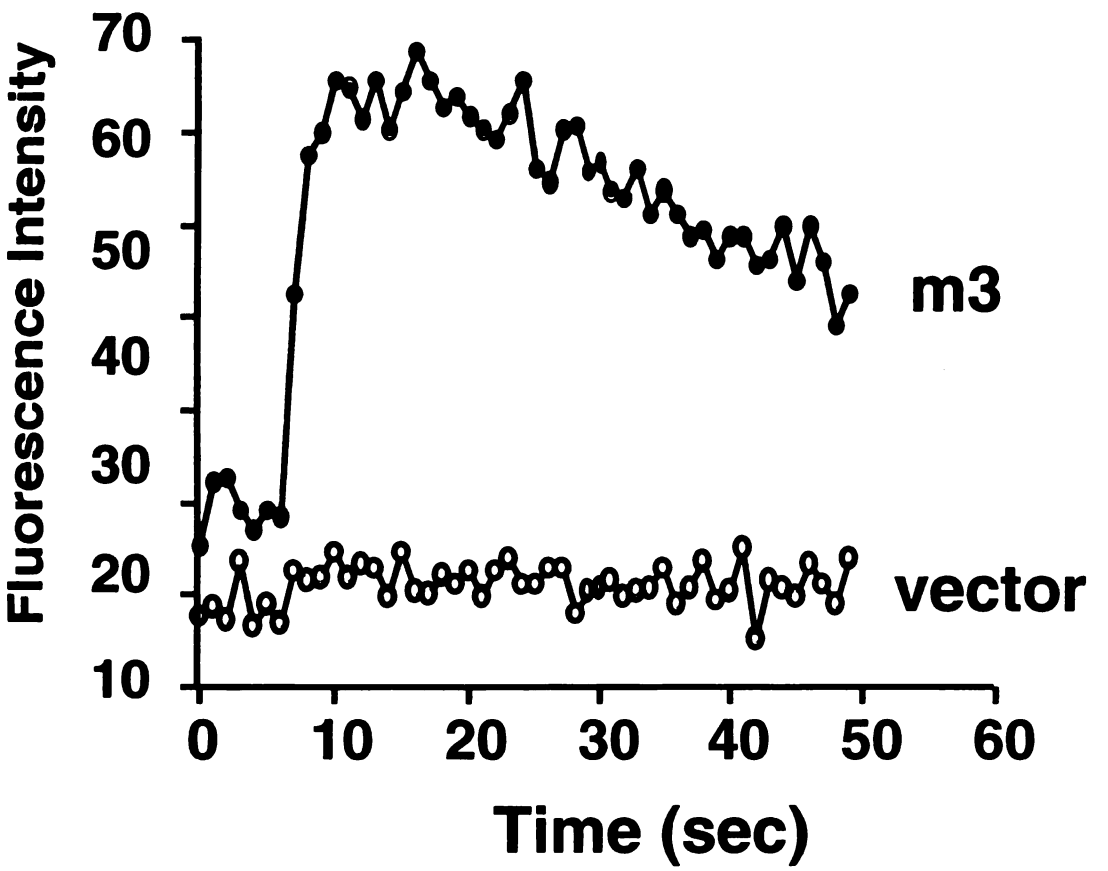


Figure 2.9B. m3 mAChR activated calcium release in Jurkat cells. Jurkat cells were cotransfected with reporter gene and cDNA encoding m3, as described in Figure 2. Same pool of cells used for SRF mediated Luciferase assay were also used in Ca^{2+} assay. Cells were loaded with 3 μM Oregon Green 488 for 30 min. in Krebs-HEPES buffer and rinsed with same buffer containing 0.5% BSA, 100 μM CCh was injected at time 0 sec and fluorescence intensity monitored by fluorometer.

E. Effect of Giq5 Chimera in Restoring Ca^{2+} and SRF Activation by m2 mAChR

To further rule out the involvement of Gq in signaling of m1 mAChR to the nucleus, a Gq/Gi chimera was used. In this chimera Giq5, an exchange of the COOH termini between Gi and Gq switches the G protein coupling specificity of Gi coupled receptor, to Gq (Kostenis *et al.*, 1998). Cells were transfected with the Gq/Gi chimera and the m2 mAChR. Gi coupled m2 receptor alone does not mobilize Ca^{2+} nor SRF activation in Jurkat T cells. After cotransfecting with Giq5 chimera, CCh stimulated a robust Ca^{2+} release in m2 mAChR expressing cells comparable to m1 transfected cells (Figure 2.10). The same pool of cells failed to stimulate SRF activation, which further proved that Gq was not involved in SRF activation in Jurkat T cells (Figure 2.11). Giq5 transfection alone has no effects on Ca^{2+} release or SRF activation.

F. G13 Mediated SRF Activation in Jurkat T Cells

The abilities of various G protein subunits to regulate SRF gene transcription were determined by cotransfecting Jurkat cells with the reporter gene plasmid and cDNA encoding the Gα subunits and their constitutively active counterparts. To account for the differences in expression efficiency, we normalized the data using a dual-luciferase plasmid (Promega, Madison, WI). Our results showed similar pattern with or without normalization, and the data in the figures presented were not normalized. The results showed that cells overexpressing normal Gα subunits did not activate SRF, nor did constitutively

active Gα12. However, constitutively active Gα13 subunit stimulated robust SRF activation (Figure 2.12). The results indicate that differential regulatory pathways may exist among the same family of Gα12/13 class. Constitutively active Gαq showed much lower level of stimulation compared to Gα13, which may partially account for m1 mAChR mediated SRF activation.

G. Effect of Constitutively Active Gα13 on m1 mAChR Mediated SRF Activation

The possibility that m1 mAChR activates SRF through Gα13 dependent pathway was investigated. When m1 mAChR was cotransfected with constitutively active Gα13 mutant, SRF mediated luciferase expression showed a higher level of activation than m1 mAChR or Gα13 alone. This suggests that m1 may work additively with Gα13 in activation of SRF. Alternatively, m1 may activate SRF through other as yet unidentified pathways (Figure 2.13).

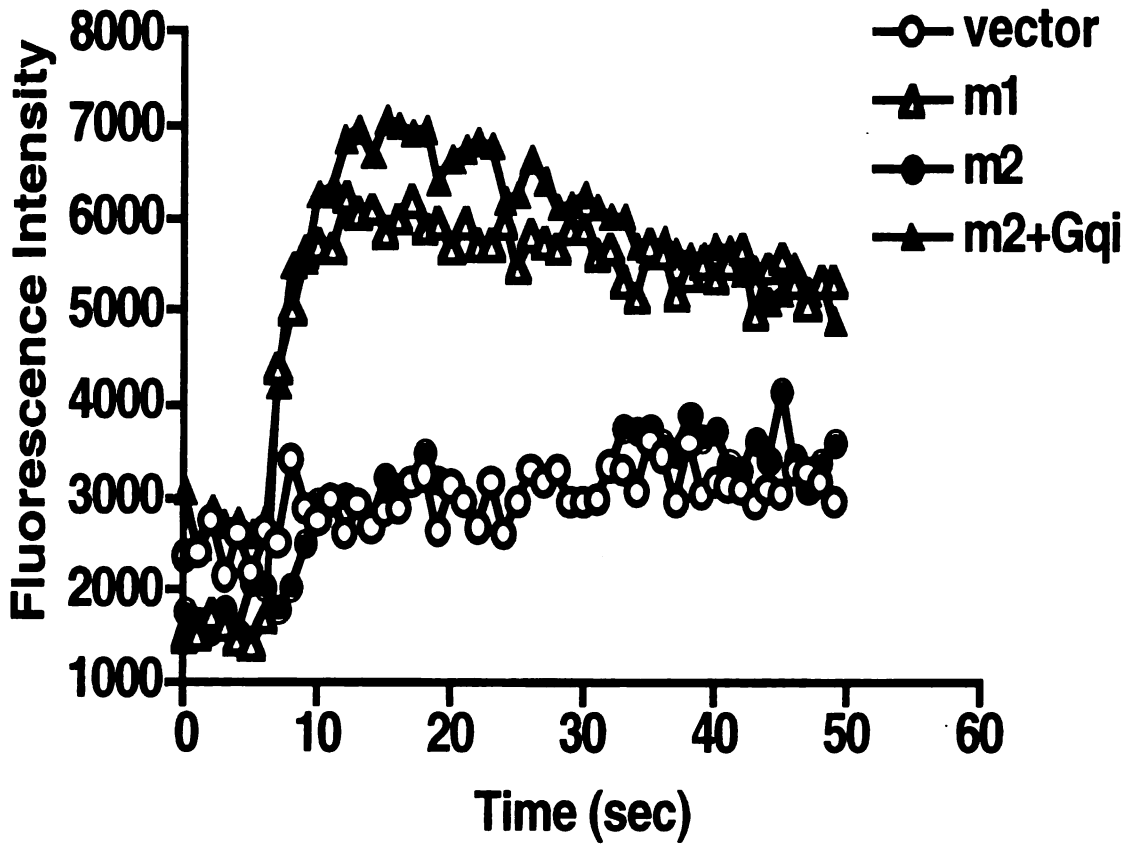


Figure 2.10. Giq5 chimera restores Ca^{2+} release in m2 transfected cells. Jurkat T cells were cotransfected with 2 μg m1 or m2 receptor plasmid, 0.2 μg of SRE.L.-Luciferase plasmid and 0.2 μg of Giq5 DNA. 18 h after transfection, cells were loaded with 3 μM Oregon Green 488 for 30 min in Krebs-HEPES buffer and rinsed with same buffer containing 0.5% BSA, 100 μM CCh was injected at time 0 sec and fluorescence intensity monitored by fluorometer.

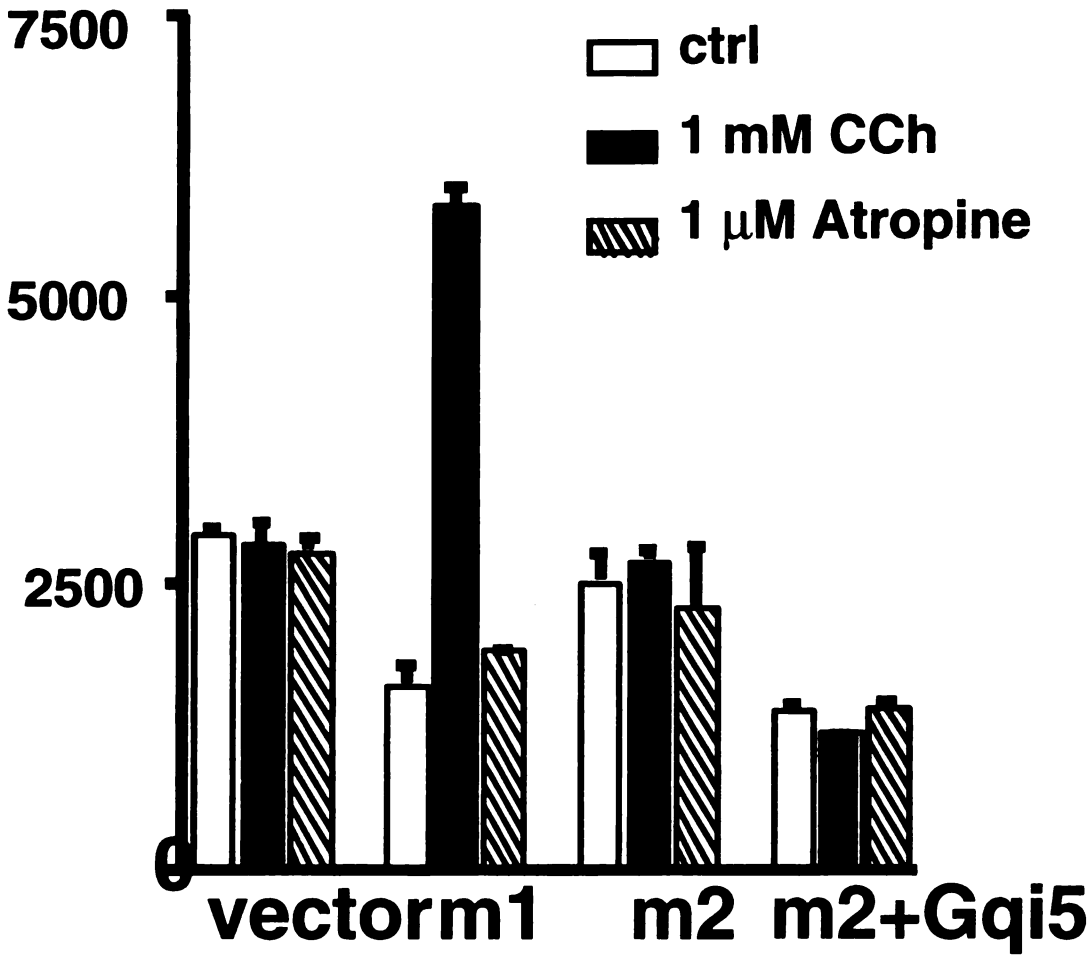


Figure 2.11. Giq5 did not restore SRF activation in m2 transfected cells. Cells were transfected as described in Figure 2.9. Aliquots of cells from the same pool used for Ca^{2+} assay were stimulated by carbachol, and luciferase activity was measured after 4 h. Experiments were done at least twice and each point is average of triplicate. Error bars represent S.D.

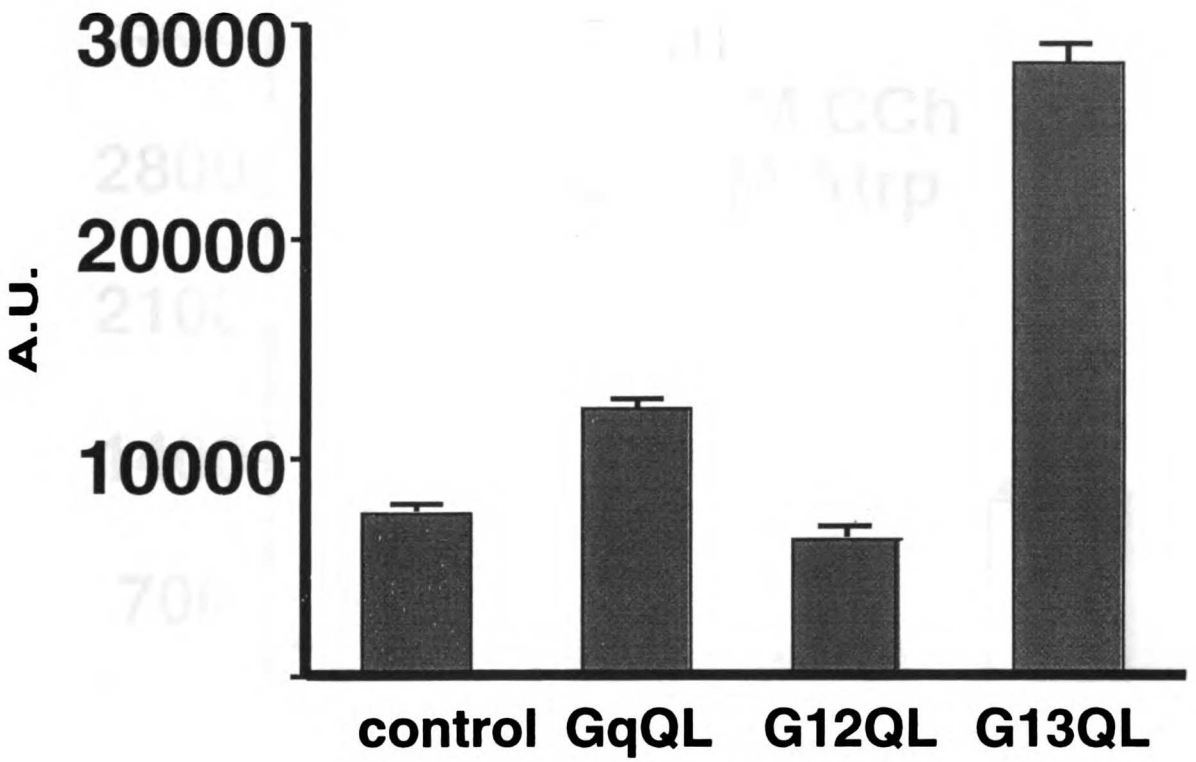


Figure 2.12. Regulation of SRF-mediated gene transcription by G protein subunits. Jurkat T cells were cotransfected with 0.2 μg of SRE.L-Luciferase reporter plasmid, 2 μg of m1 mAChR expression plasmid, and various activated forms of G protein subunits. 18 h later, cells were lysed, and luciferase activities were determined. Experiments were carried out in triplicate and repeated three times. The representative experiments are shown, and error bars represent S.D. values.

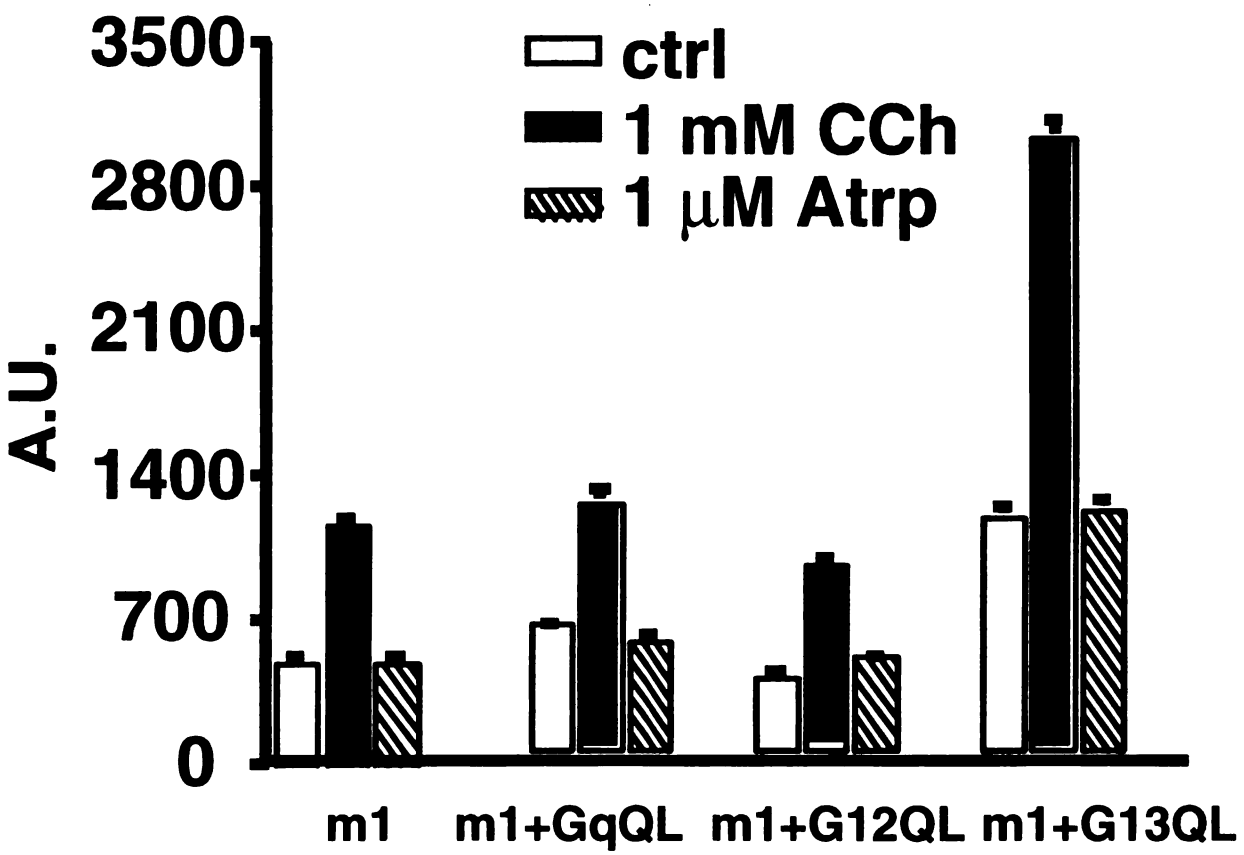


Figure 2.13. Synergistic effect of m1 and constitutively active G13 in SRF activation. Jurkat cells were cotransfected with 2 μg m1 plasmid, constitutively active G13 and reporter genes. 18 h after transfection, cells were treated with medium or 1 mM CCh, and luciferase activities were determined. Experiments were carried out in triplicate and repeated three times. The r epresentative experiments are shown, and er ror bars represent S.D. values.

H. Other Components in the SRF Pathway

It has been demonstrated that kinases and other signaling proteins can regulate the SRF pathway. To test the involvement of kinases and phosphates in SRF activation, a panel of inhibitors was used in the same transfection system. MAPK, PKA, PKC and tyrosine kinase inhibitors had no effect on SRF activation. Interestingly, calmodulin (CaM) and calcineurin inhibitors W7 and FK506 both completely inhibited the SRF dependent gene transcription mediated by m1 mAChR at pharmacologically specific concentrations but had no effect on constitutively active G13 mediated SRF activation (Figure 2.14 and 2.15).

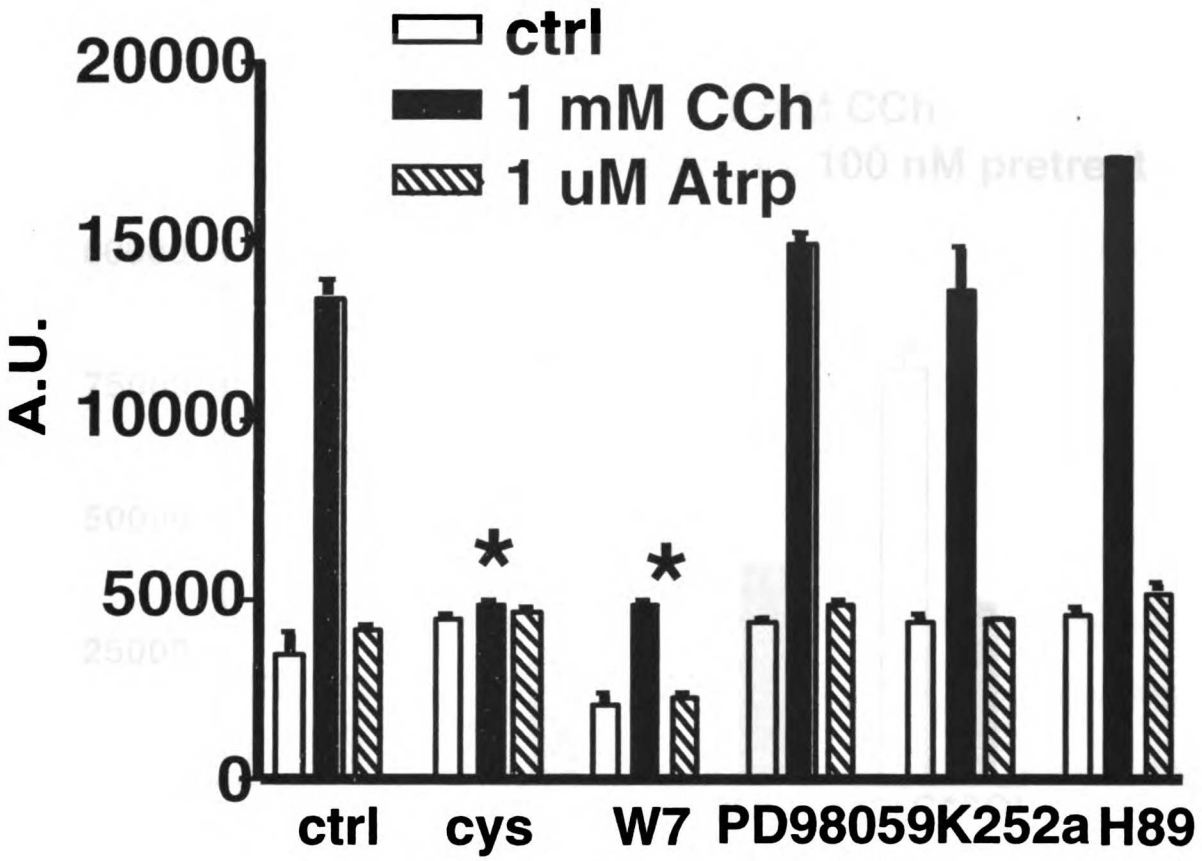


Figure 2.14. Effects of inhibitors on m1-mediated SRF activation. Jurkat cells were cotransfected with 2 μg m1 mAChR plasmid and reporter genes. 18 h after transfection, cells were pretreated with inhibitors for 30 min, and then stimulated with medium or 1 mM CCh. Luciferase activities were determined after 4 h. Experiments were carried out in triplicate and repeated three times. The representative experiments are shown. Error bars represented S.D. values.

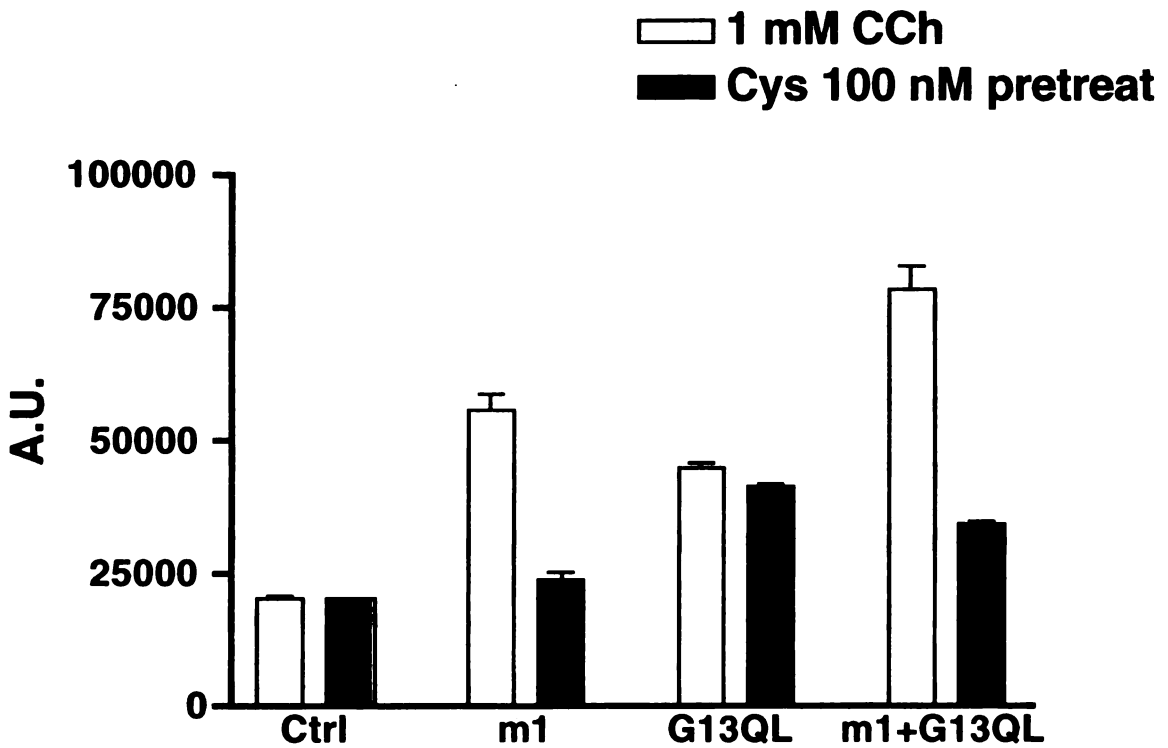


Figure 2.15 Effects of FK506 on m1 and G13QL mediated SRF activation. Jurkat cells were cotransfected with 2 μg m1 mAChR plasmid, constitutively active Gα13 and reporter genes. 18 h after transfection, cells were pretreated with 10 nM FK506 for 30 min, and then stimulated with medium or 1mM CCh. Luciferase activities were determined after 4 h. Experiments were carried out in triplicate and repeated three times. The representative experiments are shown. Error bars represented S.D. values.

