Title
Characterization and regulation of muscarinic acetylcholine receptor signaling by calmodulin

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Publication Date
2004

Peer reviewed|Thesis/dissertation

CHARACTERIZATION AND REGULATION OF MUSCARINIC ACETYLCHOLINE RECEPTOR SIGNALING BY CALMODULIN

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Ap

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ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my advisor, Wolfgang Sadée, for his guidance and support throughout this entire experience. I would also like to thank my committee, Deanna Kroetz and Mark von Zastrow, for their advice and understanding, without which it would have been impossible to complete this project. I would also like to extend a warm thank you to all members of the Sadée Lab, especially Pascale and Audrey, for stimulating conversations, listening and helpful suggestions.

The completion of this project would have been impossible without the love and support of my family and friends. I especially want to thank my parents, Linda and Scott, my sisters Laurie and Emily, and my brother-in-law Carl. Without your encouragement I would never have finished. And to Denise and Mike, for suggestions, support and, most importantly, allowing me a spot on the sofa and making San Francisco fun. This has been an incredible experience.

Chapter 4 has been published previously: Julie L. Lucas, Joseph A. DeYoung and Wolfgang Sadée. AAPS PharmSci 2001; 3(4) article 31.

CHARACTERIZATION AND REGULATION OF MUSCARINIC ACETYLCHOLINE RECEPTOR SIGNALING BY CALMODULIN

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December 2004

ABSTRACT

Calmodulin (CaM), a well-characterized calcium sensor, binds directly to GPCRs, including the μ opioid receptor (MOR) on the i3 loop. CaM binding to MOR interferes with G-protein coupling. Furthermore, melittin and mastoparan, tetradecapeptides found in wasp venom, bind both G_α and CaM, suggesting that the two binding sites are similar and may overlap. Therefore, CaM may bind numerous GPCRs, and act as a second messenger. Due to a putative CaM binding motif on the C-terminal portion of the i3 loop, a region important in G-protein binding, the hM1 muscarinic receptor (hM1) was selected as a candidate to better elucidate CaM-i3 loop interactions.

Studies with peptides derived from the hM1 i3 loop demonstrate that CaM is capable of binding hM1, and a 5-residue sequence essential for calmodulin binding was defined. Studies with hM1 peptides suggest the presence of a second, adjacent CaM binding site. Mutagenesis studies identified two mutant peptides that display reduced binding to CaM. The results suggest that calmodulin can bind to an M1 region implicated in G-protein coupling.

Microarray studies revealed that stable transfection of wild type or CaM-binding-deficient mutant hM1 into HEK-293 cells has distinct effects on gene expression patterns even though G-protein coupling was not impaired. Furthermore, addition of CaM to membranes from cells expressing hM1 inhibits [^{35}S]GTPγS incorporation, suggesting that

CaM regulates hM1 G-protein coupling. These data support the hypothesis that CaM, through its direct interaction with hM1, regulates signal transduction.

Variations in the MOR sequence disrupt CaM binding. To identify any SNPs on hM1 that could disrupt CaM binding, the *CHRM1* gene in 245 individuals was sequenced. Nine SNPs in the coding region of *CHRM1* were identified, but only a single allele, C417R, was found to contain a nonsynonymous SNP. Due to its location, C417R is unlikely to affect CaM binding, but previous studies show that the highly conserved C417 is important for agonist binding and coupling. The extraordinary sequence conservation of *CHRM1* was unexpected as M1-knockout mice show only minimal functional impairments. This study supports a broader trend suggesting that CaM is a key regulator of GPCR signaling at the receptor level.

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Chapter 1

Introduction

G-protein coupled receptors are classically described as being able to signal through the activation of a heterotrimeric G-protein. In recent years it has become apparent that GPCRs also interact with and signal through various other proteins. This work describes the interaction between the hM1 muscarinic acetylcholine receptor and the ubiquitous calcium sensor calmodulin, and highlights the potential role for this interaction in regulating G-protein coupling and cell signaling.

MUSCARINIC RECEPTORS

Function and Expression Muscarinic receptors belong to the G-protein coupled receptor (GPCR) superfamily. They are class I rhodopsin-like GPCRs. Like all GPCRs, they have 7 transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus. There are 3 extracellular loops (e1, e2 and e3) and three intracellular loops (i1, i2, and i3). There are five muscarinic receptors in humans, hM1-5. M1, M3 and M5 couple to G_q to mobilize Ca^{2+}, and M2 and M4 couple to $G_{i/o}$ to inhibit adenylyl cyclase. The M1 receptor is the major postsynaptic receptor in the brain; it is found in the hippocampal and cortical