V. DISCUSSION

The ability of muscarinic acetylcholine receptors to activate SRF mediated gene transcription and the involvement of G protein subunits in SRF activation were investigated. The findings that Gq coupled m1 mAChR activated SRF through a RhoA mediated pathway is consistent with previous reports (Fromm *et al.*, 1997; Mao *et al.*, 1998). The fact that Gi/o coupled m2 mAChR receptor did not activate SRF was consistent with the observation that the Gi and Go were not involved in SRF regulation (Gutkind *et al.*, 1997).

The inability of m3 mAChR receptors that are thought to be identical to m1 in activating SRF indicates that SRF may be activated by a Gq-independent pathway. Mao et al. have established a Gq dependent activation by m1 mAChR in NIH3T3 cells (Mao *et al.*, 1998), while Fromm et al. proposed a G12/13 dependent pathway in a similar system (Fromm *et al.*, 1997). In the present work, the Gq-independent activation of SRF was supported through several lines of evidence: first Gq coupling of both m1 and m3 were intact while they showed differential regulation of SRF dependent gene expression. Second, the use of the G protein construct Giq5, which switches the m2 receptor coupling from Gi to Gq, restored the Ca^{2+} response, but not SRF activation.

The discrepancy between the present results and those of Mao et al. (Mao *et al.*, 1998) could be explained by the differences among cell lines used. Most probably, cell lines express different proteins, including G proteins and other regulatory proteins. It has been extensively reported that even one

subfamily of G proteins can be regulated distinctively in different systems ((Bourne, 1997; Simon *et al.*, 1991; Strathmann & Simon, 1991)).

The failure of Gq coupled m3 mAChR to activate SRF is consistent with the notion that not all Gq coupled receptors activate SRF in Gq/11-dependent pathways. It has been shown that not only Gαq, but also other members of G proteins such as Gα12, Gα13 can activate SRF (Mao *et al.*, 1998). Constitutively active Gα subunits were used to dissect the involvement of Gα subunits. In our system, only constitutively active Gα13 not Gα12 activated SRF, the differential role of G12 family member has also been shown recently in stress fiber and focal adhesion formation (Buhl *et al.*, 1995). Furthermore, Gα13 and m1 receptors additively activated SRF, which suggests alternative pathways may exist in this signaling event (Brown *et al.*, 1997).

It has been reported that PKC and MAPK can activate SRF through ternary complex factor (TCF) pathway (Johnson *et al.*, 1997). To delineate the alternative signaling pathways leading to transcriptional activation, we tested the effect of a panel of kinase and phosphates inhibitors on m1 mAChR mediated SRF activation.

Neither PKC or PKA inhibitor, nor the MAP kinase inhibitor PD 98059, blocked CCh-induced activation of SRF. In addition, m1 mAChR induced SRF-Luc activity was not mediated via the mitogen activated protein kinase pathway, since CCh did not stimulate MAPK activity measured by ERK reporter gene (Data not shown). Interestingly, SRF was sensitive to calcium modulating

protein calmodulin (CaM) inhibitor W7, while insensitive to calmodulin kinase II or IV inhibitors. This indicates a role for CaM as an independent signaling molecule in regulating nuclear signaling while coinciding with the observation that activated m1 mAChR causes relocalization of CaM from membrane to cytosol (Wang et al., unpublished results). CaM has been implicated in binding cytoskeletal and membrane proteins that may be directly involved in actin reorganization. In the case of SRF activation, it might directly act on Rho or its effectors.

The differential sensitivity of m1-mediated and G13-mediated SRF activation to calcium/CaM regulated phosphatase calcineurin inhibitor CsA or FK506 is intriguing. CaM phosphatase signaling leading to SRF activation has not been reported previously. This pathway may be specific to Jurkat T cells and cells with similar lineage. It has been reported that reporter gene expression driven by multiple AP-1 sites was selectively sensitive to CsA and FK506 in Jurkat T cells (Jain *et al.*, 1993; Kuo & Leiden, 1999).

In conclusion, nuclear signaling of m1 has been demonstrated to be Gαq, Gαi independent. SRF signaling can also be mediated via constitutively active Gα13. These studies suggest the possibility that multiple independent pathways may be involved in the nuclear signaling of muscarinic receptors (Figure 2.16). Our study also highlights the complexity of the GPCR signal transduction system.

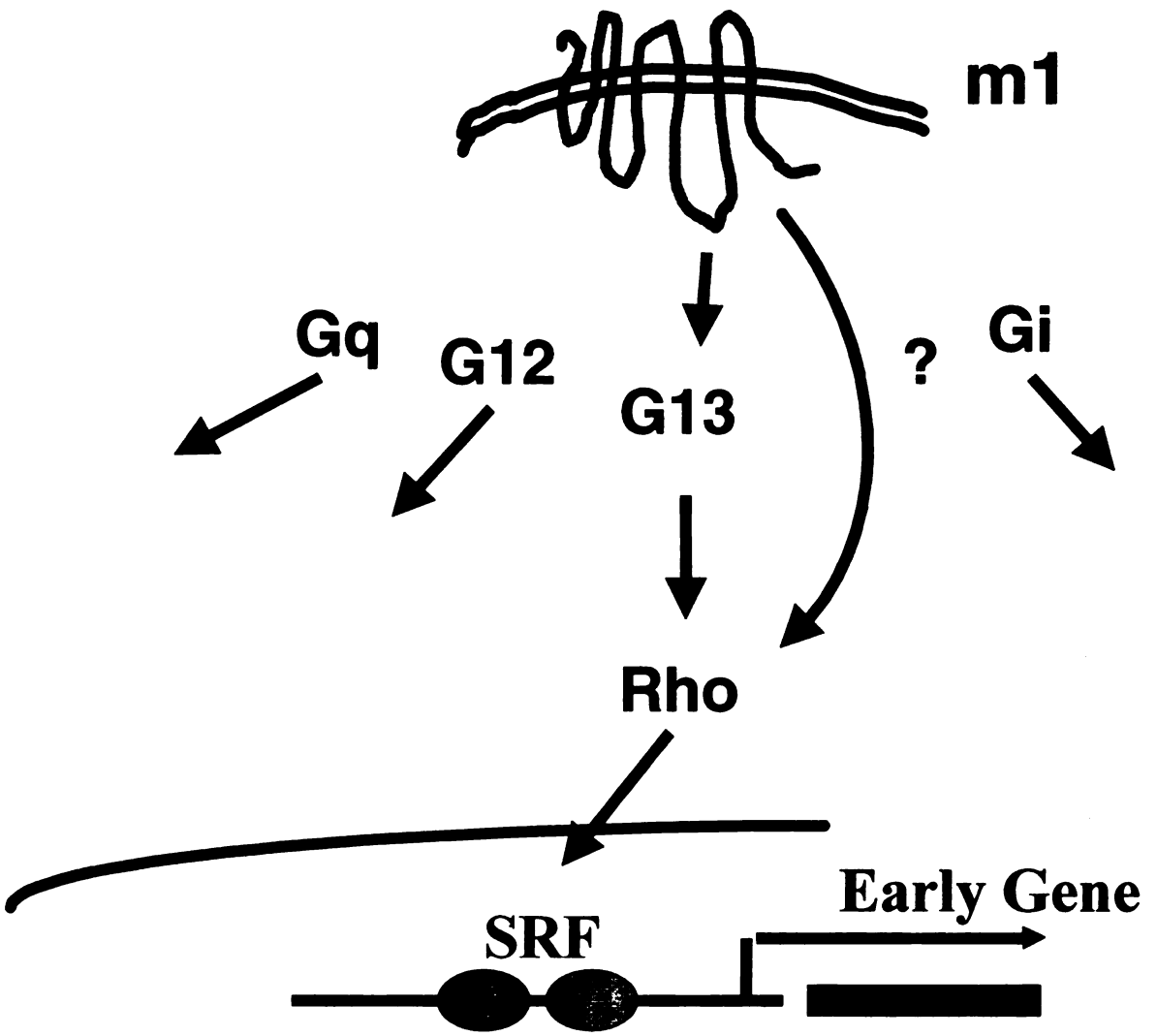


Figure 2.16. Proposed model for SRF activation in Jurkat cells.

CHAPTER 3

CHARACTERIZATION OF SRF ACTIVAITON BY m1 mAChR-MULTIPLE RECEPTOR DOMAINS INVOLVED IN SRF ACTIVATION

I. SUMMARY:

In chapter 2 we demonstrated that Gq coupling and nuclear signaling of muscarinic receptors were not connected. Notably, m1 and m3 mAChR showed distinct nuclear signaling measured by SRF activation. In this chapter we investigated whether Gq coupling and nuclear signaling of SRF by muscarinic receptors can be mutationally separated, and whether internalization/endocytosis plays a role in the SRF activation. Since the large third intracellular (i3) loop from many GPCRs has been implicated in G protein coupling and internalization of many GPCRs, we tested whether the i3 loop was involved in SRF activation using m1/m3 chimera. m3 mAChR chimera with i3 loop from m1 AChR stimulated SRF activation, however m1 mAChR mutant with i3 loop deletion could still result in SRF activation, which suggests that domains other than i3 were involved, and factors other than Gq coupling contributed to the SRF activation. It has been reported that internalization serves as an entry point for multiple G protein crosstalk and nuclear signaling in G protein coupled receptors. To investigate whether receptor internalization plays a role in SRF activation, we investigated the ability of a collection of mutants with different internalization profile, to activate SRF in Jurkat T cells. Among them, mutant d284-d392, which internalizes better than the wild type receptor, activated SRF

to a similar level as wild-type m1, while an internalization defective mutant d 344-358 was also able to stimulate SRF activity. Thus, internalization is not a factor in SRF activation.

m1 mAChR mutants d284-288, V127A and L131A that failed to induce SRF-dependent transcription were, nevertheless, able to induce Ca^{2+} mobilization. This further suggests that SRF activation and Gq coupling involved different regulatory pathways.

From studies using m1 mutants, we identified two mutants with constitutive activity of SRF pathway. These mutants were the C-terminal truncation mutant K447 and constitutively active mutant E360A measured by G protein coupling. The identification of these constitutively active mutants and the high throughput nature of reporter gene assay may provide a powerful tool for identification of inverse agonists for muscarinic receptors.

II. INTRODUCTION

Previously, we investigated the nuclear signaling pathway stimulated by muscarinic receptors using serum response factor driven luciferase reporter gene assay. We have shown that m1 mAChR stimulated SRF activation in human Jurkat T-Ag cells in a Rho-dependent and Gαq independent pathway consistent with previously published work. Among heterotrimeric G proteins, only constitutively active Gα13 activated SRF in Jurkat cells, and it also additively activated SRF with m1, which suggests that there are alternative SRF pathways in m1 mAChR activation of SRF. Further studies indicated that novel signaling molecules such as CaM and CaM-Ca^{2+} dependent phosphatase calcineurin were involved in m1 mAChR stimulated SRF activation, and had no effect on G13 mediated SRF activation. The separation of G protein with the muscarinic receptor nuclear signaling prompted us to further study the receptor domains involved in SRF activation.

Different families of GPCRs use a number of different domains both to bind their ligands and to activate G proteins. Their coupling to G proteins is regulated by splicing, RNA editing and phosphorylation. Mutagenesis and receptor subtype chimera studies, as well as two-dimensional crystallization of rhodopsin, have led to a model of a common 'central core', composed of seven transmembrane helical domains, and its structural modifications during activation. There is an emerging picture of increasing complexity in signal transduction by GPCRs (Bockaert & Pin, 1999).

Regions within the muscarinic acetylcholine receptor responsible for the specificity of G protein coupling have also been extensively studied. Both loop regions and C-terminal have been implicated in G protein coupling (Felder *et al.*, 1992; Moro *et al.*, 1993; Wess, 1998).

Since both m1 and m3 mAChR subtypes couple to the same Gq protein and effectors, it is of interest to explore other differences among these subtypes in an effort to understand the functional relevance of subtype diversity. Receptor internalization initiates a second wave of signal transduction in which the "endocytosed" receptor functions as a critical structural component of a mitogenic signaling complex. In CHO and HEK cells, m1 and m3 mACRs manifested differences in agonist-induced receptor redistribution from the cell surface: in CHO cells, m3 became rapidly and extensively internalized following agonist occupancy, whereas m1 stayed partially on the surface. Studies also showed that different residues were involved in receptor trafficking for these two receptors (Shockley *et al.*, 1999). Receptor internalization may act to physically translocate the receptors with bound agonist into intracellular vesicles and bring them in contact with other signaling factors in the cytosol or at the nuclear membrane (Pierce *et al.*, 2000). Furthermore, internalization can act as an initial step in the retrograde signaling system. Recently more evidence has become available to support the role of receptor internalization in GPCR signaling. GPCR mediated ERK1/2 activation has been shown to be endocytosis dependent (Pierce *et al.*, 2000). Several factors involved in receptor internalization have been shown to play a role in nuclear signaling of

some receptors, especially small GTPase. Stimulation of β2-adrenergic receptors results in assembly of a protein complex containing activated *c-Src* and the receptor (Burstein *et al.*, 1998). Thus, it appears that receptor internalization plays a dual regulatory role in receptor mediated signaling.

In this chapter, a panel of m1 mAChR mutants with distinct signaling behaviors was screened for their ability to activate SRF. The results indicate a lack of correlation between internalization and SRF activation among these mutants.

III. EXPREMENTAL PROCEDURES

A. Cell Culture and Transfection

Jurkat Leukemic T cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin in 5% CO_2 incubator at 37°C. Transfections were performed using LipofectAMINE Plus (Life Technologies, Inc.) according to manufacturer's instructions. Jurkat T cells ($1x10^7$) were cotransfected with 0.2 µg SRE.L-Luciferase plasmid and 2 µg of either empty vector pSG5a vector, or muscarinic receptor DNA plasmid. The transfection was stopped after 5 h by adding medium containing 5% serum.

B. DNA Constructs and m1 mAChR Mutants

The genes encoding the human muscarinic cholinergic subtypes receptors (m1-3 mAChRs) in pSG5 vector were obtained from a human placental genomic library as previously described (Maeda *et al.*, 1990). Construction of mutants was described previously (Moro *et al.*, 1993).

Rho constructs were gifts from Dr. Marc Symons (The Picower Institute for Medical Research). SRE.L-Luciferase reporter plasmid (Buhl *et al.*, 1995) and C. botulinum C3 ADP-ribotransferase (C3) were kindly provided by Dr. Songzhu An (UCSF).

C. Reporter Gene Assay

18 h later after transfection, cells were replated in 96 well plates and stimulated with medium or reagents as indicated for 4 h at 37°C. Cells were then lysed by Reporter Lysis Buffer (Promega, Madison WI), and luciferase activities were measured by luminometer. (Molecular Devices)

D. Receptor Internalization Assay

The levels of m1-3 mAChR expressed by the cells and their internalization were determined by measuring the binding of [^{3}H]-NMS to the cell surface receptors of intact cells and by [^{3}H]-QNB to the total cellular receptors, as previously described. Briefly, transfected cells were incubated with 1.5-2.0 nM [3H]-N-methyl scopolamine (NMS) or 1.5-2.0 nM [3H]-QNB in PBS at 12°C for 90 min. After labeling, cells were placed on ice, filtered (S&S #32 glass fiber filter) and rinsed with ice-cold PBS three times. The radioactivity on the filters was measured by scintillation counting.

E. Intracellular Ca^{2+} Measurement

The assay for intracellular calcium release is described in detail in Chapter 4. Briefly, 18 hr after transfection, cells were washed with Kreb-HEPES buffer and loaded with 3 μM Oregon Green 488 fluorescent dye at room temperature for 30 min. After loading, cells were washed with Kreb-HEPES buffer with 0.5% BSA three times and diluted to approximately $2x10^6$ cells /ml and distributed evenly (ca. $3x10^5$ cells/well) into an opaque, white 96-well plate

(Corning Costar, Cambridge, MA). Buffer alone or buffer containing test compounds was injected sequentially into separate wells and the fluorescence intensity monitored at 1 s intervals using an excitation wavelength of 485 nm and emissions of 538 nm.

IV. RESULTS

A. Effect of m3/m1 Chimera Mutant on SRF Activation

To test whether Gq coupling and nuclear signaling of SRF by muscarinic receptors can be mutationally separated and to identify the domains involved in the nuclear signaling of muscarinic receptors, we tested two chimeric receptors between m3 and m1 mAChRs. One chimeric receptor containing m3 receptor with i3 loop of m3 (residue 282 to 463) exchanged with that of m1 (residue 230 to 349) showed significant SRF activity (Figure 3.1 A and B).

B. Effect of Intracellular Loop 3 Deletions on SRF Activation

To test whether intracellular loop 3 is sufficient for SRF activation, and to mutationally separate domains involved in nuclear signaling, a m1 mAChR mutant with i3 loop deletion from 232-358 was tested for SRF activation. Surprisingly, its SRF activation was intact (Figure 3.2), and its Gq activation measured by calcium influx was also intact (data not shown). Taking together the results from figures 1 and 2, suggest that either i3 loop was sufficient for restoring SRF activation by m3, but not necessary for m1 mAChR mediated SRF activation or the unexpected conformational changes caused by chimera

favored SRF activation. Since the large third intracellular (i3) loop has been implicated in other signaling regulation, especially internalization, we compared the internalization pattern of m1, m3 and m3/m1 mAChR chimera.

C. Internalization of m1 and m3 mAChRs

The reporter assay lasted for 4 h, and substantial percentage of receptors underwent internalization during this process. To account for the differences in m1 and m3 mAChR signaling, the role of internalization in nuclear signaling was also investigated. Cells transfected with m1 and m3 chimera were treated with or without 1 mM carbachol in serum-free medium for different times. After carbachol treatment, cells were washed and 3H-NMS and 3H-QNB binding were measured. The time courses of receptor internalization for m1 and m3 mAChR were distinctively different (Figure 3.3). m1 mAChR showed minimal internalization during 4 h period, while 50% of m3 mAChR receptors internalized.

D. Internalization vs. SRF Activation in m1 mAChR Mutants

To further investigate whether receptor internalization plays a role in SRF activation, we tested the SRF activation of a panel of m1 mAChR mutants with different internalization efficiency. Among them, d344-358 was an internalization defective mutant, and d232-358 showed >50% internalization within 4 hr, however, both were able to activated SRF in Jurkat T cells. Thus, internalization did not appear to be a factor in SRF activation (Table 3.1).

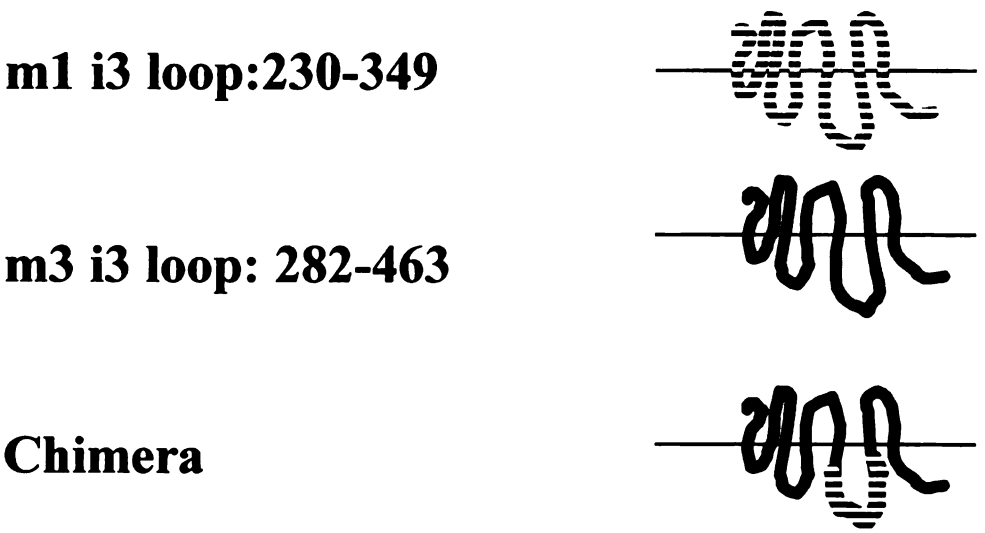


Figure 3.1A. m3 chimera with m1 i3 loop restored SRF activation by CCh. Graphic depiction of m1, m3 and chimera m3/m1 mAChRs.

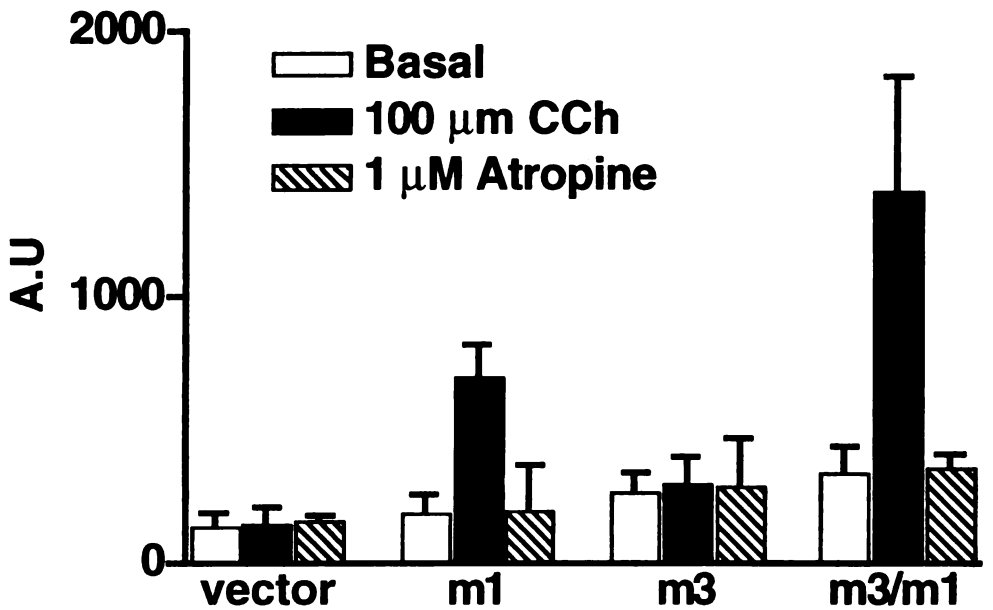


Figure 3.1B. m3 chimera with m1 i3 loop restored SRF activation by CCh. Jurkat cells were cotransfected with 0.2 μg of SRE.L-luciferase reporter plasmid, and 2 μg pSG5 vector, m1, m3 or m3/m1 chimera cDNA. 18 h later, aliquots of $1x10^5$ cells in 0.15 ml were transferred to 96-well plates and treated with media (control), 100 μM carbachol or carbachol (CCh) with 1 μM Atropine as indicated. Cells were lysed 4 h later and luciferase activity was measured. Experiments were done twice with the representative experiment shown and each point is average of triplicate. Error bars represent S.D.

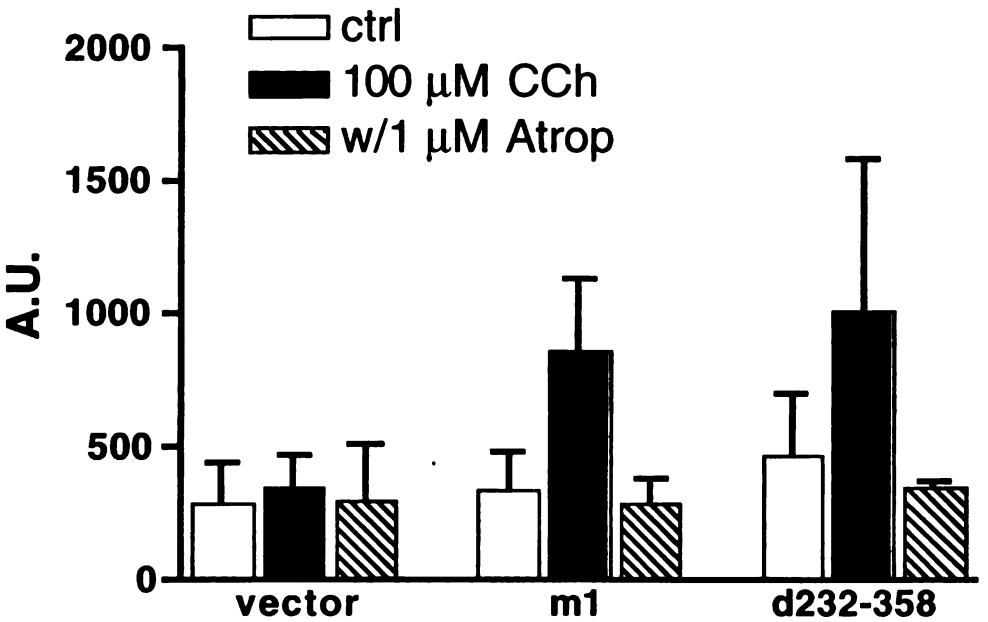


Figure 3.2. m1 mAChR mutant with i3 loop deletion activated SRF. Jurkat cells were cotransfected with 0.2 µg of SRE.L-luciferase reporter plasmid, and 2 µg pSG5 vector, m1 or m1 i3 loop deletion mutant receptor cDNA. 18 h later, aliquots of $1x10^5$ cells in 0.15 ml were transferred to 96-well plates and treated with media (control), 100 µM CCh or CCh with 1 µM Atropine as indicated. Cells were lysed 4 h later and luciferase activity was measured. Experiment were done at least two times and each point is average of triplicates, error bars represent S.D.

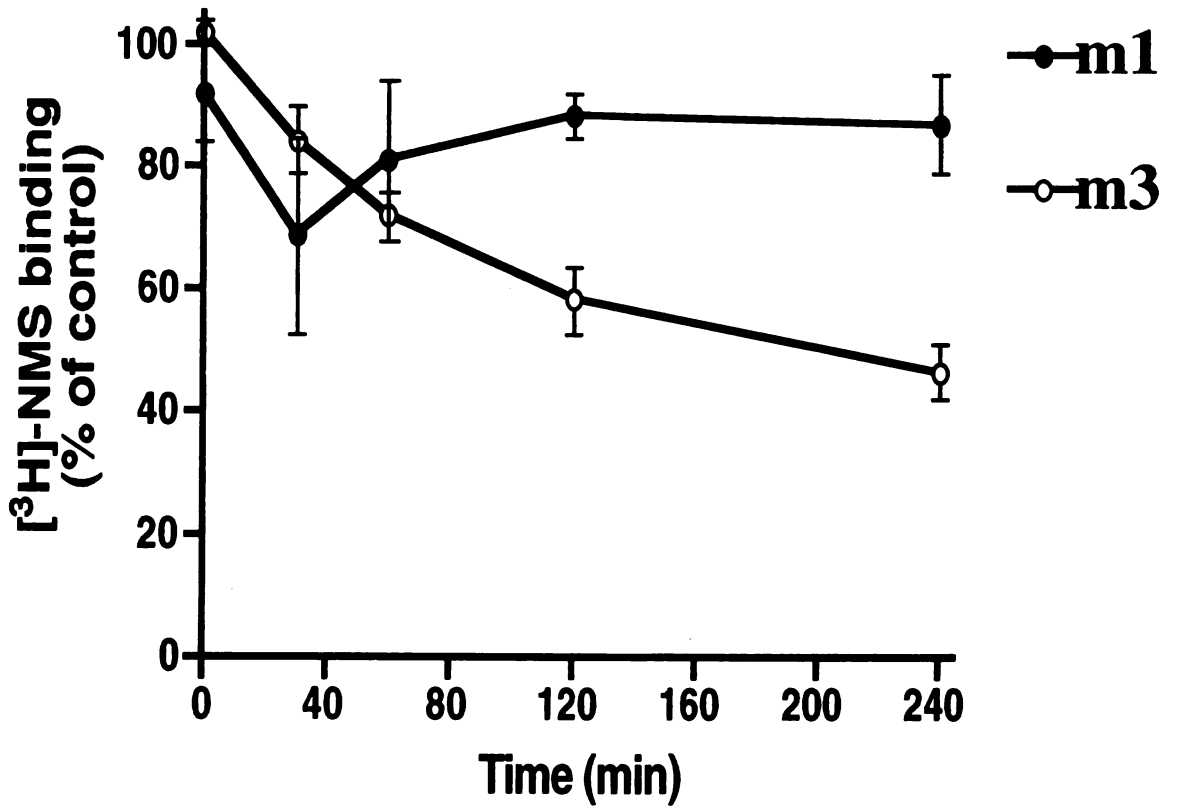


Figure 3.3. Internalization of m1 and m3 mAChRs after treatment with 1 mM carbachol. Jurkat T cells transfected with 2 μg of m1 or m3 plasmid and 0.2 μg of reporter gene were treated with 1 mM carbachol for varying times. After washing with ice-cold PBS, cell surface receptor binding was assessed using the hydrophilic ligand [3H]-NMS. Each point is average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments. Open circles: m3 binding; filled circles: m1 binding.

Table 3.1. Carbachol induced internalization and SRF activation by m1 mAChR receptor wild-type and mutants. Jurkat cells were cotransfected with 0.2 μg of SRE.L-luciferase reporter plasmid, and 2 μg of respective mutant receptor cDNA. Internalization was measured by the ratio between cell surface receptor binding using the hydrophilic ligand [^{3}H]-NMS and total receptor binding using the lipophilic ligand [^{3}H]-QNB. SRF activation was measured as described. Each point is mean ± SD of 2-3 experiments.

	Internalization (% of control)	SRF activation (fold increase)
m1 wild-type	15±7	4.0±1.2
d284-292	12.5±6	6.0±2.1
d284-290	53±21	4.7±1.5
d284-288	97±4	7.1±2
d288-292	83±12	3.2±0.2
d344-358	<5±0.7	7.5±1.9
d232-358	67±12	4.0±2.3

E. Receptor Domains Involved in SRF Activation

We screened m1 mAChR mutants with deletions or mutations in other receptor regions. Among these mutant receptors, d284-288, V127A, L131A that failed to induce SRF-dependent transcription (Figure 3.4), were able to induce Ca2+ mobilization to an extent comparable to that stimulated by wild-type m1 mAChR (data not shown). This further suggests that SRF activation and Gq coupling involve different regulatory pathways.

F. Constitutive SRF Activity by E360A and K447 mutants

Studies on m1 mAChR mutants identified several mutants that constitutively activated SRF. One point mutation in i3 loop, E360A and m1 C-terminal truncation mutant K447 showed elevated SRF activation level compared to wild-type m1 mAChR, and the activation was specifically inhibited by inverse agonist atropine, which exhibited similar IC50 in antagonizing IP production as in SRF activation (Figure 3.5 A and B.). E360A has previously been shown to be constitutively active in IP production (Hogger *et al.*, 1995).

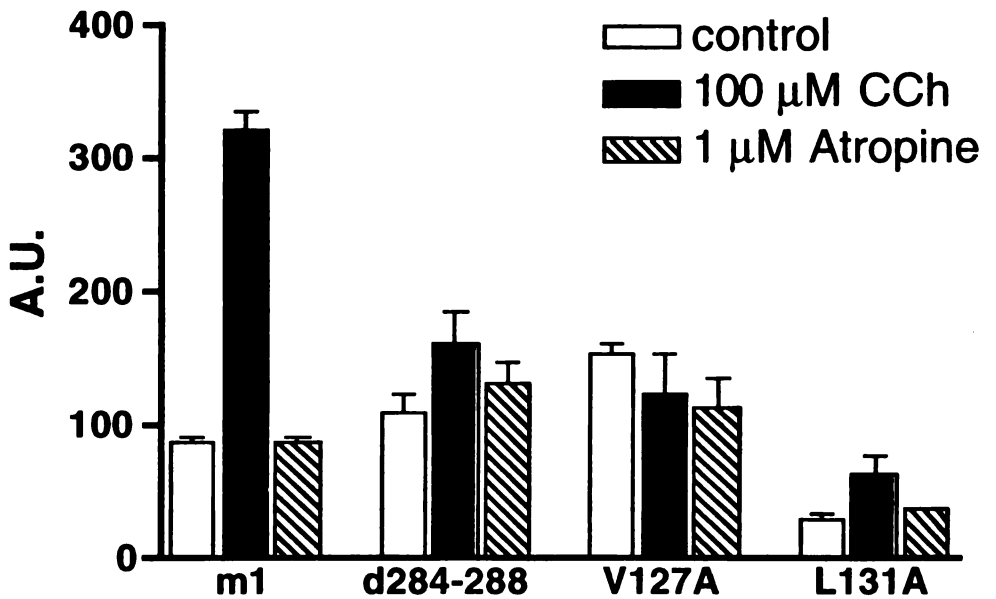


Figure 3.4. m1 AChR mutants failed to activate SRF. Jurkat T-Ag cells were cotransfected with 2 μg of m1 mAChR receptor or mutants expression plasmid and 0.2 μg of SRE.L-luciferase plasmid. 18 h later, cells were stimulated with 100 μM CCh and luciferase expression measured after 4 h. Each point is average of quadruple points. Error bars represent S.D. The curve is representative of three independent experiments.

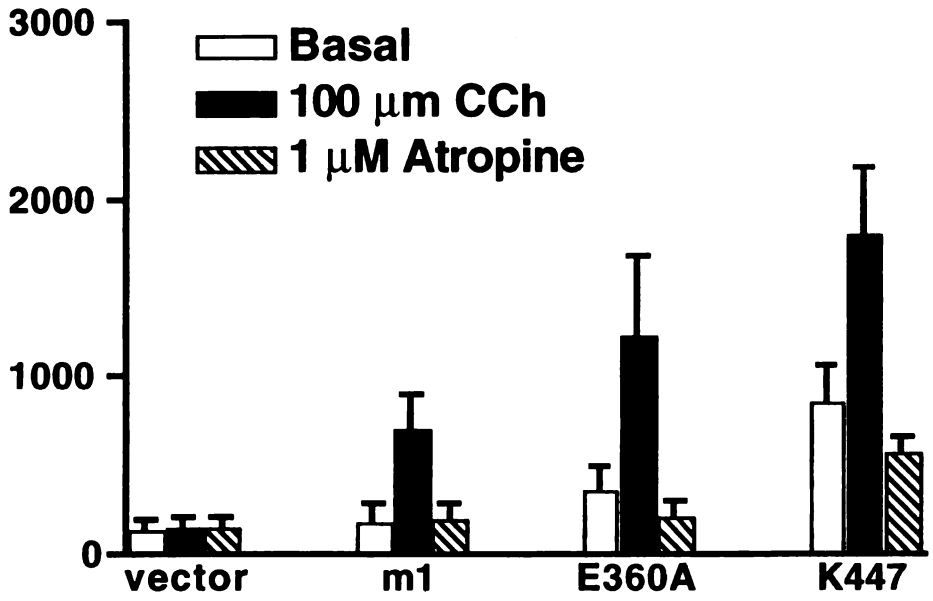


Figure 3.5 A. CCh induced SRF activation by m1 AChR and constitutively active mutants E360A and K447. Jurkat T-Ag cells were cotransfected with 2 μg of m1 mAChR receptor or mutants plasmid and 0.2 μg of SRE.L-luciferase plasmid. 18 h later, cells were stimulated with 100 μM CCh and luciferase expression measured after 4 h. Each point is average of quadruple points. Error bars represent S.D. It is a representative of three independent experiments.

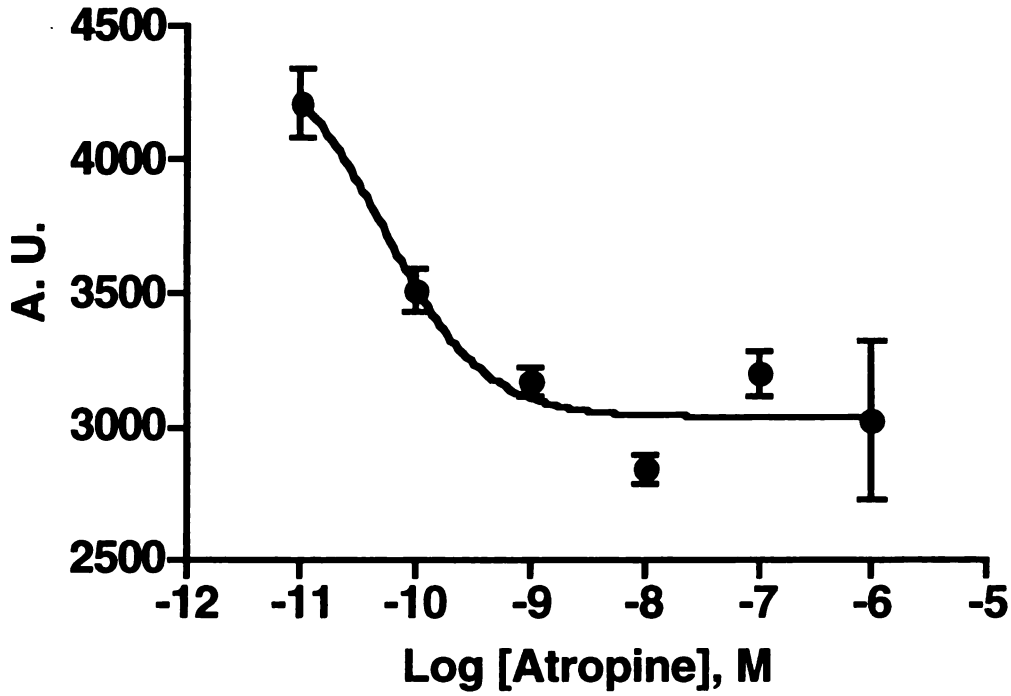


Figure 3.5 B. Concentration dependent inhibition of E360A constitutive activity by increasing concentration of atropine. IC50 is 5 nM. Each point is average of quadruple points. Error bars represent standard deviation.

V. DISCUSSION

In the previous chapter, we demonstrated that m1 AChR signals to the nucleus by activating serum response factor through a Gi and Gq independent pathway. The mechanisms and components involved in this process are not well understood. In this study, we examined m1 AChR mutants to address the following questions: 1) what confers the difference between m1 and m3 AChR nuclear signaling; 2) can the domains involved in Gq coupling be distinguished from the SRF activation domains; 3) does internalization play a role in SRF activation.

The role of i3 loop in SRA activation of muscarinic receptors remains paradoxical. While, the replacement of i3 loop rendered the SRF activity to m3 AChR; deletion of the corresponding i3 loop from m1 mAChR did not affect its SRF activation. There are two possible explanations for this phenomenon: 1). i3 loop is sufficient by not required for SRF activation or/and 2). other signaling differences or conformational restraints account for the SRF activation.

Receptor internalization has been implicated in nuclear signaling and intracellular loop regions have been closely linked to internalization (Daaka *et al.*, 1998; Hausdorff *et al.*, 1991; Lee & Fraser, 1993). It is known that RhoA acts as a negative regulator for m1 mAChR sequestration, and sequestration has been shown to be required for MAPK, Elk and Ras activation. Recent evidence has shown that internalization acts as an initial step in activating MAPK pathways (Maudsley *et al.*, 2000; Pierce *et al.*, 2000), to translocate the receptors from cell surface to intracellular vesicles and bring them in contact

with other signaling factors. However, the involvement of internalization in activating growth factor mediated pathways also showed great heterogeneity among different receptors and different cell systems (Schramm & Limbird, 1999). For example, in studies on mu-opioid receptor, Keith et al demonstrated that internalization was not involved in CREB activation (Keith *et al.*, 1996), while Keith et. al showed a correlation between ERK activation and internalization (Keith *et al.*, 1996). Since intracellular loop 3 is critical for receptor internalization (Lameh *et al.*, 1992; Moro *et al.*, 1993; Moro *et al.*, 1994), we further examined the internalization and SRF activation patterns among m1 mAChR mutants.

After four hours of treatment with carbachol, m1 mAChR failed to internalize significantly, while 50 % of m3 receptors sequestered inside the cell. To test whether internalization plays a role in SRF activation, m1 mAChR mutants with different internalization patterns were screened for their SRF activity. Both highly internalizing mutants and internalization-defective mutants were capable of activating SRF, and there was no correlation between these two events. Recent study also showed the lack of correlation between internalization and ERK activation by m3 mAChR (Budd *et al.*, 1999).

In an attempt to identify SRF activation domain, we also screened m1 mAChR mutants with mutations and deletions at different regions. Among them, intracellular loop 2 region point mutations V127A (Valine 127 to Alanine) and L131A (Leucine 131 to Alanine) showed minimal SRF activation, while activating PLC pathway. These mutants have partially impaired G protein

coupling, which may account in part for lack of SRF activation. The lack of correlation between receptor domains and SRF activation can also be explained by recent studies demonstrating that multiple loops and carboxyl terminus mediate complex roles in receptor signaling for some receptors (Felder *et al.*, 1992).

Finally, we have identified two mutations of m1 mAChR that resulted in constitutive activation in nuclear signaling. The use of these mutant combined with high throughput nature of reporter gene assay described in this chapter and chapter 4, may provide a system for identifying inverse agonists for muscarinic receptors (Lefkowitz *et al.*, 1993).

CHAPTER 4

RAPID MEASUREMENTS OF INTRACELLULAR CALCIUM USING A FLUORESCENCE PLATE READER

I. SUMMARY

Intracellular calcium is a universal second messenger that can serve as a broad-based measure of receptor activity. In this chapter, we describe a rapid and sensitive method to assay intracellular calcium ions in human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells from multi-well plates using a fluorometer equipped with on-line injectors, and examine the compatibility of visible-light excitable dyes Calcium Green-1 and Oregon Green 488 BAPTA-1. Using this assay, we were able to detect and quantify activity from muscarinic and β-adrenergic receptors endogenous to HEK293 cells, and detect calcium signals generated by activation of Gi coupled recombinant μ-opioid and dopamine D2L receptors, as well as the Gs coupled melanocortin subtype 4 (MC4) receptor. Fluorescence signals, stable in HEK293 cells, required the use of Oregon Green 488 BAPTA-1 and an inhibitor of organic anion transport in CHO cells. Under appropriate conditions, both cell types can be used to collect complete concentration-response data for a variety of receptors--including a recombinant muscarinic m1 mAChR receptor expressed in CHO cells--from a single plate of dye-loaded cells.

II. INTRODUCTION

Combinatorial synthesis and expanding genomic data bases have increased the need for rapid and broadly applicable methods to screen receptors as potential drug targets and to study regulation of intracellular free Ca^{2+} levels by living organisms. Regulation of intracellular free-calcium levels, a ubiquitous process, can serve as a facile means to screen drugs and analyze cellular signal transduction pathways *in vitro* (Tsien *et al.*, 1982). Yet large-scale drug screening and quantitative comparisons can be complicated by the instability of signals generated by fluorescent calcium indicators. Signal stability is difficult to achieve with several cell types, including Chinese hamster ovary (CHO) cells, a cell line favored in the study of ionizing radiation, hyperthermia and second messenger systems (Gottesman, 1985). Such instability compromises our ability to measure rapid quantitative calcium responses using multi-well systems or other serially formatted data collection systems.

Fluorescent signals emitted by calcium indicators can vary with time and between cell types due to sequestration or efflux of the dye, or poor hydrolysis of the acetoxymethyl (AM) esters that impedes activation and trapping of dye within the cell (Roe *et al.*, 1990). Compartmentalization or extrusion of dye across the plasma membrane is known to increase with temperature; calcium measurements, therefore, are often performed at low temperature to stem this loss (Malgaroli *et al.*, 1987). Even at reasonably low temperatures, however, fluorescence signals degrade in some cell types due to efflux, and addition of

an inhibitor of anion transport may become necessary. Wieder and Fox (Wieder & Fox, 1994), reported that in CHO cells, for example, the UV-excitable dye Indo-1 displays marked instability even at room temperature, an effect attributed to efflux. The authors offered a partial solution to this problem by selecting a mutant CHO cell line with better dye retention characteristics; but what effects such mutations have on normal cellular function remains unclear. Other means of alleviating signal instability in test cells are of interest.

The UV-excitable dye Fura-2 has emerged by default as the standard for Ca^{2+} measurements in many cell types including CHO cells (Di Virgilio *et al.*, 1989; Malgaroli *et al.*, 1987; Roe *et al.*, 1990). Empirically, this dye appears to generate more stable signals, and the ratiometric quantitation of that signal allows more careful monitoring of any potential decay. Experiments are typically performed either from cuvettes, or under a microscope. Such protocols suffer on a number of fronts. In addition to being slow and laborious, the inability to employ lower energy dyes brings its own set of disadvantages. For example, the high excitation energy used with Fura-2 can cause photodamage, light scatter, and sample auto-fluorescence. The excitation parameters of Fura-2 are also incompatible with laser-based equipment commonly used in flow cytometry, confocal microscopy, and many fluorescence plate readers. Visible-light excitable dyes reduce such problems and can be used more easily with other fluorescent dyes or combined with UV-activated "caged" compounds (Kao *et al.*, 1989).

The aim of this work was to establish conditions under which versatile visible-light excitable dyes could be employed in a high-throughput assay for making calcium measurements using live cells. The performance and stability of visible-light excitable dyes Calcium Green-1/AM and Oregon Green 488 BAPTA-1/AM in HEK293 and CHO cells were evaluated, and this information used to develop a rapid, quantitative bioassay for G-protein linked receptors from multi-well plates. Endogenous muscarinic and β-adrenergic receptors, which are expressed at low levels and activate Ca^{2+} currents in HEK293 cells (Conklin *et al.*, 1992; Premont *et al.*, 1992), were used to evaluate assay sensitivity; and recombinant Gi and Gs coupled receptors, μ-opioid, dopamine D2L, melanocortin MC4 receptors, not typically assayed using Ca^{2+} methods--were used to demonstrate assay versatility.

Despite difficulties associated with dye compatibility, CHO cells are, nevertheless, well suited for the study of transfected muscarinic receptors because they lack endogenous receptors of similar type (Shockley *et al.*, 1997). These cells are used here as part of a model system which can be used to test recombinant muscarinic receptors *in vitro*, and to demonstrate that under the right conditions even such problematic cell lines can serve as suitable candidates for serial analysis using multi-well systems.

III. EXPREMENTAL PROCEDURES

A. Tissue Culture and Stable Cell Lines

HEK293 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) F12/H16 and CHO cells (ATCC) in Ham's F12 medium--both media supplemented with 50 μg/mL streptomycin, 50 U/mL penicillin and 10% fetal bovine serum (Sigma Chemical, St. Louis, MO)--and incubated at 37°C in 5% CO_2. Stable cell lines of HEK293 cells were prepared as described elsewhere using cDNA containing the human melanocortin receptor subtype 4 (pcCMV/hMC4) (Gantz *et al.*, 1993; Quillan & Sadee, 1996); murine μ-opiate receptor (pRcCMV/μOR) (Chen *et al.*, 1993); and the human dopamine D2L receptor (pcDNA3/hD2L) (Grandy *et al.*, 1989). Stable HEK293 clonal cell lines were maintained in medium containing 200 μg/mL of G418 (Bethesda Research Laboratories, Bethesda, MD). Stable cell lines of CHO cells were prepared by co-transfecting pSG5 (Strategene, La Jolla, CA) containing an insert for the full-length coding sequence of the human m1 muscarinic receptor together with pRSVneo (Strategene), as described elsewhere (Maeda *et al.*, 1990). Stably transfected cell lines were selected with 400 μg/mL of G418 and maintained at 200 μg/mL. The CHO-m1 cell line used in this study contained 1,000 fmol of m1 mAChR per mg of total protein (Shockley *et al.*, 1997).

B. Calcium Measurements

Calcium measurements on CHO and HEK293 cells were made using a FLUOstar 97 fluorometer (BMG LabTechnologies, Inc., Durham NC, USA) equipped with dual injectors. Gain was calibrated to ensure consistency between wells and set to 80% of resting baseline intensity. The instrument linearity, precision, and cross-talk were calibrated over the range of measured fluorescence intensities using free salt forms of Calcium Green-1 and Oregon Green 488 BAPTA-1 (Molecular Probes, Eugene, OR, USA) dissolved in Krebs-HEPES buffer [118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 11.7 mM D-Glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4].

HEK293 and CHO cells were prepared for calcium measurements as follows: Cells were trypsinized and immediately quenched with culture medium containing 10% fetal bovine serum, pelleted, and then rinsed twice. Pelleted cells were resuspended in fresh medium, allowed to recover under 5% CO_2 at 37°C for 1 hr, rinsed twice with Krebs-HEPES buffer, and then loaded with 3 μM Calcium Green-1/AM or Oregon Green 488 BAPTA-1/AM (Molecular Probes) for 30 min in the same buffer containing 1% (w/v) Pluronic F-127 (Sigma Chemical, St. Louis, MO, USA), and where indicated, with 0.25 mM sulfinpyrazone (Sigma). After loading, cells were rinsed 3 times with Krebs-HEPES buffer containing 0.5% (w/v) bovine serum albumin (Sigma), diluted to approximately 200,000 cells/mL, and distributed evenly (approximately 30,000 cells/well) into an opaque white 96-well plate (Costar, Cambridge MA, USA). Buffer alone or buffer containing test compounds, was injected sequentially into separate wells

and the fluorescence intensity monitored at 1 sec intervals using an excitation wavelength of 485 nm (bandwidth 20 nm) and emissions filter of 538 nm (bandwidth 25 nm). Five baseline measurements were taken at 1 sec intervals prior to each injection.

At the end of each experiment, fluorescence intensities were calibrated for determination of intracellular calcium concentration ($[Ca^{2+}]_i$) values by permeabilizing cells with 1% Triton X-100 (Sigma), to release all the dye (F_{max}), and subsequently chelating with 10 mM EGTA (F_{min}). Calcium concentrations were calculated using the following equation: $[Ca^{2+}]_i = K_d(F-F_{min}) /(F_{max}-F_{min})$ (Kao, 1994). An equilibrium dissociation constant (K_d) of 190 nM and 170 nM was used for Calcium Green-1 (Eberhard & Erne, 1991) and Oregon Green 488 BAPTA-1 (Haugland, 1996), respectively.

C. Determination of Dye Loss Due to Leakage

HEK293 or CHO cells, loaded with dye according to procedures outlined for calcium measurements, were rinsed three times and resuspended in Kreb-HEPES buffer containing 0.5% BSA, then 800 µL aliquots divided into microcentrifuge tubes. Samples were then incubated at 20°C or 37°C, as indicated, for various periods of time. At each time point, cells were pelleted and 700 µL of supernatant removed and combined with 40 µL of 80% ethanol/H_2O (v/v). Excess supernatant was removed from the cell pellet, cells lysed by adding 40 µL of 80% ethanol/H_2O, lysate resuspended in 700 µL of

Kreb-HEPES buffer, and the fluorescence intensity of 200 µL aliquots of each sample--supernatant and resuspended cell lysate solutions--determined. Fluorescence intensity of supernatant fractions reflects dye leakage from the cell, and the combined fluorescence intensity from both supernatant and cell lysate was used to defined total amount of dye. Leakage (reported as % of total) is defined as the amount of dye measured in the extracellular supernatant over total amount of dye in both supernatant and cellular fractions. The ratio of fluorescence intensity between supernatant and total was plotted versus time.

D. Data Analysis

Numerical data from each injection run were tabulated by subtracting the change in fluorescence intensity caused by injection of buffer alone from the change caused by injection of buffer containing test compound. Concentration-response curves were generated by plotting mean values of averaged fluorescence intensity between 5 and 15 seconds, at each concentration. Sample mean fluorescence intensity and sample standard deviations (SD) were plotted and fit to concentration-response graphs using a logistic equation as described elsewhere (DeLean *et al.*, 1978; Quillan & Sadee, 1996).

IV. RESULTS

A. Testing of Visible-light Excitable Dyes in HEK293 Cells

The strength and stability of fluorescence signals produced by activation of the visible-light excitable dyes, Calcium Green-1 and Oregon Green 488 BAPTA-1, were first investigated in HEK293 cells. Cells were loaded with AM esters of either dye, divided equally into wells of a 96-well plate and allowed to equilibrate for 30 min at 37°C, then injected sequentially, well by well, with buffer or buffer containing the calcium ionophore ionomycin (RBI, Natick, MA, USA). Ionomycin uniformly elicited a robust increase in fluorescence intensity that peaked about 8-15 sec after injection, and then decreased gradually to 50-90% of maximum intensity by the end of each 44 sec observation period (Figure 4.1A).

The stability of each visible-light excitable dye was assessed by monitoring the decay in its fluorescence intensity over time. Injections of ionomycin into different wells at varying times after dye loading demonstrated that the signal intensity of both dyes remained relatively stable for over 2 hours--a length of time sufficient to make multiple measurements using the multi-well plate format. Signal variability between wells, and loss of signal strength over time, at both 37°C and 20°C, are illustrated for Oregon Green 488 BAPTA-1 in Figure 4.1B. Average variation and loss of strength over 3 hrs were less than 20%, even at the higher temperature. Instrument linearity over the range of measured intensities was independently confirmed by adding free Calcium Green-1 directly to Krebs-HEPES buffer (data not shown).

Although the loss of signal intensity in HEK293 cells was minimal, we examined its source by monitoring dye leakage from the cells. As demonstrated in Figure 1C, the small loss in signal strength could be accounted for by a minor efflux of dye from the cells. Dye loss from HEK293 cells displayed single rectangular hyperbolic kinetics and reached a maximum of approximately 10% after one hour at 37°C. These combined results demonstrate that acetoxymethyl (AM) esters of Calcium Green-1 and Oregon Green 488 BAPTA-1 are stable fluorescent indicators for measurement of calcium activity in HEK293 cells, and suitable for serial measurement using multi-well formats.

B. Dye Stability in CHO Cells

The reported incompatibility of Indo-1 with measurement of Ca^{2+} in CHO cells (Wieder & Fox, 1994) led us to suspect that Calcium Green-1 and Oregon Green 488 BAPTA-1 might also perform poorly with these cells. This suspicion was confirmed in preliminary experiments using ionomycin as described above for HEK293 cells. Both dyes generated fluorescent signals that were unstable and deteriorated rapidly, even when tests were performed at a lower temperature, and immediately after dye loading. Addition of an anion transport inhibitor, sulfinpyrazone, improved the performance of both dyes; in the case of Calcium Green-1, the improvement was not marked enough to make it a suitable candidate for the multi-well format under development (Figure 4.2A). The performance of Oregon Green 488 BAPTA-1 was significantly better, and this dye was chosen for use in further studies with CHO cells.

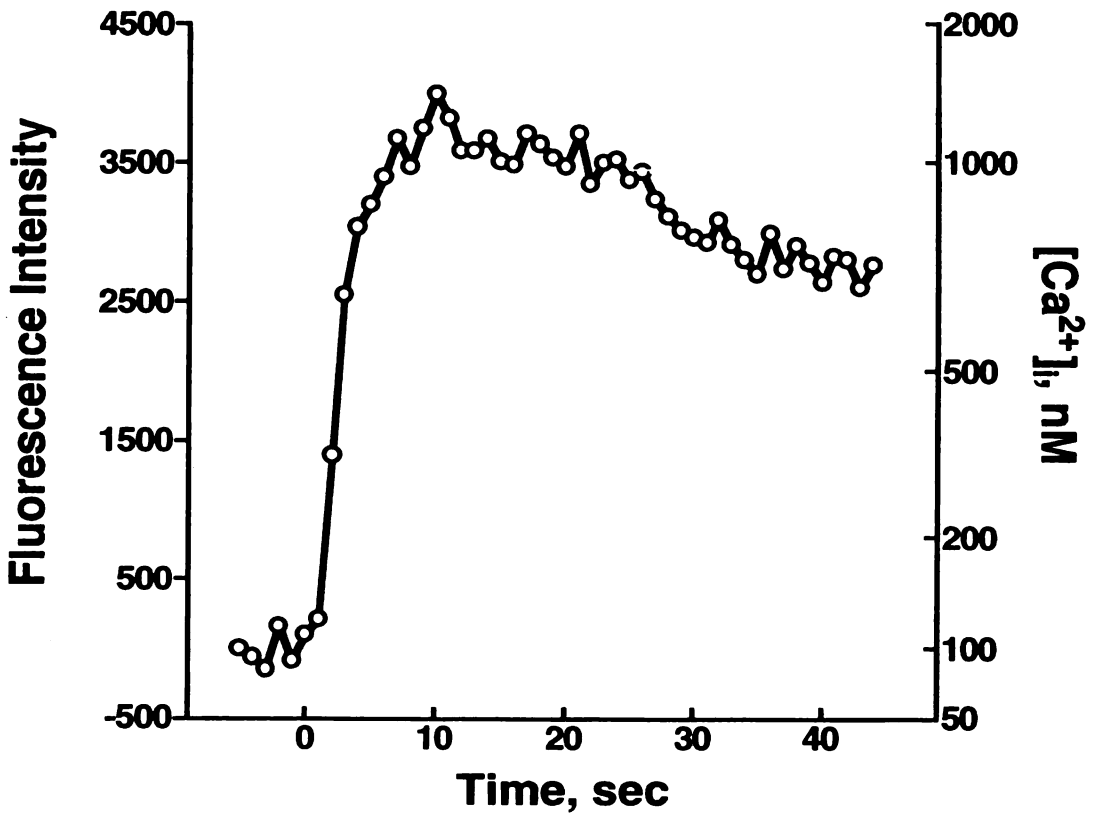


Figure 4.1A. Analysis of Fluorescence Signals and Dye Stability in HEK293 Cells. Response curve produced by injection of ionomycin (1 μM final) into one well of a 96-well plate containing HEK293 cells loaded with Calcium Green-1/AM. This composite curve was generated by subtracting a buffer injected well from a matched well injected with ionomycin. Injections, at 0 sec, were performed after cells were incubated for 30 min at 37°C.

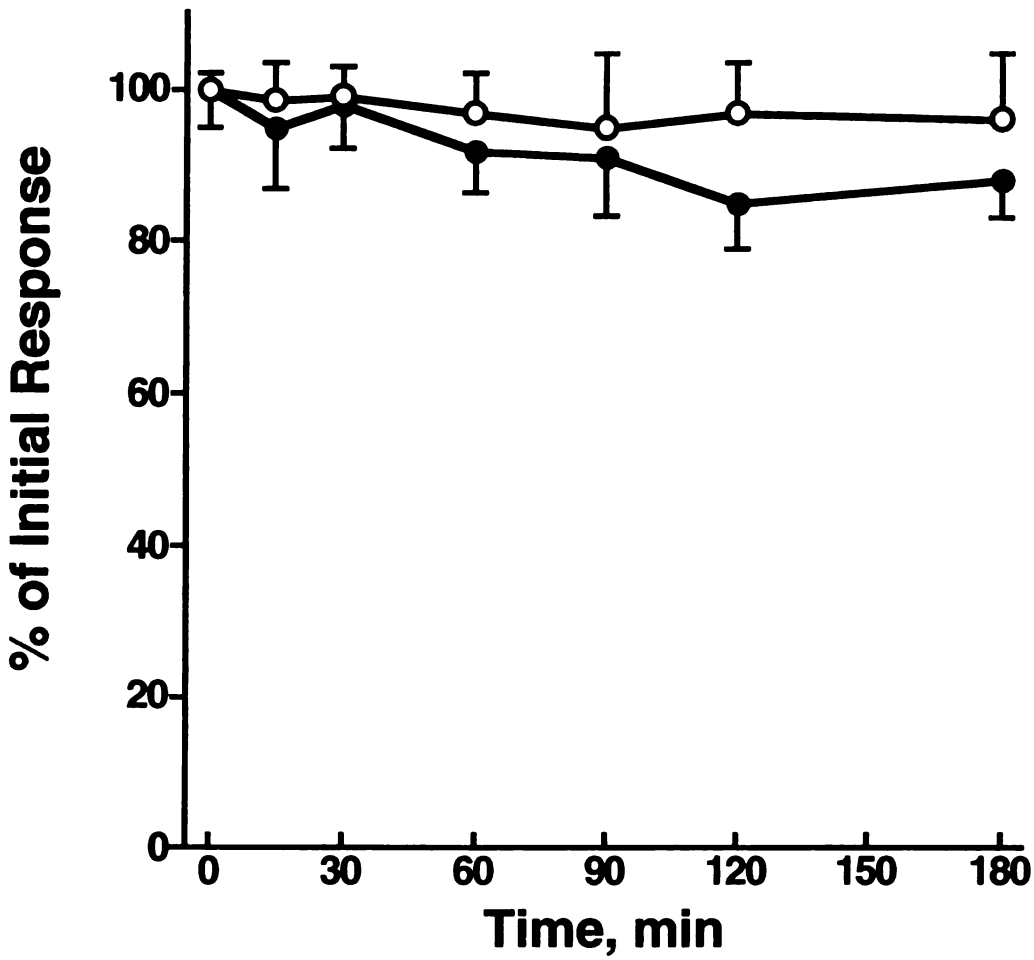


Figure 4.1B. Analysis of Fluorescence Signals and Dye Stability in HEK293 Cells. The stability of Calcium Green-1 fluorescence in HEK293 cells at 37°C (solid circles) and 20°C (open circles) was tested by comparing the amplitude of ionomycin response (illustrated in Figure 1A) over time. Fluorescence signals generated with Oregon Green 488 BAPTA-1 were equivalent in amplitude but slightly more stable over time. Each symbol represents the mean and SD of 4 independent measurements.

Although results with sulfinpyrazone indicated that dye efflux was a major cause of signal degradation in CHO cells, further tests were performed to assess the contribution of temperature and time to this decay. Supernatant fractions were collected at various times after dye loading and the total fluorescence quantified; supernatant fluorescence increased rapidly with Calcium Green-1 loaded cells and less so for cells loaded with Oregon Green 488 BAPTA-1 (data not shown). Dye leakage is highly temperature dependent and, as with HEK293 cells, displayed a single equilateral hyperbolic kinetic (Figure 4.2B). Leakage of Oregon Green 488 BAPTA-1, however, was essentially abolished in the presence of sulfinpyrazone when the temperature was lowered from 37°C to 20°C. Therefore, repeated sequential multi-well plate Ca^{2+} measurements in CHO cells are best done with Oregon Green 488 BAPTA-1 at room temperature, in the presence of the anion transport inhibitor sulfinpyrazone.

C. Analysis of Receptor Activity From 96-well Plates

After establishing compatibility of fluorescent dyes with HEK293 cells, we used the multi-well format to measure the Ca^{2+} responses evoked by activation of endogenous muscarinic receptors with acetylcholine (ACh; RBI) and carbachol (CCh; Sigma). ACh and CCh produced significant, concentration-dependent increases in the Ca^{2+} signal that could be measured from a single 96-well plate using a single batch of Calcium Green-1 loaded cells. An example of responses evoked by CCh in HEK293 cells is shown in Figure

4.3A, which gave an EC_{50} value of $2x10^{-6}$ M. Atropine (RBI) inhibited responses evoked by injection of 100 μM CCh in a concentration-dependent manner, yielding an IC_{50} value of 1.2 nM as expected for this high potency muscarinic antagonist (Figure 4.3B). These results corroborate the muscarinic receptor origin of the fluorescence signals observed.

The sensitivity and versatility of this method of using Ca^{2+} to study G-protein coupled receptor function in HEK293 cells were probed further by measuring the Ca^{2+} responses evoked by stimulation of endogenously expressed β-adrenergic receptors, recombinant Gi-coupled μ-opioid and dopamine D2L receptors, and the Gs-coupled MC4 receptor. Traces and concentration-response curves obtained for μ-opioid and β-adrenergic receptors are shown in Figure 4.4. Ligands effective in evoking Ca^{2+} signals in transfected cells--morphine (RBI), quinpirole (RBI), and α-MSH (Sigma), respectively--had no effect on wild-type, non-transfected cells (data not shown). This demonstrates that Ca^{2+} measurements can be applied to receptors like these, typically viewed as more closely associated with cAMP modulation. These techniques could simplify screening, analysis and study of these receptors *in vivo*.

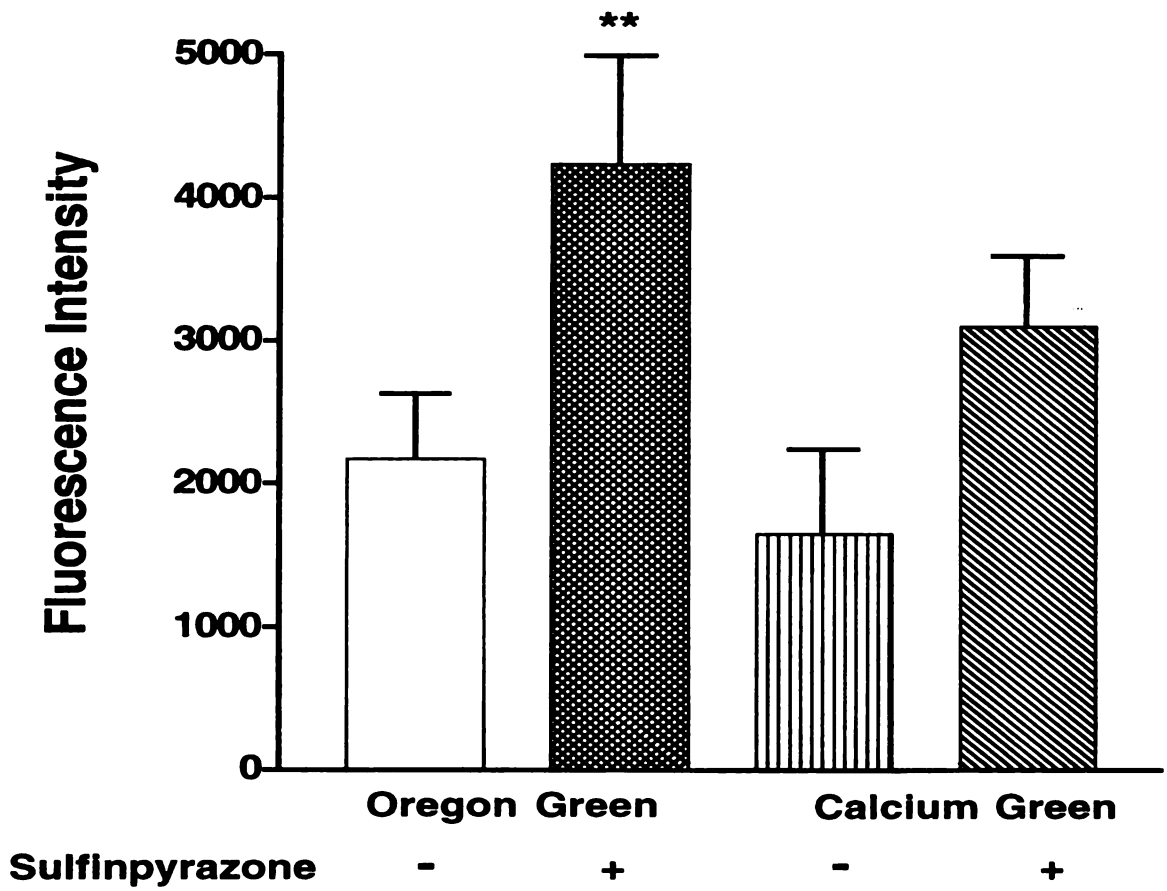


Figure 4.2A. Stability of Visible Light Excitable Dyes in CHO Cells. Signals generated with Calcium Green-1 and Oregon Green 488 BAPTA-1, although stable in HEK293 cells, degrade rapidly in CHO cells. Response amplitude to injection of ionomycin (1 μM), performed within 10 min of dye loading, was significantly improved with addition of 250 μM sulfinpyrazone. ** $P < 0.002$ versus all other groups (students t-test; bivariate, unmatched pairs).

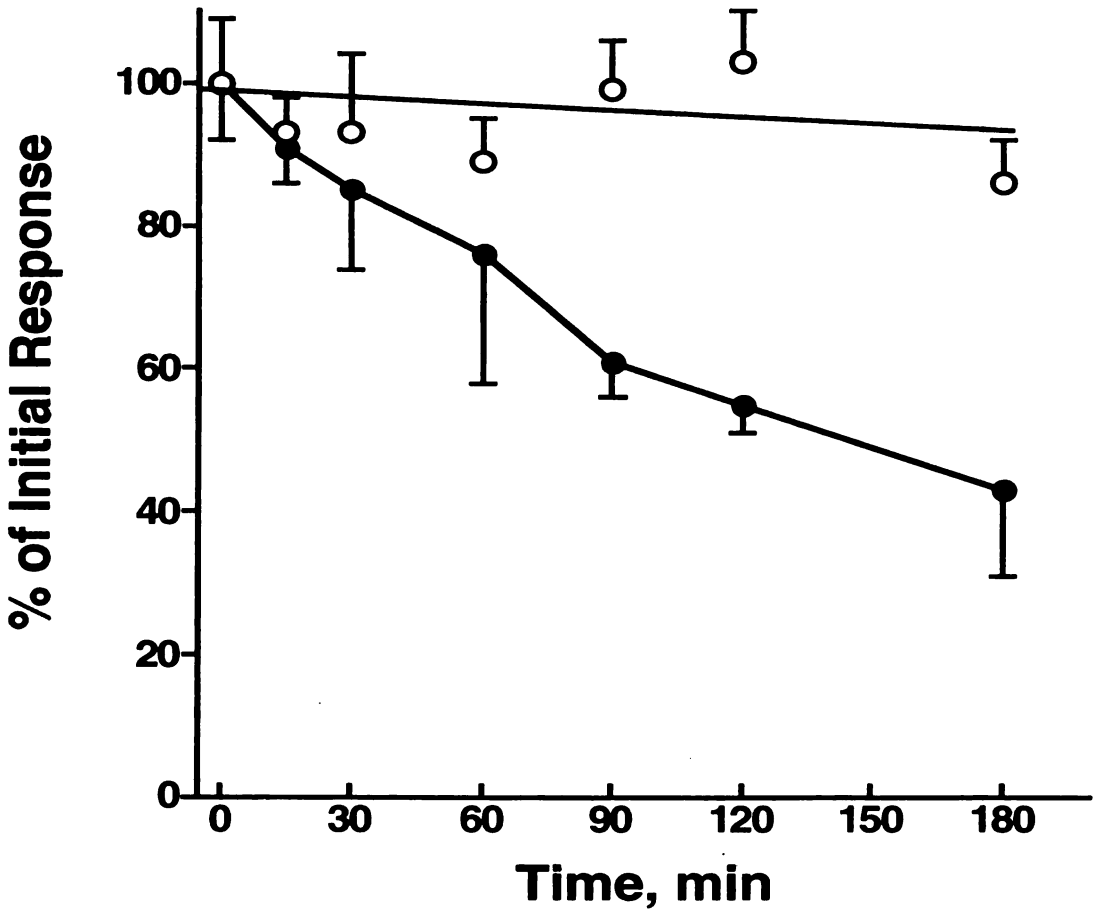


Figure 4.2B. Stability of Visible Light Excitable Dyes in CHO Cells. The stability of fluorescence in CHO cells loaded with Oregon Green 488 B APTA-1 is significantly affected by temperature even in the presence of 250 μM sulfinpyrazone. Leakage of the dye from cells to supernatant, rapid at 37°C (solid triangles), was negligible when the temperature was reduced to 20°C (open triangles).

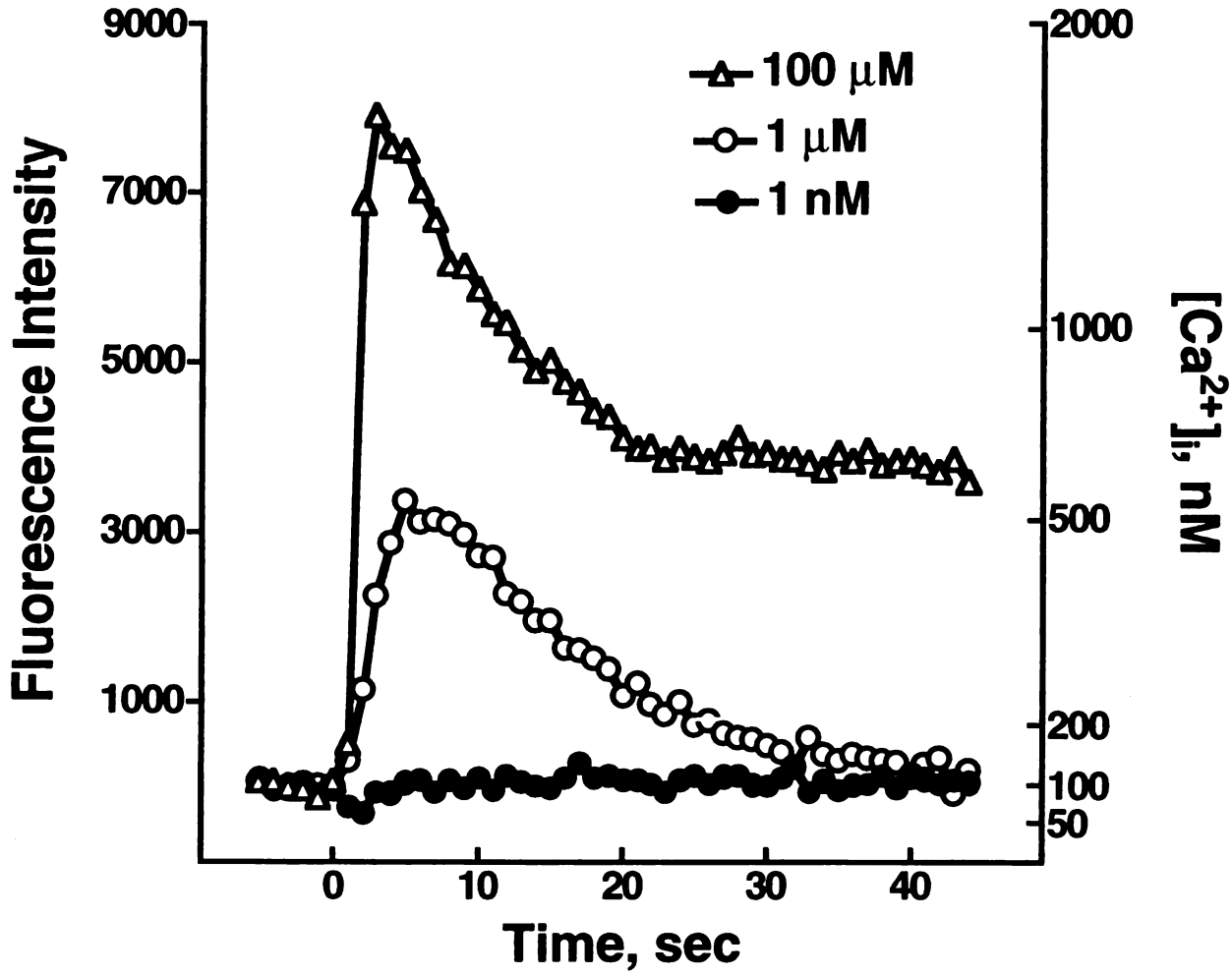


Figure 4.3A. Test of Muscarinic Receptors Endogenous to HEK293 Cells. (A) Muscarinic receptors, expressed at low levels in HEK293 cells, were stimulated with increasing concentrations of carbachol. Signals generated with Calcium Green-1 at 37°C were dose-dependent, robust, and reproducible.

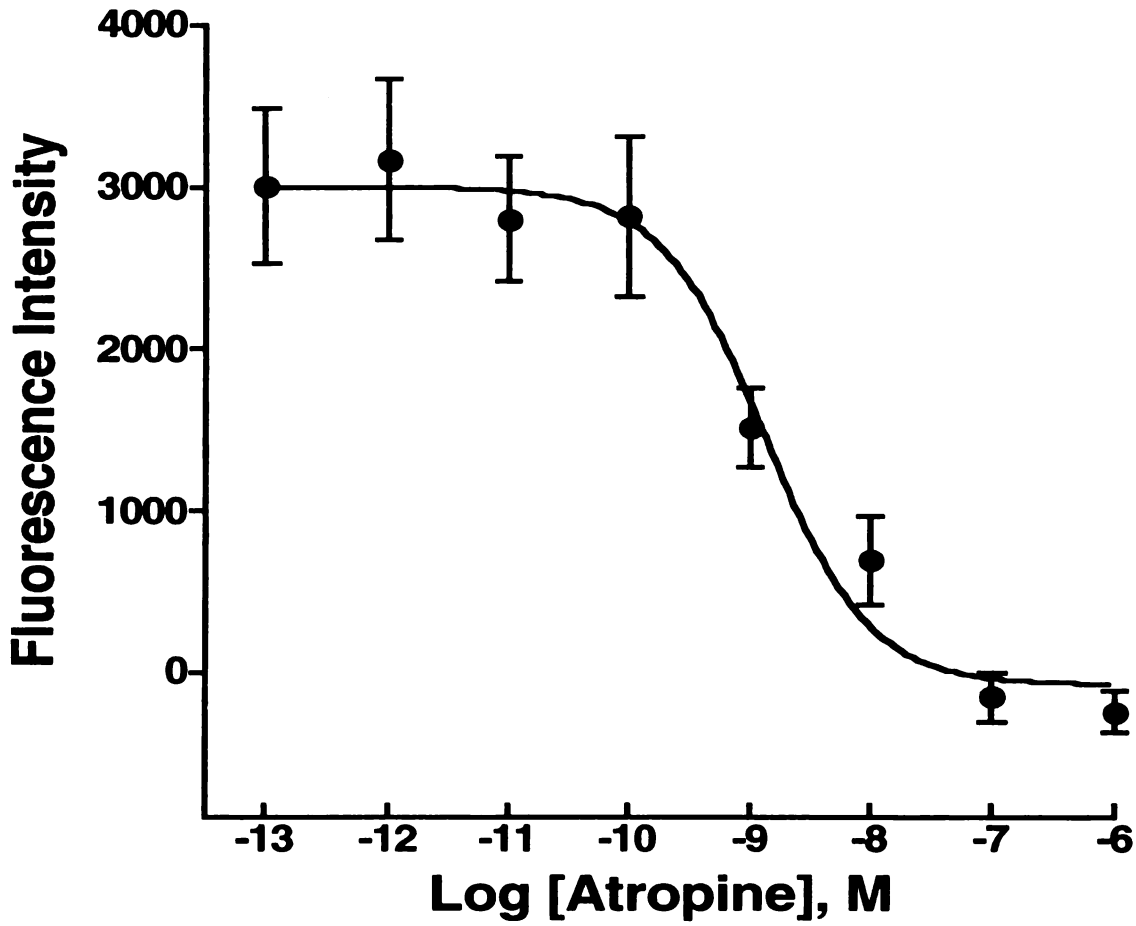


Figure 4.3B. Test of Muscarinic Receptors Endogenous to HEK293 Cells. A n inhibition curve of carbachol (100 μM) by atropine was generated from a single 96-well plate of HEK293 cells loaded with Calcium Green-1. The IC_{50} of atropine was 1.3± 0.2 nM; n=4.

Similar experiments to those described for HEK293 cells were performed using CHO cells stably expressing a recombinant human muscarinic m1 mAChR receptor. Oregon Green 488 BAPTA-1 was then used to measure the calcium responses evoked with ACh and CCh. An example of the fluorescent signal produced by activation of the m1 mAChR receptor in CHO cells is shown in Figure 5.5. In non-transfected cells these responses were absent, and in transfected cells were dose-dependent inhibited by atropine (data not shown). Measurements made from a single pool of Oregon Green 488 BAPTA-1 loaded CHO cells gave an EC_{50} of 4.4 μM for ACh, comparable to the EC50 values reported previously using Fura-2 (Danthuluri et al., 1988; Roux et al., 1997).

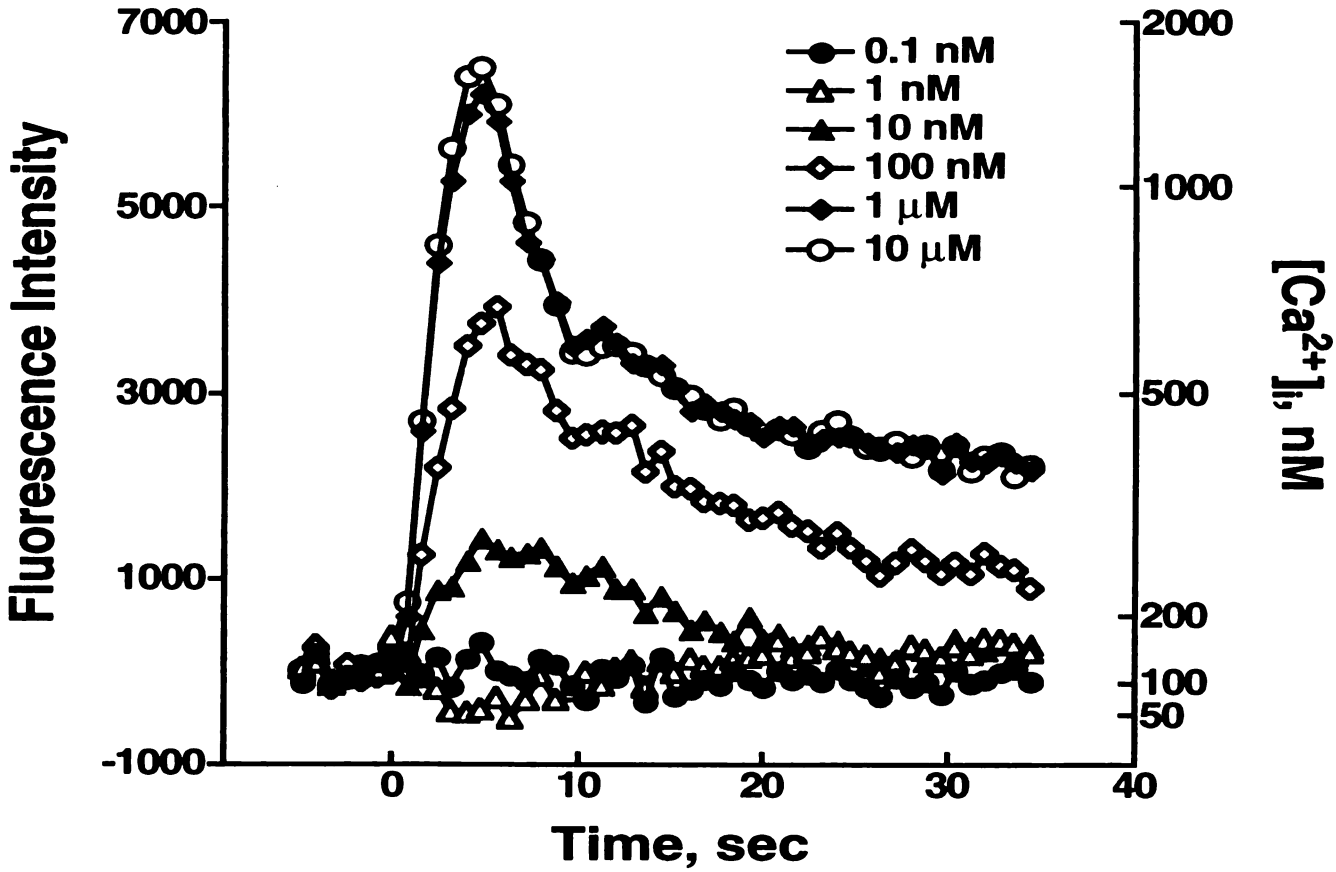


Figure 4.4A. Gi and Gs Coupled Receptors Activate Calcium Flux in H EK293 Cells. S timulation of a recombinant Gi-coupled μ-opioid receptor with morphine produces a dose-dependent increase in Calcium Green-1 fluorescence intensity. Similar responses were observed with a recombinant dopamine D2L receptor (not shown).

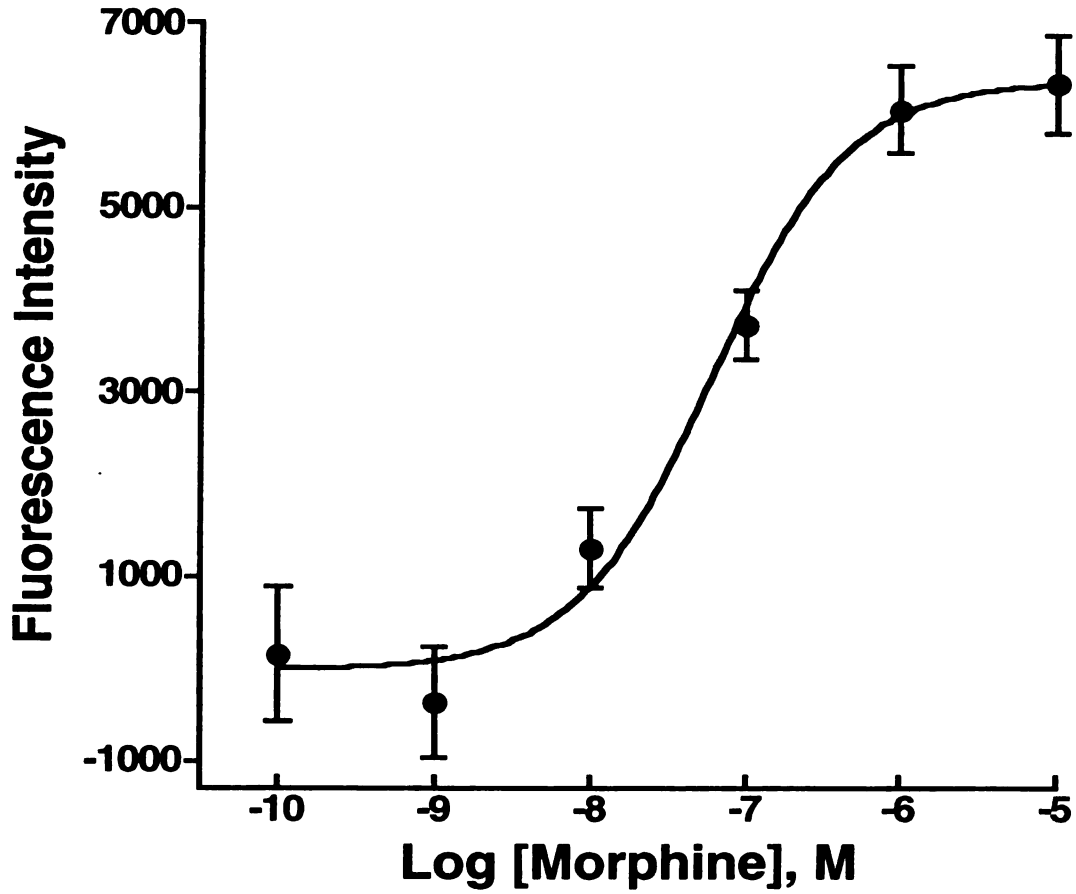


Figure 4.4B. Gi and Gs Coupled Receptors Activate Calcium Flux in HEK293 Cells. A concentration-response curve generated by stimulation of the μ-opioid receptor showes morphine with an EC_{50} of 62 (SD 0.7) nM.

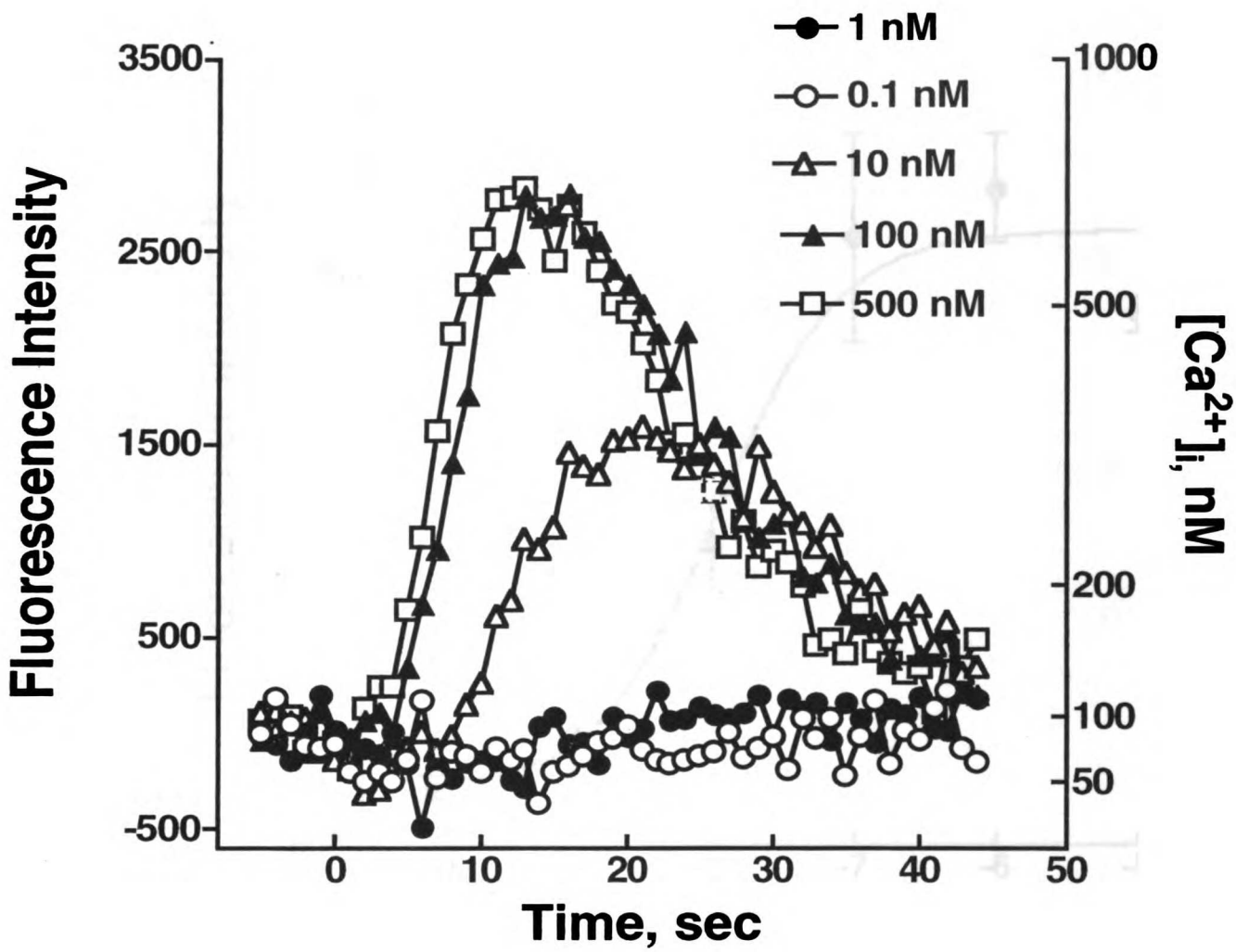


Figure 4.4C. Gi and Gs Coupled Receptors Activate Calcium Flux in H EK293 Cells. Calcium flux activated by stimulation of endogenous Gs-coupled β-adrenergic receptors with increasing concentrations of isoproterenol.

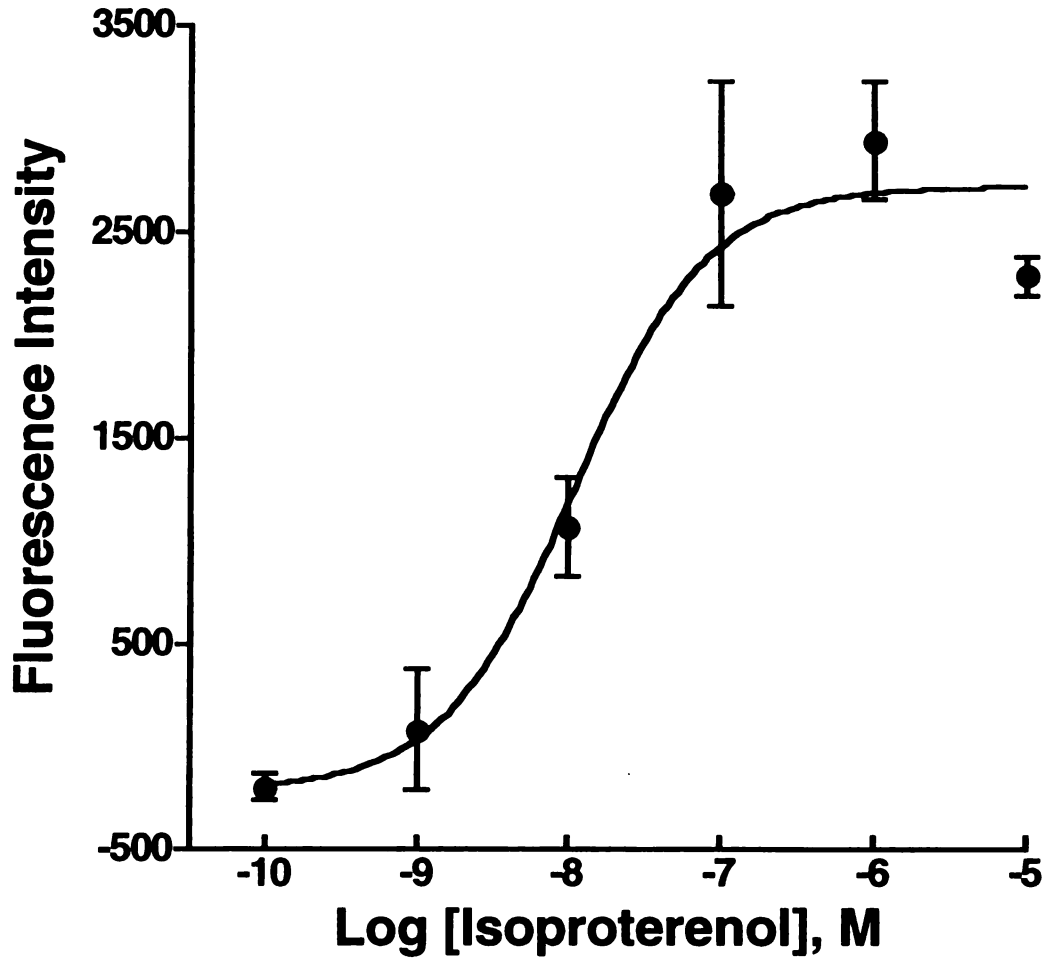


Figure 4.4D. Gi and Gs Coupled Receptors Activate Calcium Flux in HEK293 Cells. Calculated EC_{50} for isoproterenol was determined to be 1.1 (SD 0.6). (A-D) Each point represents a mean and SD (where indicated) of n=4.

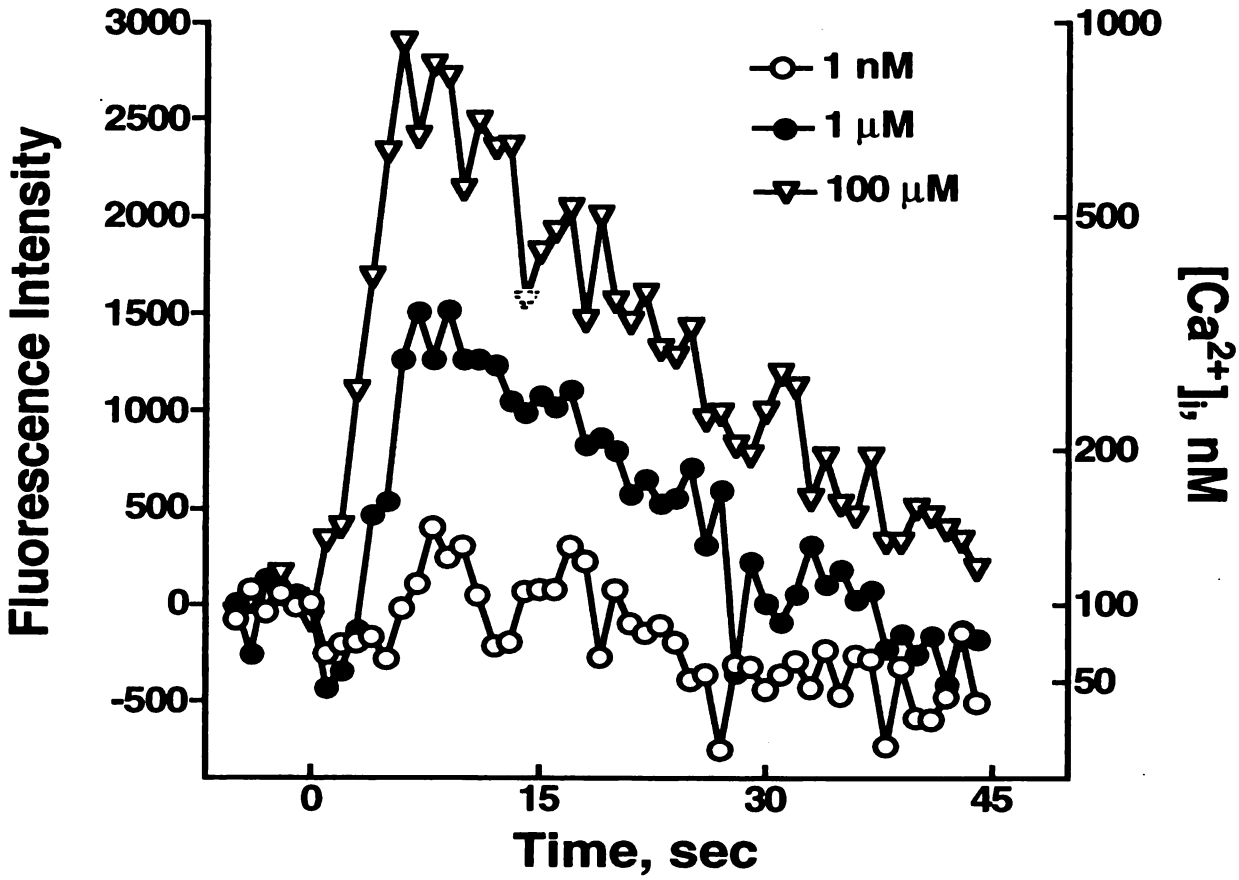


Figure 4.5A. Testing of a Recombinant muscarinic M1 receptor in CHO cells. CHO cells provide a good platform from which to s tudy recombinant muscarinic receptors since they lack endogenous s ites. Using Oregon Green-488, sulfinpyrazone (250 μM), and a temperature of 20°C, it is possible to measure calcium responses in CHO cells transfected with a recombinant muscarinic m1 receptor from 96-well plates.

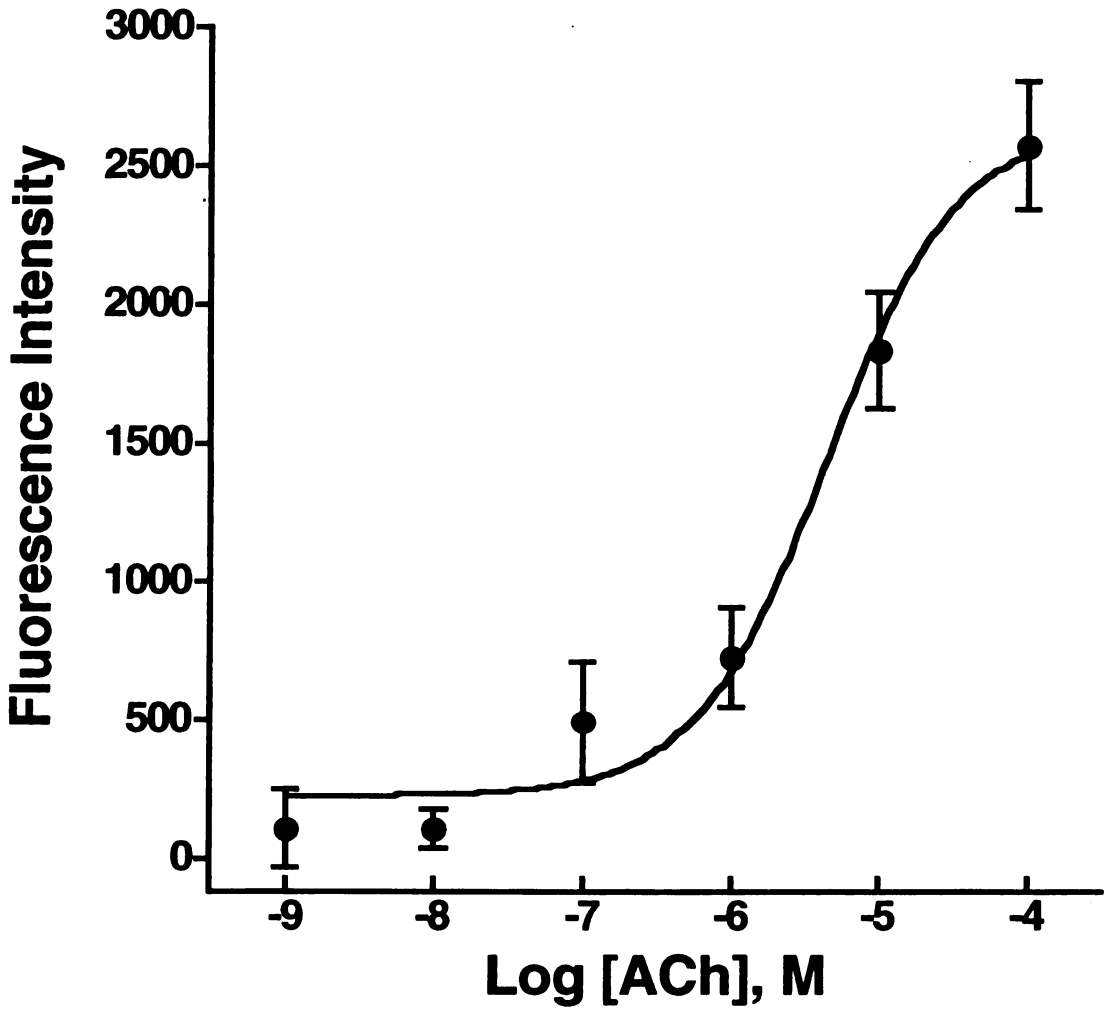


Figure 4.5B. Testing of a Recombinant muscarinic M1 receptor in CHO cells. A concentration-response curve generated by stimulation of the m1 receptor indicates an EC_{50} of 4.4 (SD 1.1) μM. Each point represents a mean and SD (where indicated) of n=4.

V. DISCUSSION

We have evaluated the performance of two visible-light excitable fluorescent dyes, Calcium Green-1/AM and Oregon Green 488 BAPTA-1/AM, in HEK293 and CHO cells, and demonstrated a sensitive fluorometric assay useful for making quantitative measurements of rapid Ca^{2+} responses using a multi-well plate format. Multi-well formats permit rapid, routine collection of data, as demonstrated here, and facilitate analysis of a wide variety of G-protein coupled receptors. These methods should simplify large-scale screening and make analysis of calcium responses less expensive and more widely available to pharmaceutical and biological scientists alike.

In HEK293 cells both Calcium Green-1 and Oregon Green 488 BAPTA-1 performed well for extended periods of time; calcium could be measured from 96-well plates at room temperature and even at 37°C. Since cellular processes involved in second messenger regulation can be differentially affected by changes in temperature, the option of performing experiments at different temperatures can be useful. In CHO cells, Oregon Green 488 BAPTA-1 was stable enough for multi-well formats if an inhibitor of anion transport was used in conjunction with low temperatures. At 37°C and in the absence of the inhibitor, both dyes are extruded rapidly from CHO cells; this factor should be considered if Ca^{2+} is to be measured in studying thermal regulation (Gottesman, 1985). It is unclear from these results why the two dyes behave so differently with respect to cellular efflux since they are remarkably similar in structure; our results do

suggest, however, that Oregon Green 488 BAPTA-1 should be considered for cell lines where leakage is a major problem.

The ability to detect and analyze endogenous muscarinic and β-adrenergic activity in HEK293 cells demonstrates that multi-well calcium assays can be sensitive and convenient ways to monitor receptor activity. As routine measures of receptor activity, current protocols for inositol phosphate and cAMP measurement are slow, expensive, laborious, and hampered by low levels of receptor expression. For example, low expression of muscarinic receptors endogenous to HEK293 cells leads to a barely measurable 2-fold increase in inositol phosphate levels, whereas in cells overexpressing a recombinant m1 mAChR muscarinic receptor, increase is more than 20-fold (unpublished observation, KL). Sensitivity of the multi-well system illustrated here is reflected in the robust Ca^{2+} responses easily measured in non-transfected cells.

β-adrenergic receptors activate the Gα subunits of heterotrimeric G-proteins which stimulate adenylyl cyclase and cause increases in cytoplasmic cAMP levels. While Gq-mediated pathways are those more commonly associated with intracellular Ca^{2+} flux, in HEK293 cells β receptor activation has been shown also to cause a minor, transient rise in cytoplasmic Ca^{2+} levels (Skeberdis et al., 1997). The ability to measure these subtle responses demonstrates not only the sensitivity of this multi-well format, but also its convenience as a means to analyze Gs-linked receptors. Since Ca^{2+} responses were also visible in cells transfected with melanocortin receptors and upon stimulation with forskolin (data not shown), Ca^{2+} flux due to Gs stimulation is not

unique to β-adrenergic receptors. HEK293 cells, therefore, may provide a simple and inexpensive way to screen an array of Gs-coupled receptors without need to resort to chimeric Gα proteins to transduce Ca^{2+} signals.

Ca^{2+} fluctuations in response to activation of Gi-linked receptors, in addition to Gq- and Gs-linked ones, can be measured with this system, as demonstrated with transfected μ-opioid and D2L dopamine receptors. The current understanding of cellular mechanisms involved in generating calcium flux upon activation of these receptors is limited; improved data collection techniques should help unravel the transduction pathways and their relationship to enigmatic processes like addiction (Aley & Levine, 1997; Spencer *et al.*, 1997).

The need for high-throughput screening and analysis will undoubtedly continue to drive the evolution of devices and methods for measuring receptor activity. Parallel injection systems--systems which inject multiple or all wells simultaneously (Dooley *et al.*, 1998)--will reduce the importance of signal stability in some instances but will not eliminate it entirely. Multi-well plates processed serially with robotic devices, for example, will still require stability for efficient processing, and issues pertaining to temperature will remain even if thermal regulators are available. For those who do not need or cannot afford such instrumentation, the serial injection multi-well systems can be a convenient and effective way to screen and analyze calcium signals *in vitro.*

CHAPTER 5

NOVEL BIOASSAY FOR IMMUNOSUPPRESSANT DRUG SCREENING AND CLINICAL MONITORING

I. SUMMARY

This chapter describes the integration of cell surface receptor and nuclear signaling to provide a reporter gene assay. This assay is suitable for screening of immunosuppressant compounds, and is adaptable to miniaturization and high-throughput screening.

Immunosuppressants act through inhibition of T cell proliferation. T cell transcription factors have been identified and targeted for genetic and pharmacological manipulation of T cell immunity. Among them, NFAT (nuclear factor of activated T cells) was first identified as an inducible nuclear protein complex that translocates to the nucleus upon activation. The translocation step has been targeted by a group of immunosuppressant compounds, including FK506 and Cyclosporine A (CsA). We developed a luciferase reporter gene assay measuring the level of NFAT expression driven by muscarinic receptor activation or intracellular stimulants in Jurkat T cells. In this assay, the IC50 of FK506 and Cyclosporine A were 30 nM and 1 nM respectively. Meanwhile, NFAT specific inhibitors were discriminated from immunosuppressants that act on different targets. Rapamycin, which interferes with the progression of T cells from G1 to S phase of the cell cycle, failed to inhibit NFAT activation.

To illustrate the versatility of this assay, we applied this assay to the analysis of FK506 in patient blood samples and obtained a pharmacokinetic profile similar to that obtained with conventional LC-MS. This activity driven assay simultaneously monitors the parent compound and active metabolites. Thus, the assay provides a clinical readout of total drug activity. This 96-well high-throughput assay can be used for both screening of new immunosuppressants and for monitoring of blood levels of immunosuppressants in patients.

II. INTRODUCTION

Inhibition of T lymphocytes development and function plays a vital role in suppressing immune response. Immunosuppressant drugs inhibit immune response by blocking transcription of genes that are critical for mounting immune response, such as cytokine genes (Mattila *et al.*, 1990). Gene transcription is tightly regulated by signal transduction pathways from cell-surface receptors, intracellular signaling molecules, to nuclear transcription factors (Peters *et al.*, 1993). Recent gene targeting studies in mice have identified unique roles for a number of transcription factors in T cell development and function (Kiani *et al.*, 2000).

NFAT (nuclear factor of activated T cells) was first identified as an inducible nuclear protein complex that could bind to a sequence motif present in the distal antigen receptor response element of the human IL-2 promoter (Shaw *et al.*, 1988). NFAT proteins are retained in the cytoplasm of resting T cells in a serine phosphorylated form. Three different steps of activation have been defined: dephosphorylation, nuclear translocation, and increase in affinity for DNA. After T cell activation, NFAT protein is dephosphorylated by the protein phosphatase, calcineurin. Calcineurin controls the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells by interacting with an N-terminal regulatory domain conserved in the NFAT family (Figure 5.1) (Cantrell, 1996; Weiss & Littman, 1994).

Calcium- and calmodulin- dependent calcineurin is activated by receptor stimulation and calcium mobilization. As a major upstream regulator of NFAT

protein, calcineurin has become the target of several successful immunosuppressants, such as Cyclosporin A (CsA) and FK506. The isolation of CsA from cultures of the fungus *Tolypocladium inflatum* and its subsequent elucidation of immunosuppressive properties by Borel et al. (Borel *et al.*, 1977) was of great clinical consequence. Together with the discovery of FK506 (Kino & Goto, 1993), their introduction to the clinic led to extraordinary improvements in organ transplantation (Schreiber & Crabtree, 1992; Sigal & Dumont, 1992).

However, most patients suffered serious adverse effects and developed life-long dependence to these drugs. The adverse effects mainly result from the non-selective inhibition of ubiquitous phosphatase activity. The identification of the involvement of transcription factor NFAT was an important step forward (Shaw *et al.*, 1988), and a family of four NFATs with distinct sequences and specificity has since been discovered (Kiani *et al.*, 2000; Kuo & Leiden, 1999). By targeting T cell specific NFAT, it is possible to identify novel immunosuppressants with fewer side effects.

We used Jurkat T cells transfected with luciferase reporter plasmid constructs driven by a basic promoter element (TATA box) joined to four tandem repeats of the NFAT binding sequence. The activation of the NFAT by stimuli that increase free calcium in the cytoplasm induces luciferase gene expression. Activation of NFAT can be measured by levels of luciferase activity.

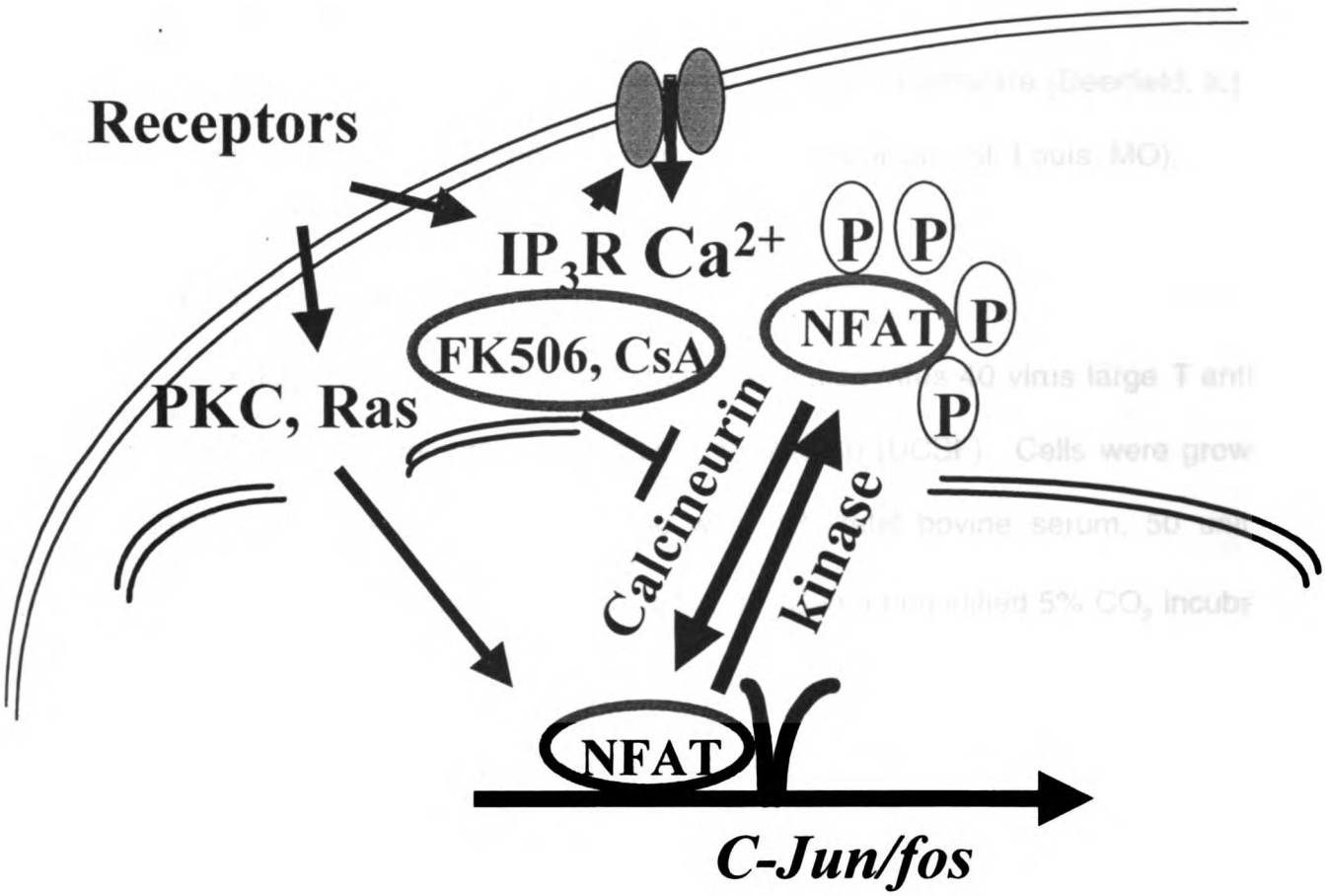


Figure 5.1. Model for activation of NFAT proteins. NFAT inteacts with calcineurin and stays in the cytoplasm in resting T cells; the binding of CaM triggered by increase of intracellular calcium ($[Ca^{2+}]_i$) leads to a conformational change and activateion of calcineurin; activated calcineurin dephosphorylates NFAT; dephorsphorylation results in NFAT nuclear import and increase in DNA binding activity; withdrawal of calcium or inhibition of calcineurin leads to rephorsphorylation and returning of NFAT to the cytoplasm. (Adapted from Crabtree, Cell, 96: 611-614,1999).

III. EXPREMENTAL PROCEDURES

A. Materials

FK506 (Tacrolimus) was provided by Fujisawa Healthcare (Deerfield, IL). All other reagents were purchased from Sigma Chemicals (St. Louis, MO).

B. Cell Culture

Jurkat Leukemic T cells containing the simian virus 40 virus large T antigen (TAg-Jurkat) were obtained from Dr. Songzhu An (UCSF). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml Streptomycin at 37 °C in a humidified 5% CO_2 incubator.

C. DNA Constructs and Transfection

Human muscarinic cholinergic subtype 1 (hm1 mAChR) receptor construct described in the previous chapters was used for co-transfection. Cells in 6-well plate (10^6 cells per well) were co-transfected either with 0.5 μg of pNFAT-Luc (Stratagene, La Jolla) DNA/well plus empty vector or co-transfected with 1 μg hm1 mAChR. Transfections were carried out using the LipofectAmine™ Plus kit (Life technologies, Inc.) according to the manufacture's instructions. 18 h later, cells were treated with receptor ligand or activators for 4 h, then lysed using Reporter Lysis Buffer (Promega). Luciferase activity was measured using luminescence plate reader (Molecular Devices). When inhibition studies were conducted, the indicated compounds were added at the

same time as the ligand CCh. A similar inhibitory effect was obtained, whether the test compound was added before or at the same time as the CCh.

D. Biological sample preparation and analysis using LC-MS

Blood samples were extracted from human subjects enrolled in FK506 clinical trial in the General Clinical Research Center of the University of California, San Francisco. 0.5 ml blood samples were precipitated with 1 ml methanol/0.2 M ZnSO4 (80/20) containing 50 μg/L internal standard ascomycin. 500 μl extracted supernatants were injected into the LC/LC-MSD system and analyzed.

E. Biological sample preparation and analysis using cell-based assay

0.5 ml blood samples were precipitated with 1 ml methanol and supernatants were concentrated under nitrogen gas. 500 μl of serum free medium was added to reconstitute the extracts and applied directly onto the cells.

F. Data Analysis

Statistical analyses were performed using GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, CA).

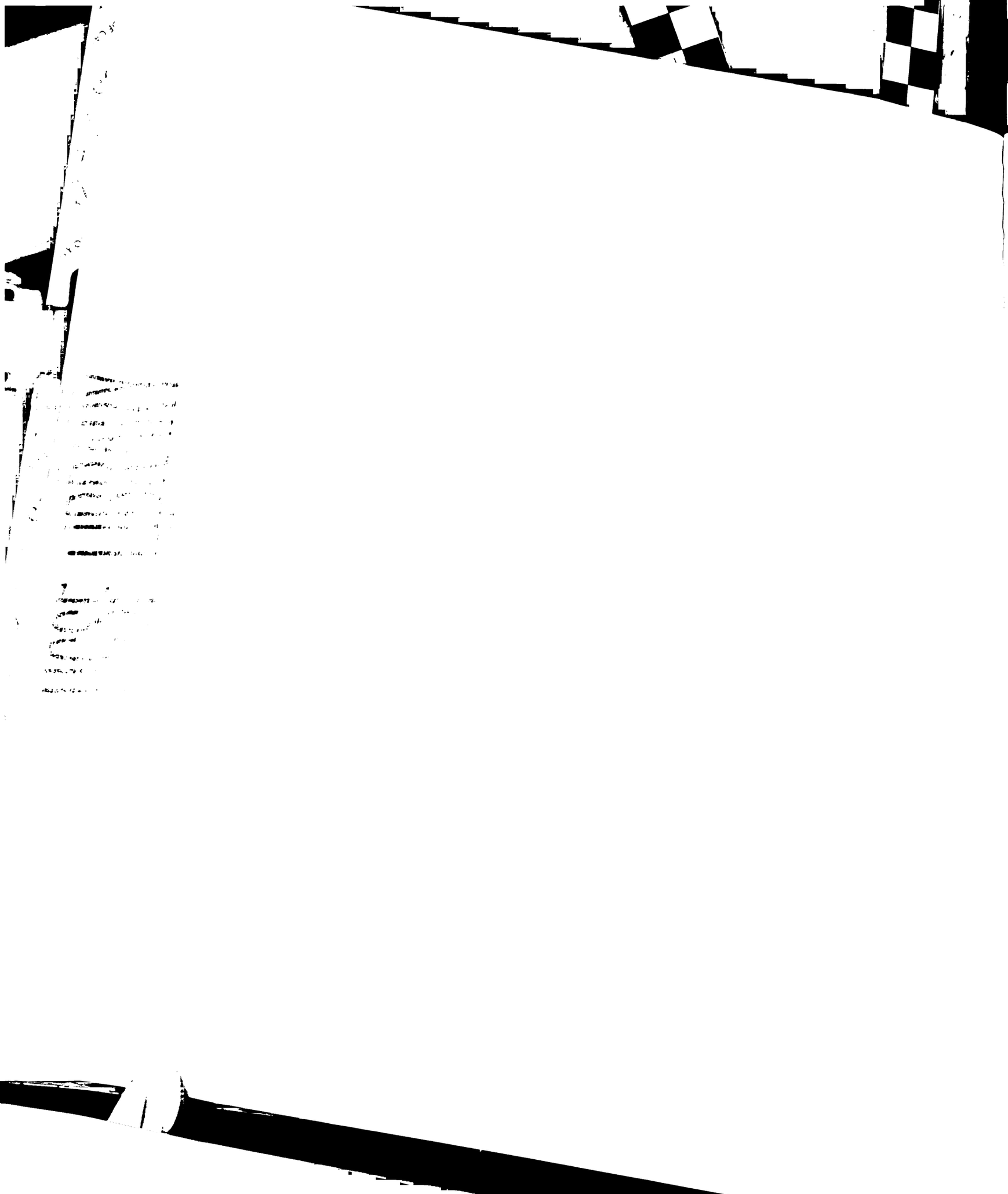
IV. RESULTS

A. Stimulation of NFAT-Luc by Muscarinic Receptors and by Intracellular Stimuli

The NFAT luciferase reporter plasmid constructs were driven by a basic promoter element (TATA box) joined to four tandem repeats of the NFAT binding sequence. First, the activity of NFAT expression vector was demonstrated with the use of surface receptor independent stimuli. Stimuli that increase free calcium in the cytoplasm activate NFAT and induce luciferase gene expression. A combination of the calcium ionophore, ionomycin and phorbol ester (PMA) mimics the effects of TCR cross-linking on gene transcription. PMA enhances the calcium sensitivity of NFAT-dependent reporter gene expression. Over 50 fold increase in luciferase activity can be achieved with the combination of 1 μM ionomycin and 1 μM PMA (Figure 5.2).

In parallel to receptor independent NFAT activation, intracellular Ca^{2+} mobilization triggered by G protein coupled receptors, such as muscarinic receptor mAChR, also rapidly dephorsphorylates NFAT protein and activates NFAT. 100 μM Carbachol (CCh) stimulated Jurkat cells co-transfected with hm1 and NFAT-Luc reporter gene (hm1/NFAT) to a similar extent as PMA and ionomycin. Control cells transfected with empty vector showed no activity upon CCh stimulation (Data not shown). This stimulation was inhibited by muscarinic receptor antagonist atropine (Figure 5.3).

To select an assay format for inhibition and screening, we also compared the stimulation profile between PMA / ionomycin and mAChR. The PMA /



ionomycin combination exhibited the maximum activation even at low concentration. The major known effect of PMA in T cells is to activate protein kinase C, Ras, and Raf (Crabtree, 1999). PMA-activated kinases may also directly phosphorylate NFAT, and increase the sensitivity to calcium. The calcium ionophore ionomycin increases the cell membrane permeability, and in combination with PMA induced strong but less differentiated stimulation. In contrast, the receptor mediated NFAT showed clear dose-dependent pattern with greater sensitivity and less toxicity to the cells, and we chose this system for further study. EC50 for CCh stimulated NFAT was 10 μM, similar to hm1 mAChR activated IP production and Ca^{2+} mobolization (Data not shown).

B. NFAT Inhibition by CsA and FK506

In order to test whether the reporter plasmid was indeed capable of detecting specific inhibition on NFAT pathway, cells transiently transfected with pNFAT-Luc and hm1 mAChR plasmid were tested with known immunosuppressants. CCh induced a 50 fold increase in NFAT mediated luciferease activity. This stimulation was inhibited by CsA and FK506 in a concentration dependent manner. IC50 for CsA and FK506 were 30 nM and 1 nM respectively (Figure 5.4 A&B), consistent with previously published data (Sigal & Dumont, 1992). FK506 inhibited both receptor mediated and intracellular stimulant PMA stimulated NFAT activation with similar IC50s (Figure 5.5 A&B). The reporter gene assay closely reflects the relative potency of two immunosuppressant compounds, FK506 and CsA (Peters *et al.*, 1993).

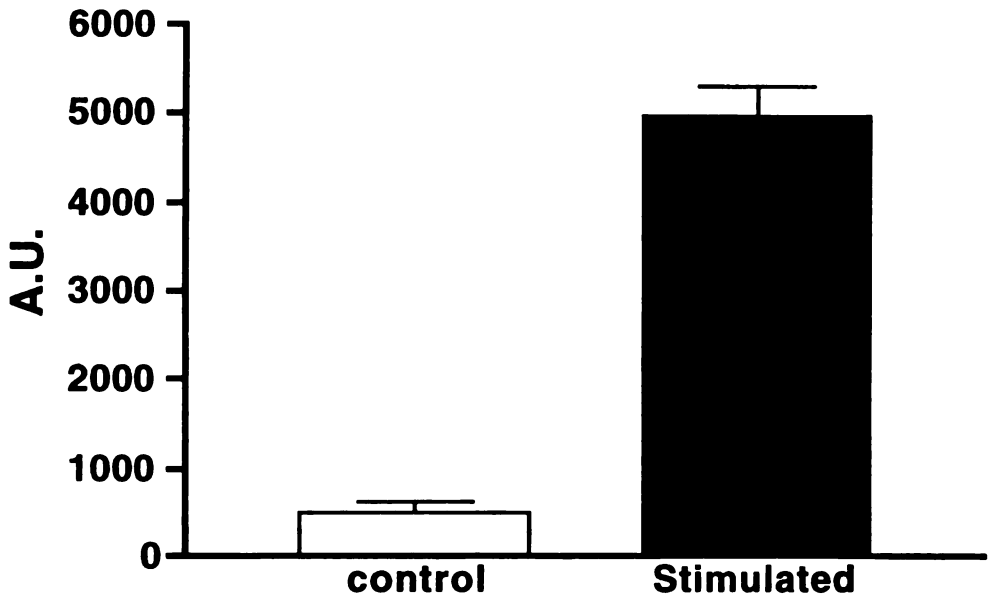


Figure 5.2. Activation of NFAT by inomycin and phobol ester. Jurkat cells transfected with pNFAT-luc were treated with a combination of PMA and inomycin (1μM ea.) for 4 hr. Cells were then lysed and luciferase activity was determined. Data shown here is from a representative experiment carried out at least three times with triplicate. Error bars represent S.D.

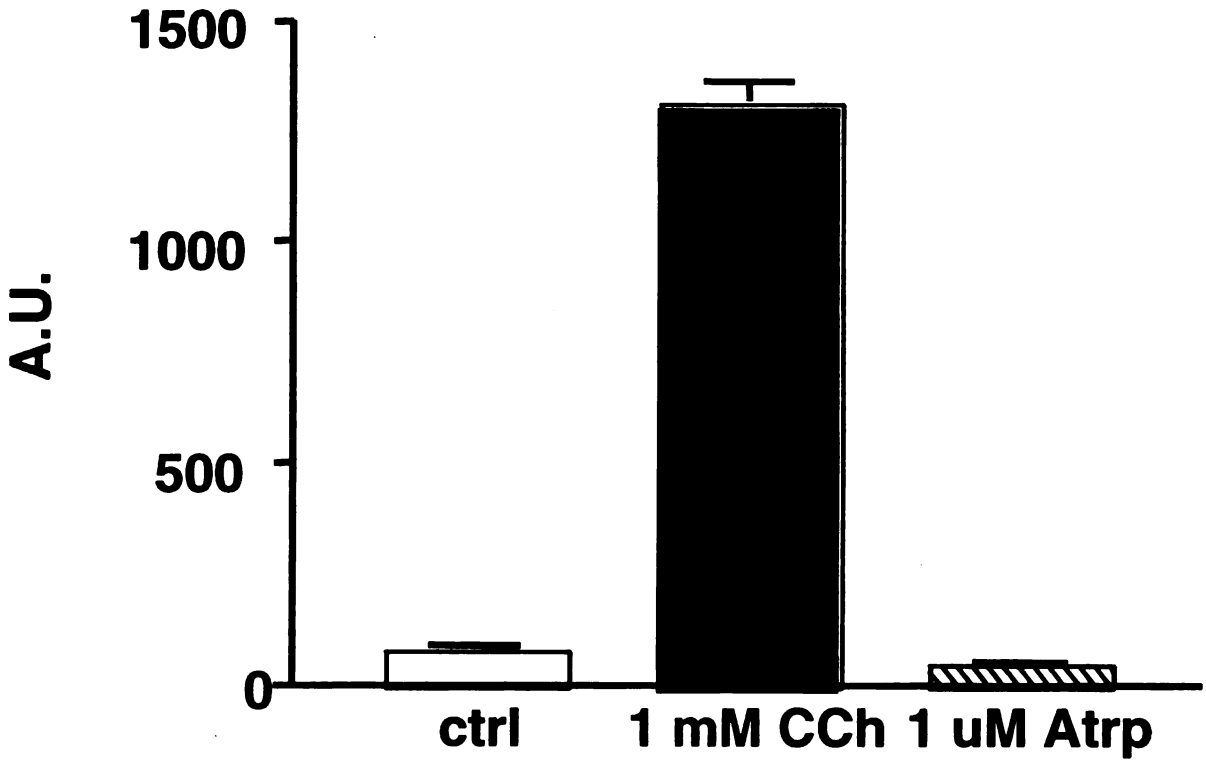


Figure 5.3. Activation of NFAT by m1 mAChR. Jurkat cells transfected with hm1 mAChR and pNFAT-luc plasmid were treated with 100 μM carbachol for 4 hr. Cells were then lysed and luciferase activity was determined. Experiments were done at least three times with triplicate. A representative experiment is shown. Error bars represent standard deviation.

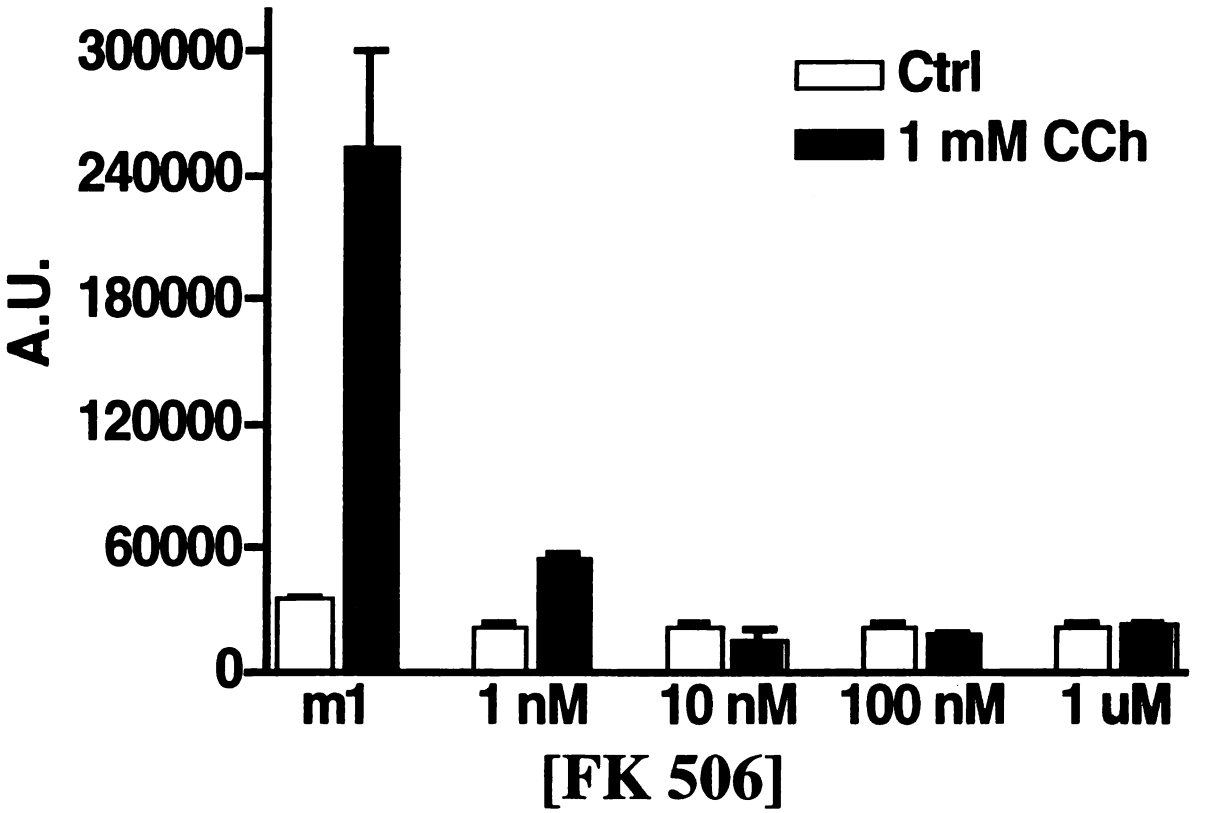


Figure 5.4A. Inhibition of NFAT activity by CsA. Jurkat cells transiently transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with indicated compounds for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.

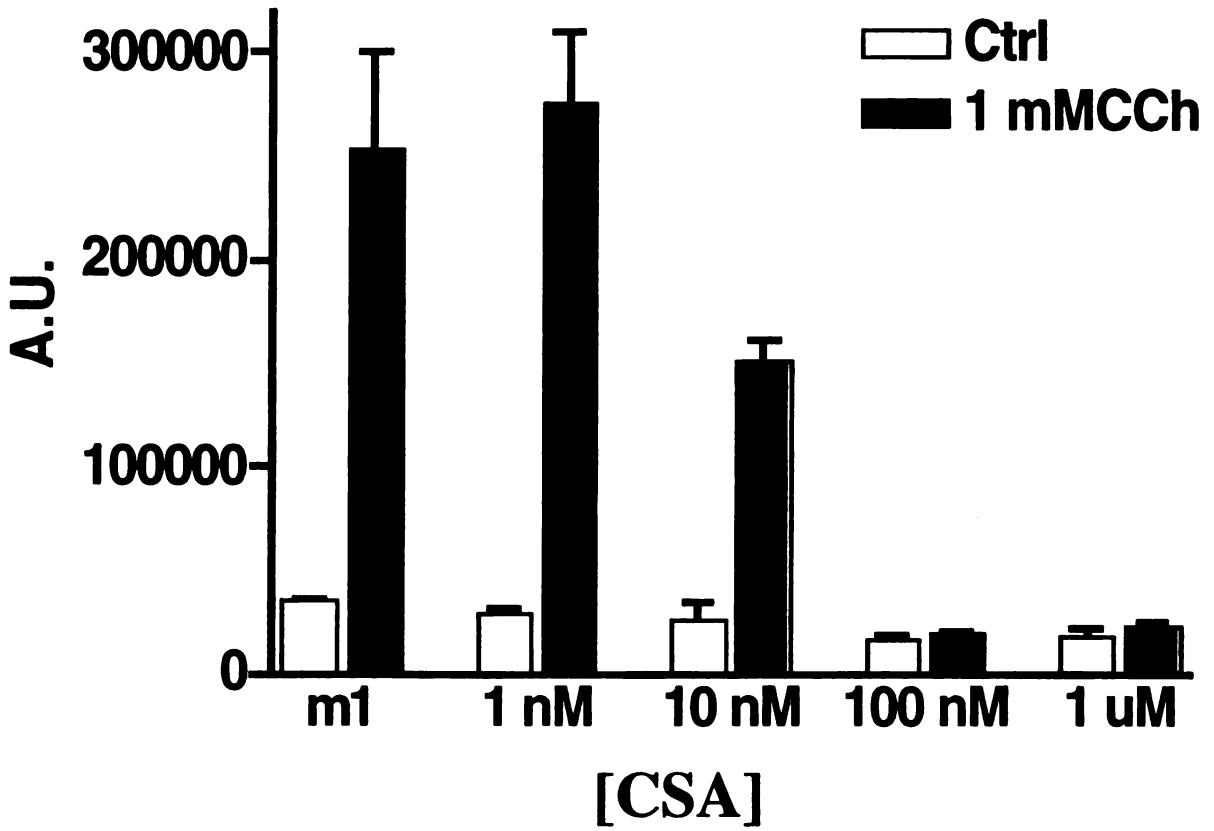


Figure 5.4B. Inhibition of NFAT activity by FK506. Jurkat cells transientlt transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with indicated compounds for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.

C. Pathway Specific Inhibition of NFAT activity

To verify the specificity of NFAT activation pathways, we tested another member of immunosuppressant family, rapamycin. Both FK506 and rapamycin interfere with signal transduction pathways required for T cell activation and growth. The distinct inhibitory effects are mediated through the formation of pharmacologically active complexes with FK506 binding proteins (FKBPs). The FKBP12-FK506 complex specifically binds to and inhibits calcineurin. The FKBP12-rapamycin complex interacts with a target protein termed the mammalian target of rapamycin (mTOR), and interferes with the progression of T cells from G1 to S phase of the cell cycle (Abraham & Wiederrecht, 1996). Using cells co-transfected with pNFAT-luc and m1 mAChR plasmid, Rapamycin failed to inhibit CCh stimulated NFAT activity at 10 nM concentration. Only at 100 nM concentration, rapamycin showed non-specific inhibition of NFAT activity (Figure 5.6). In comparison, its IC50 for inhibiting FK506 binding of FKBP was around 1 nM.

D. FK506 Detection in Biological Samples

In addition to using the assay described in this chapter for screening of immunosuppressant compounds, we tested the feasibility of its use for detecting currently available immunosuppressants, particularly FK506, in biological samples. Due to its narrow therapeutic index and highly variable pharmacokinetics, routine monitoring of FK506 concentrations in blood is required. We compared our cell-based assay with the method currently used

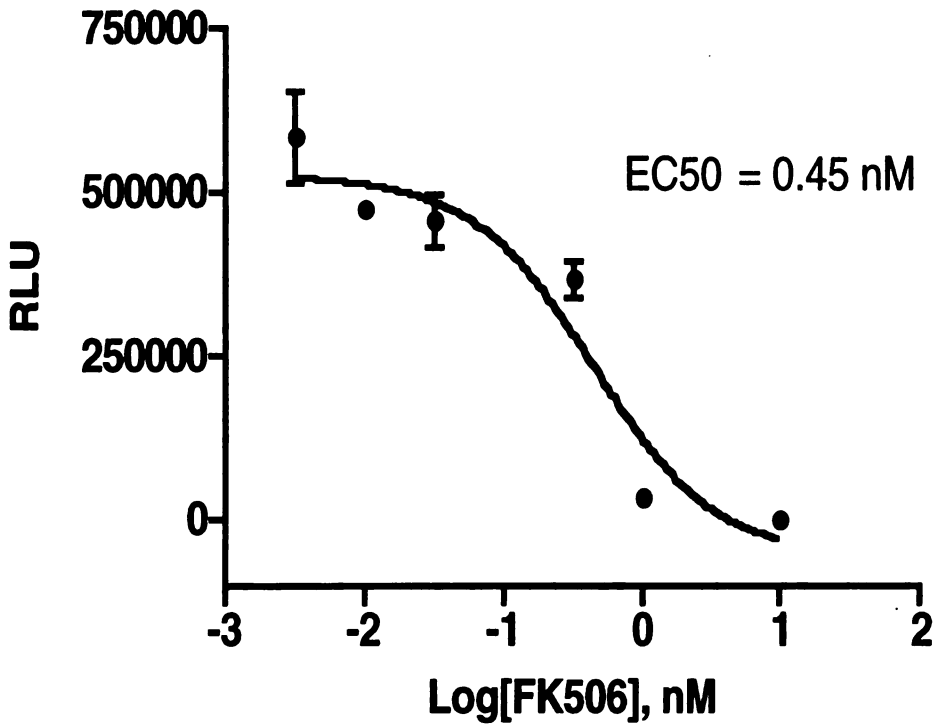


Figure 5.5A. Effect of FK506 on PMA mediated NFAT activity. J urkat cells transientlt transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with FK506 at indicated concentration for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.

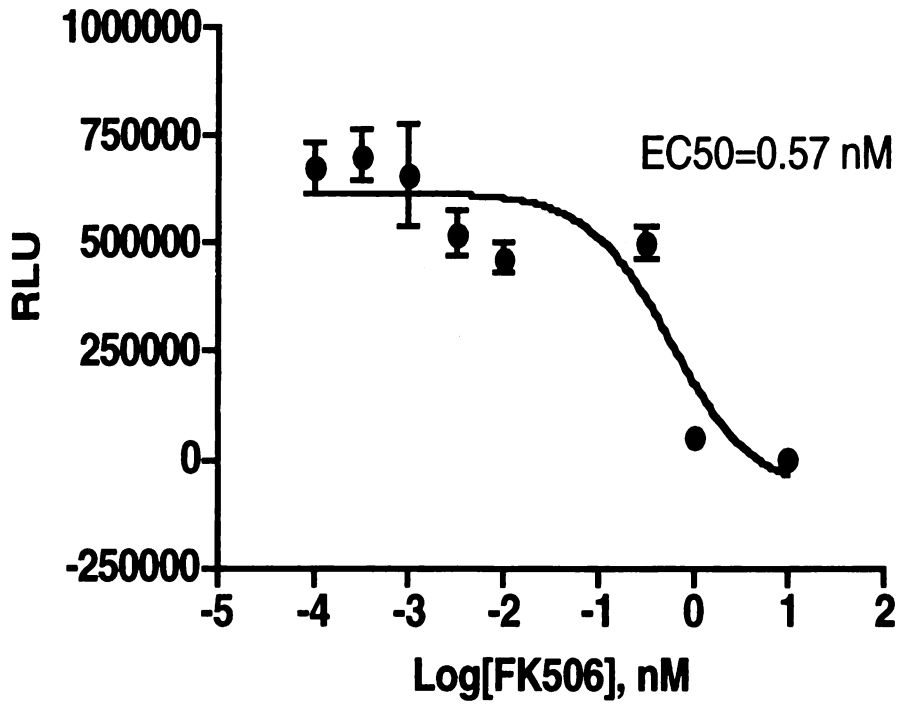
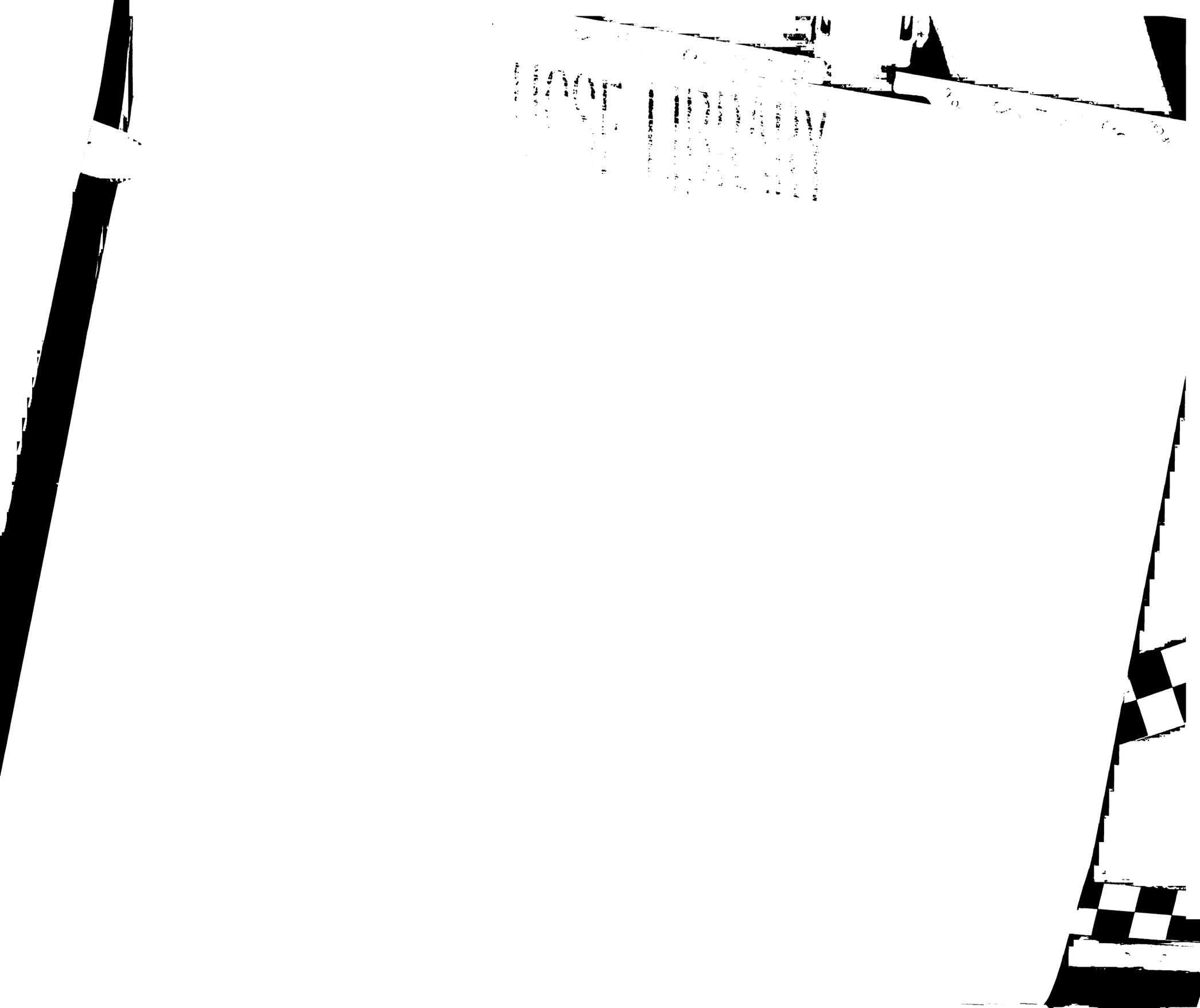


Figure 5.5B. Effect of FK506 on m1 mAChR mediated NFAT activity. Jurkat cells transientlt transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with FK506 at indicated concentration for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.



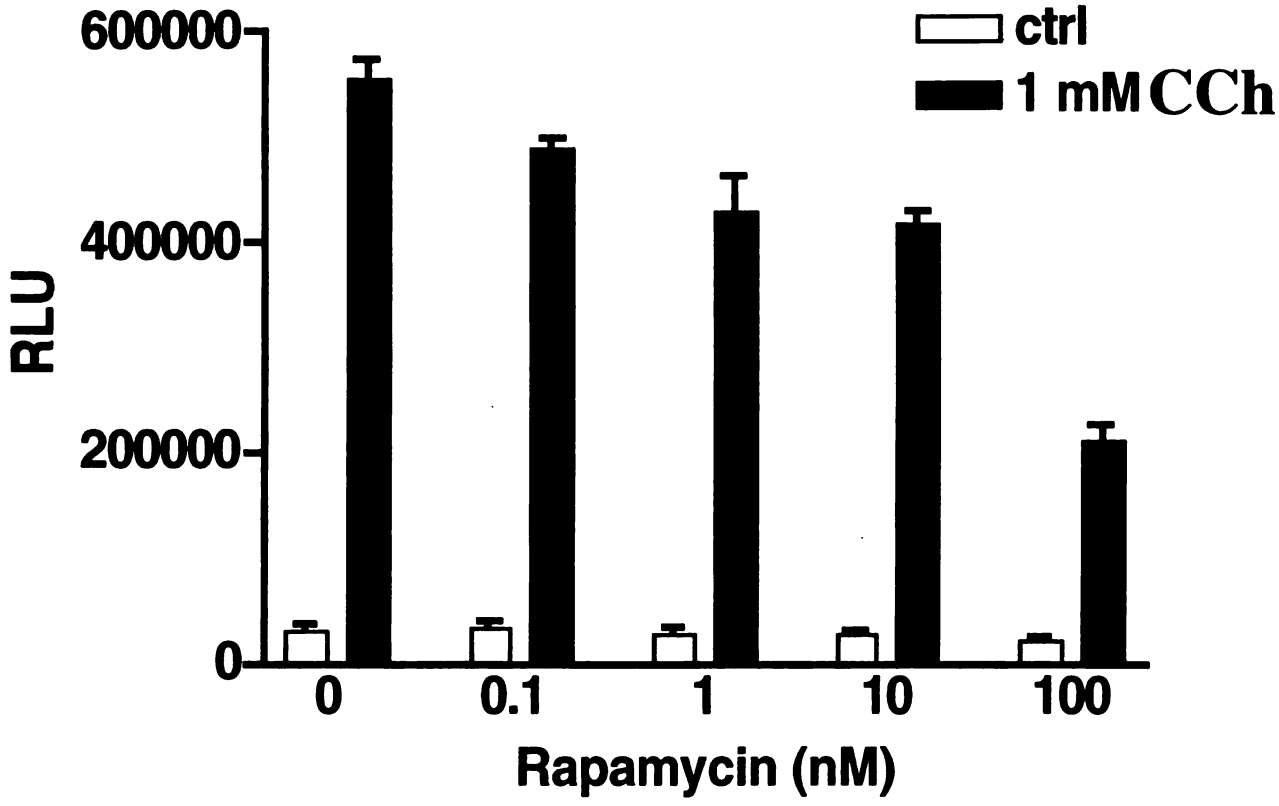


Figure 5.6. Lack of Inhibition of NF AT activity by rapamycin. J urkat cells transiently transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with rapamycin at indicated concentration for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.

for monitoring FK506 and its metabolites in clinical trial (Christians & Bennet, 1999). The latter is based on simultaneous automated solid/liquid extraction and HPLC/electrospray mass spectrometry (LC/LC-MSD).

Using muscarinic receptor mediated cell system, we first constructed a standard curve with blank blood spiked with different concentration of FK506 (Figure 5.7). FK506 showed maximum inhibition at 80 nM blood concentration. Its linearity range is 1-60 nM with an intra-assay CV of 10%, compared to LC/MS, which has linearity range from 0.3-300 nM. Table 5.1 shows the comparison between these two methods. It appears that the reporter assay has narrower detection range than LC-MS method. Given that the therapeutic range for FK506 is between 12-24 nM, this bioassay is well suited for clinical monitoring.

We then tested the blood sample obtained from patients participating in FK506 clinical trial at UCSF clinical center. Figure 5.8 shows the comparison of concentration time curve measured by two methods respectively. Concentration obtained from bioassay showed higher profile than the LC method, especially after the peak hour. One explanation is the possibility of the existence of active metabolites that can also inhibit NFAT activity, and thus, can be detected in this assay.

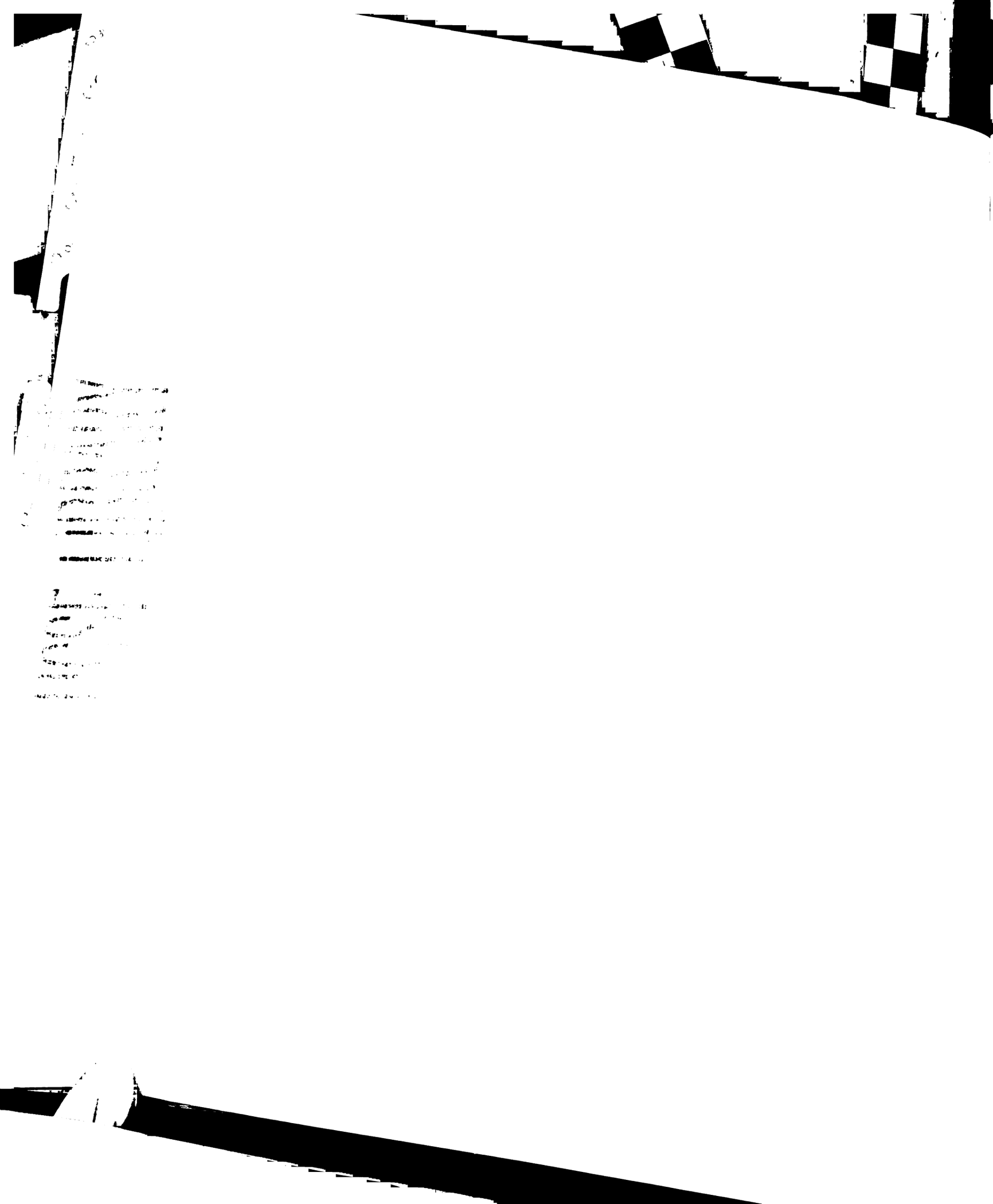
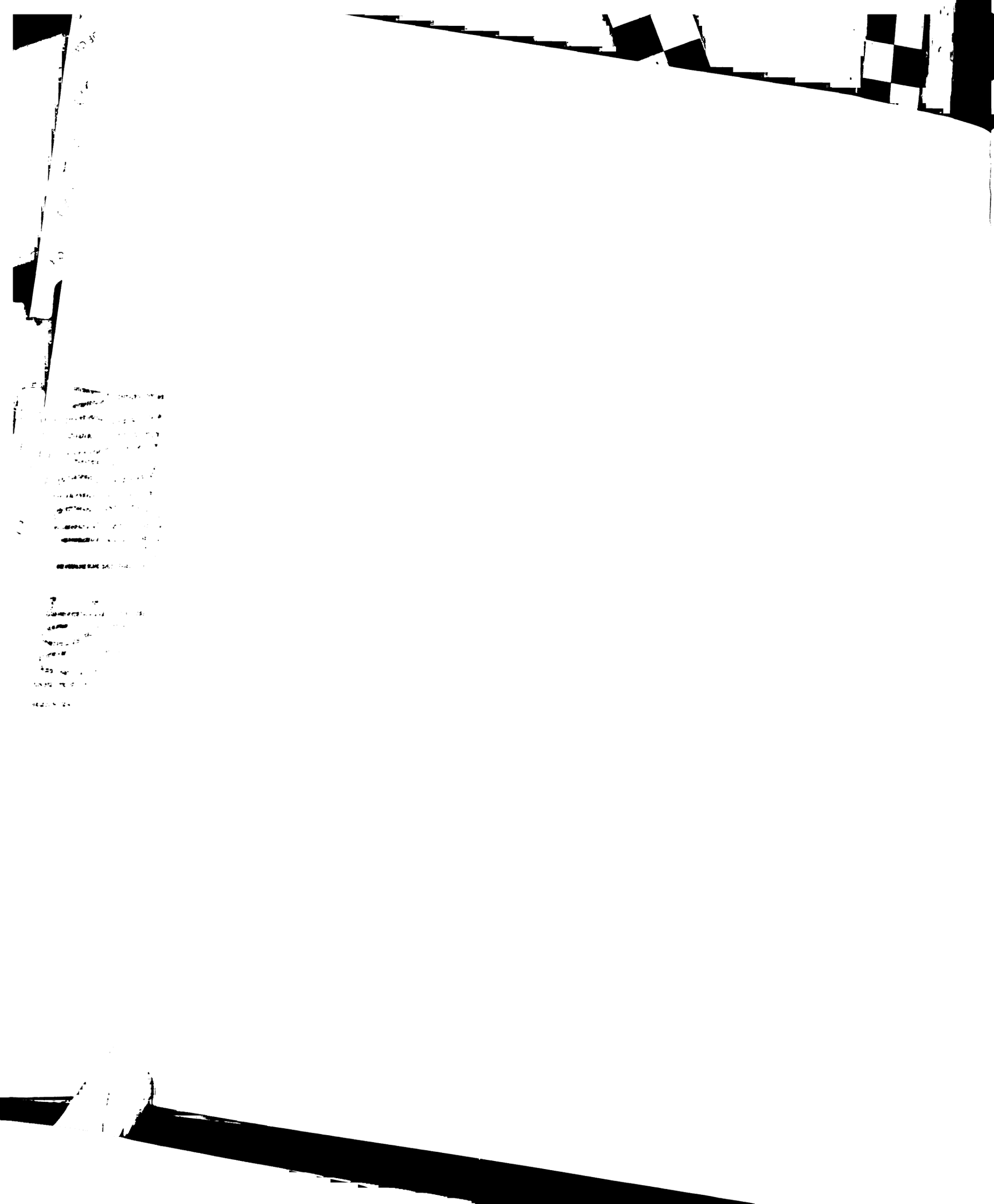


Table 5.1. Methods and characteristics for measurement of FK506.

Type of assay	Cell-based	LC/LC-MSD
Technology	Activity-based assay	Extraction/separation
Lineal range(nM)	0.1-60	0.3 - 300
Therapeutic range (nM)	10-20	10-20



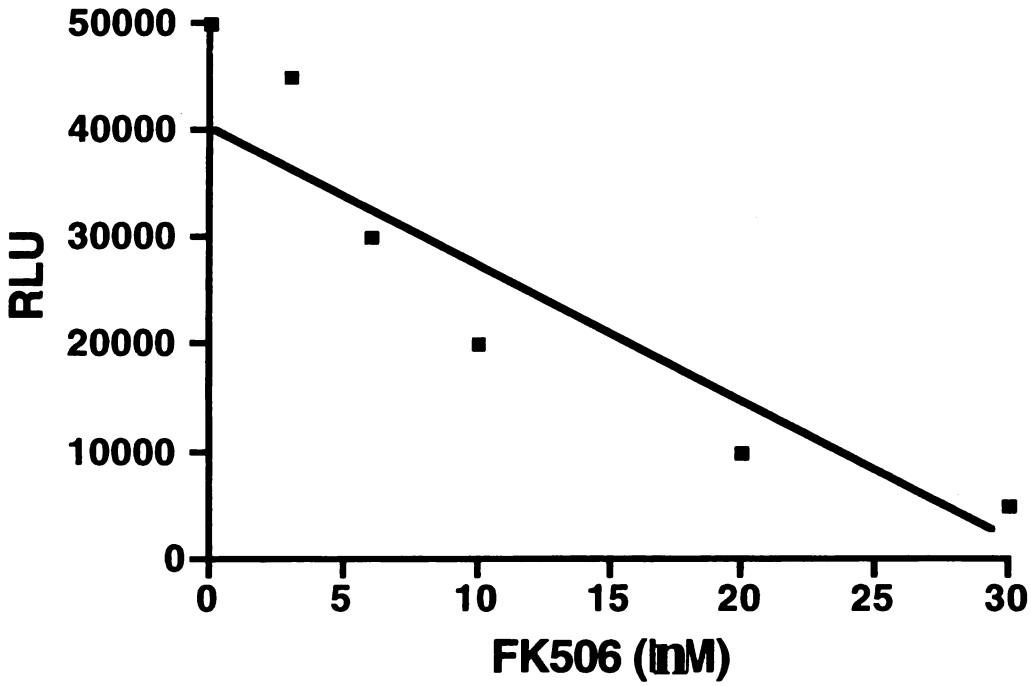
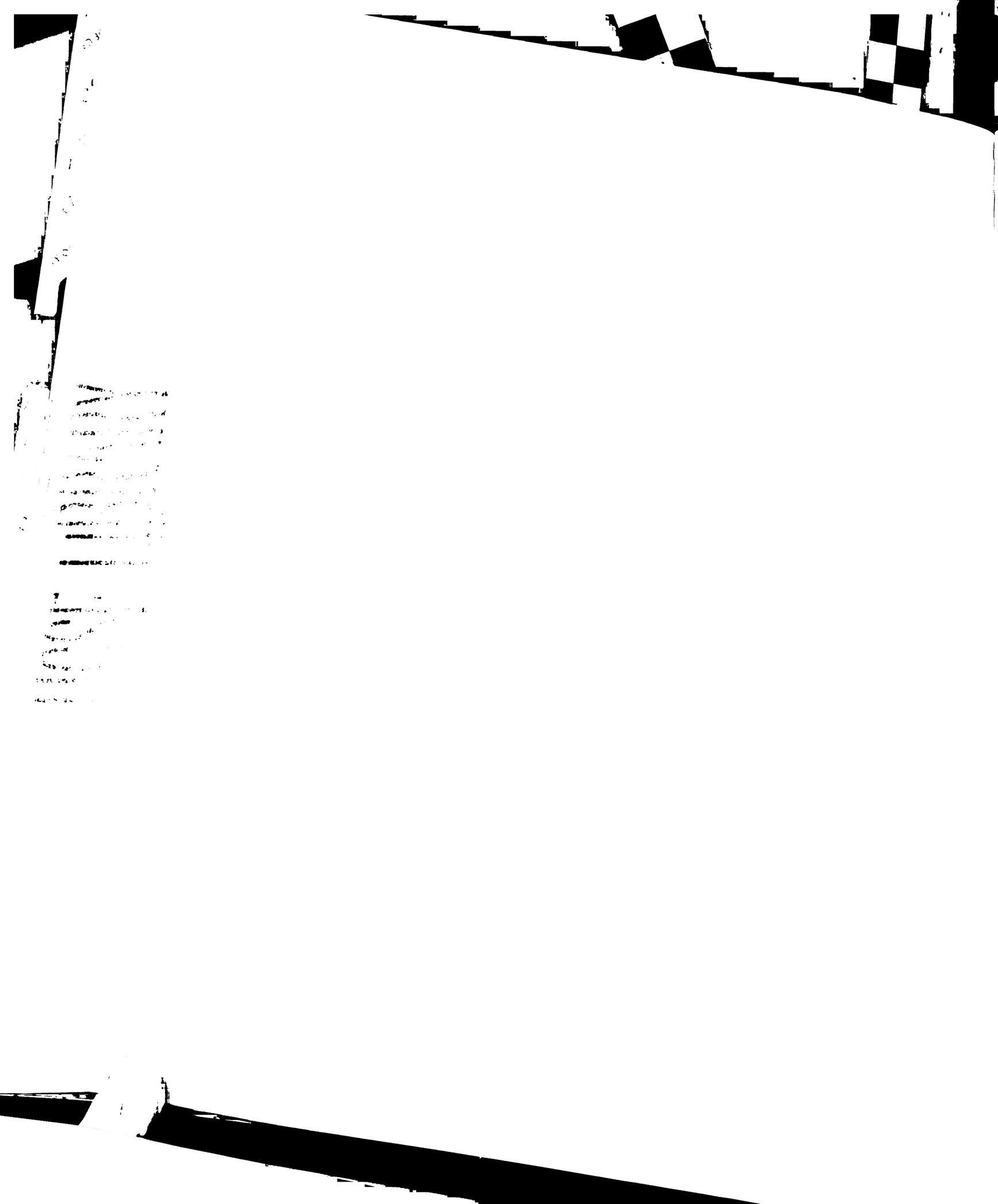


Figure 5.7. Standard curve for FK506 blood extracts detection using hm1-NFAT cell system. Jurkat cells transiently transfected with hm1 mAChR receptor and pNFAT-luc were plated onto 96-well plates. Cells were pretreated with FK506 extracts from spiked blood samples at indicated concentration for 15 min, followed by 1 mM CCh stimulation for 4 hr. The cells were then lysed and luciferase activity determined. Each point is the average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments.



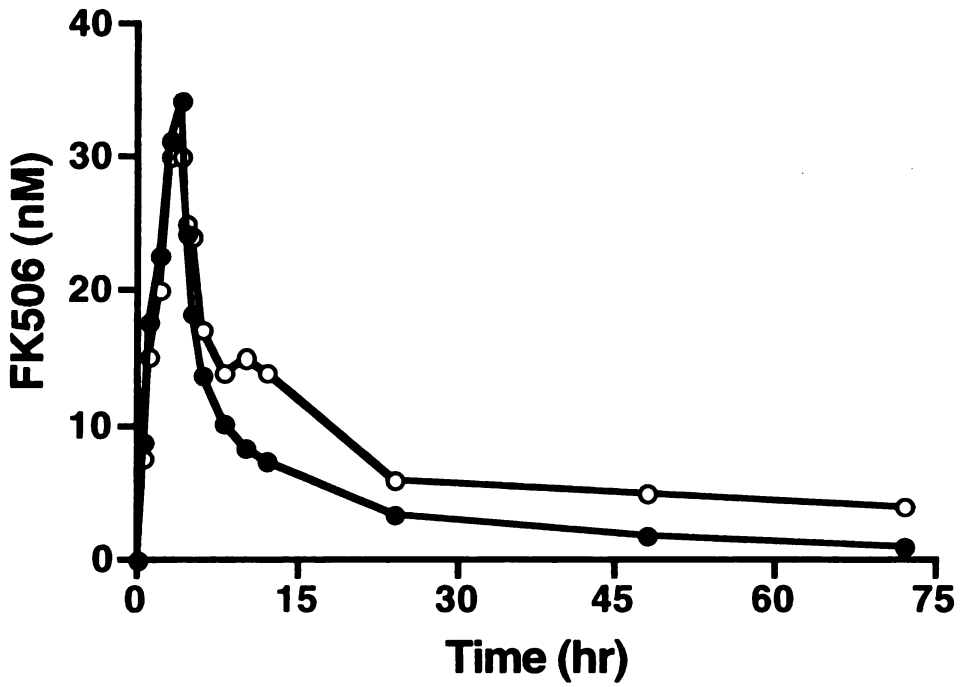


Figure 5.8. Comparison of FK506 concentrations in one patient after IV infusion using both LC-MS and bioassay. Blood samples were processed as described in methods section and subjected to LC-MS (solid circle) and NFAT bioassay (open circle) respectively. The curve is representative of three independent experiments.

V. DISCUSSION

Immunosuppressant compounds are indispensable in combating organ transplant and autoimmune diseases. FK506 and cyclosporin A (CsA) have revolutionized the field of graft transplanting. FK506 and CsA act by blocking a Ca^{2+}-mediated signaling pathway leading to NFAT activation. However, the toxicity of these drugs, which arises from their ability to inhibit calcineurin in cells outside the immune system, has limited their use in other clinical situations (Dumont *et al.*, 1992; Sigal *et al.*, 1991; Sigal & Dumont, 1992). Compounds that target NFAT directly, without affecting the phosphatase activity of calcineurin, may lack the toxicity of CsA and FK506 while retaining immunosuppressive effects.

A reporter gene system was established to assess the activity of NFAT. It is based on transient co-transfection with a plasmid that expresses luciferase gene under the control of the NFAT promoter and human muscarinic acetylcholine receptor subtype 1. Stimulation of mAChR with agonist carbachol led to a significant increase in luciferase expression, as did the intracellular stimuli PMA and ionomycin. Because the activity of NFAT is triggered most efficiently by the cell surface receptors, we further characterized the m1 mAChR-NFAT system.

To demonstrate the specificity of this assay, we tested known immunosuppressants FK506 and CsA. They inhibited NFAT controlled luciferase activity with potencies consistent with the literature. To further explore the versatility of this activity-driven bioassay, we measured FK506

concentration in blood sample from patients given FK506 by *i.v.* infusion. We were able to obtain a similar profile and comparable sensitivity between these two assay methods.

In summary, an NFAT activity based assay was developed based on select interference with NFAT activation. It may potentially aid in finding more effective and less toxic imunosuppressants.

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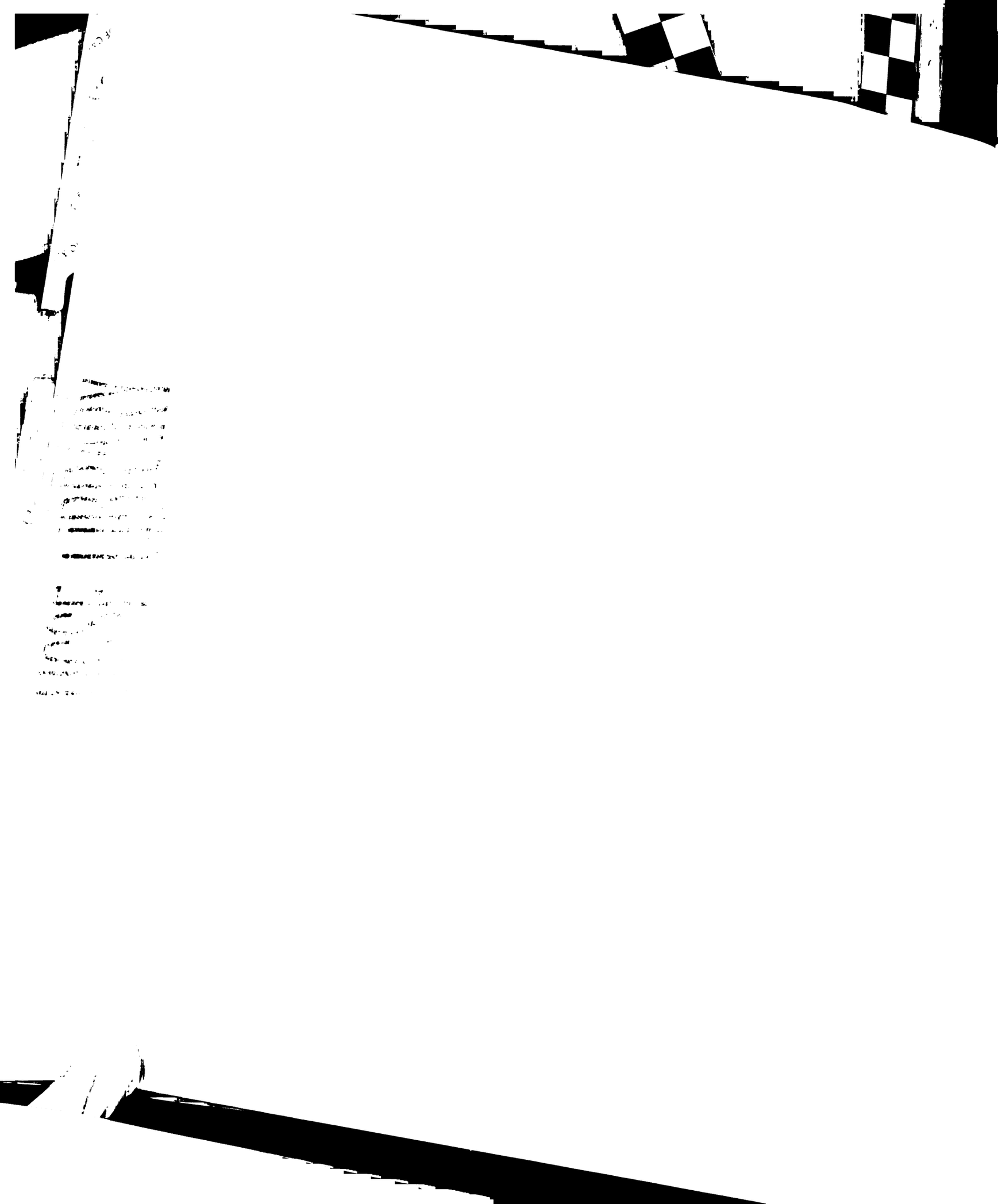

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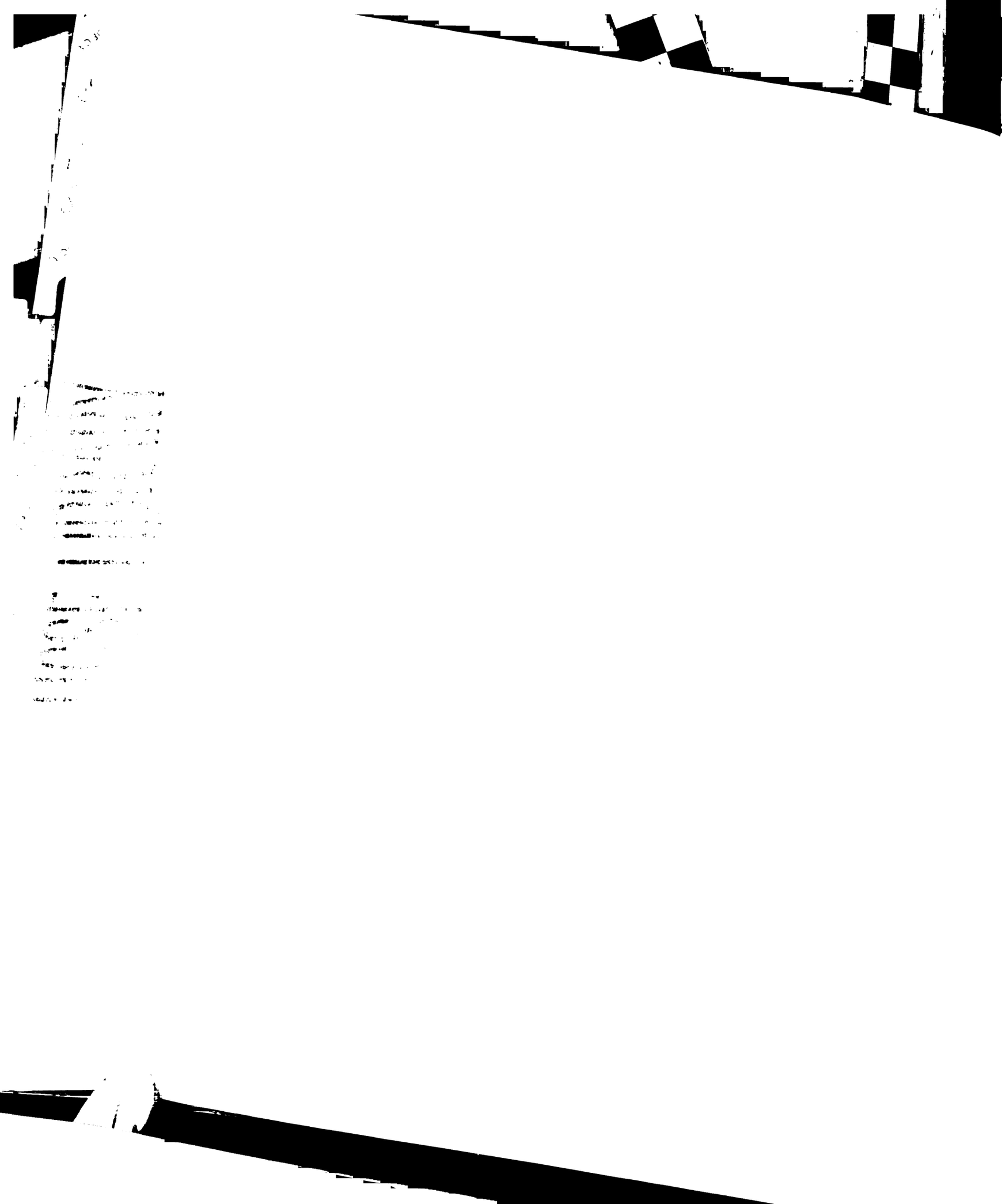

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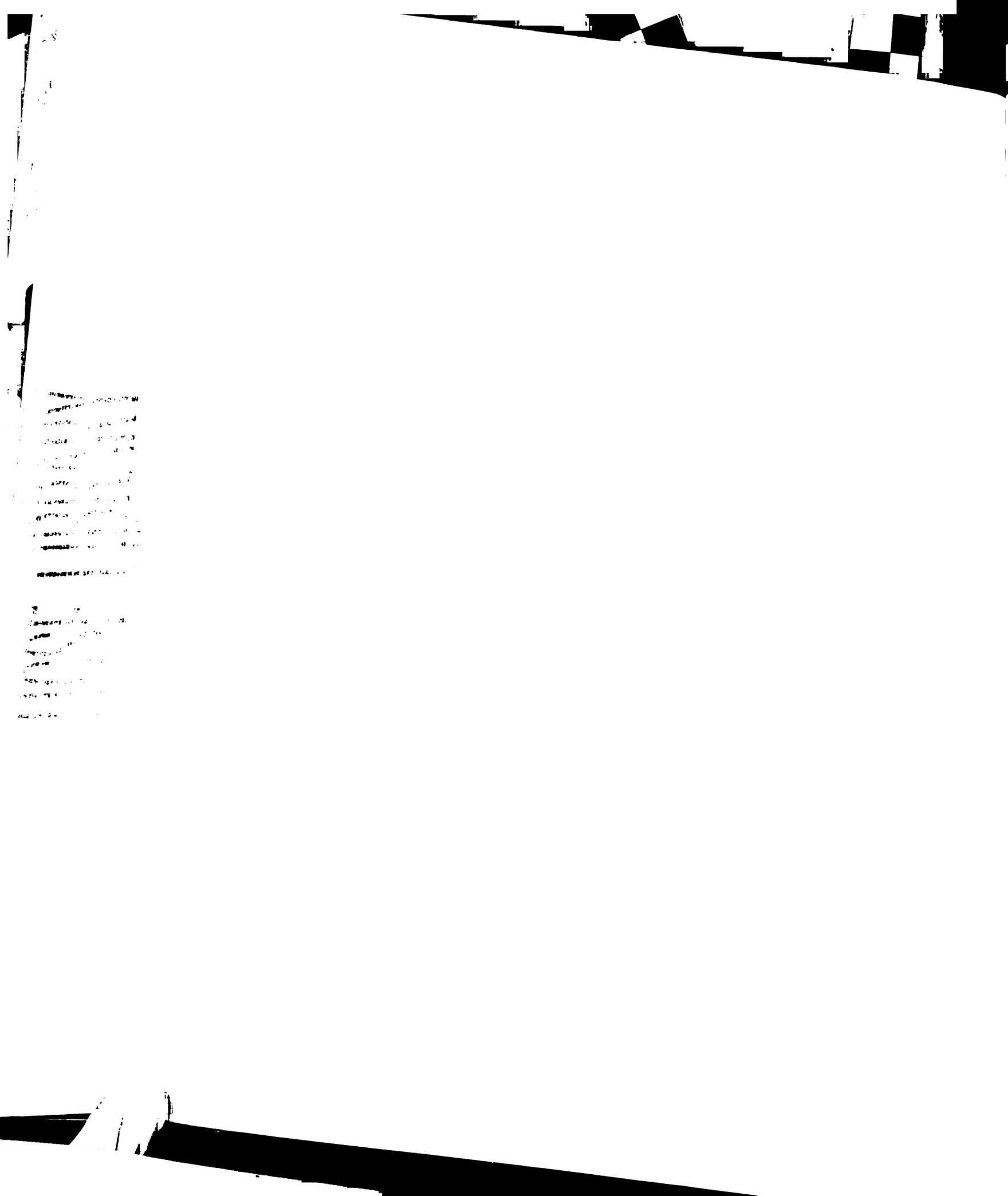

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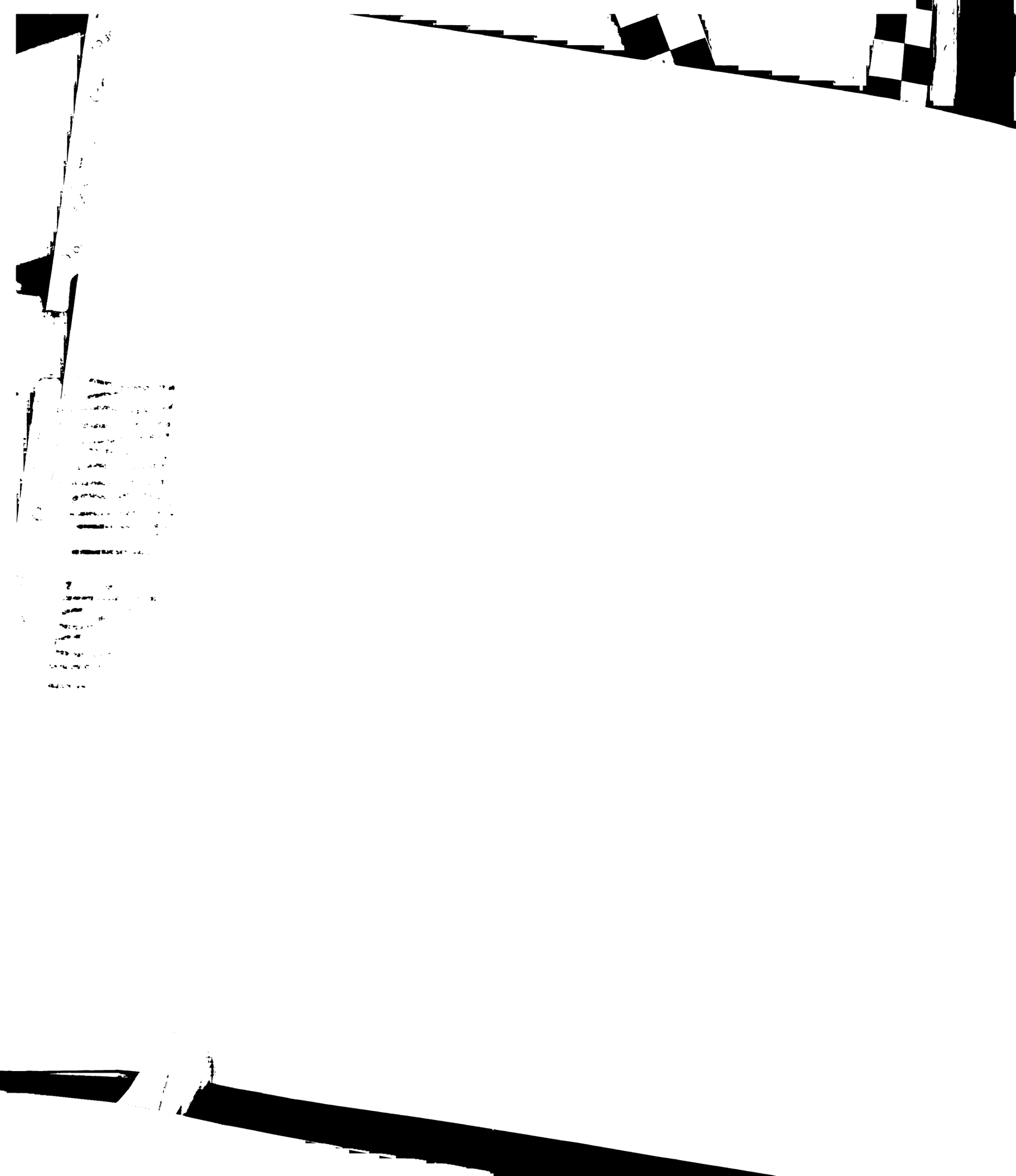

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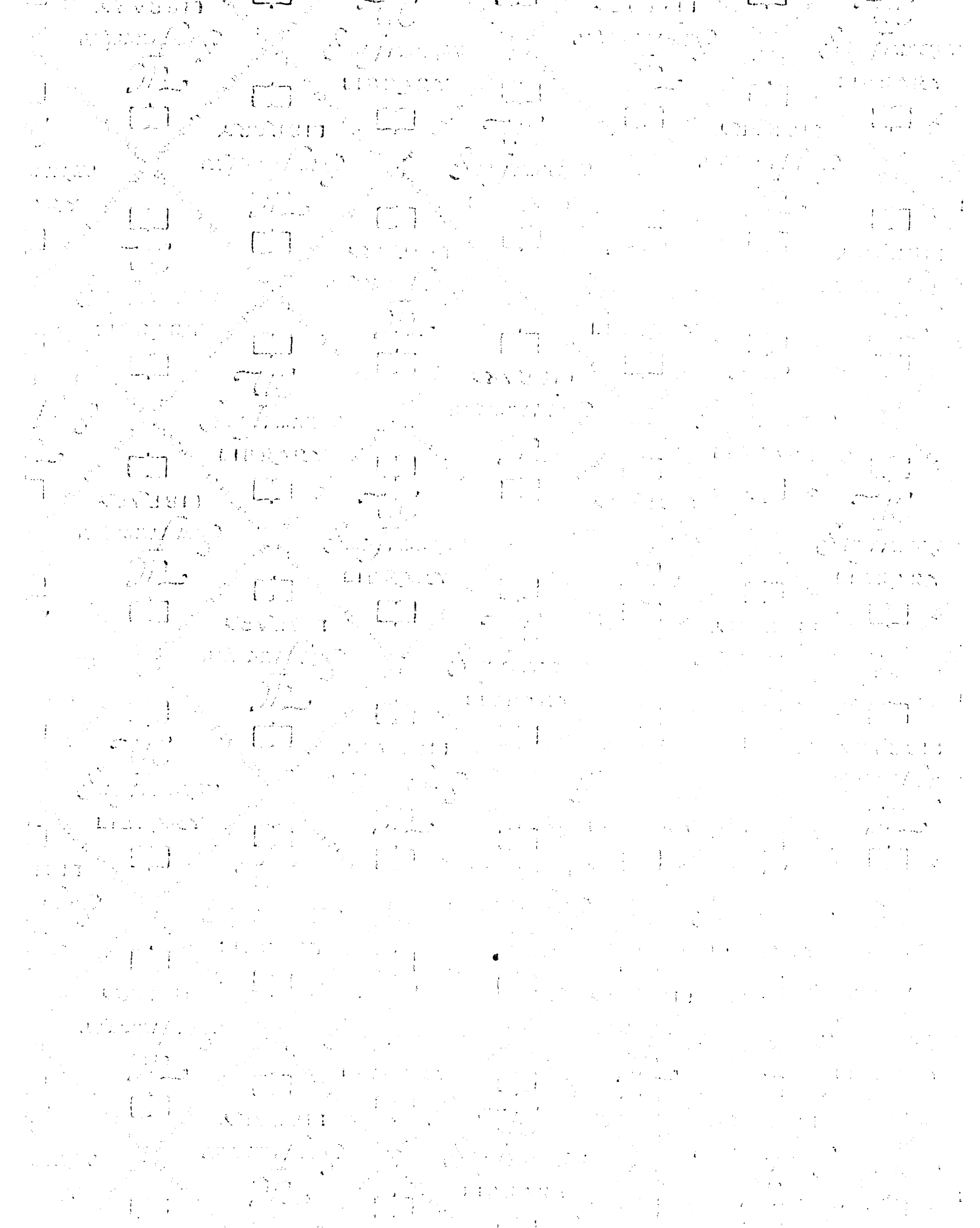

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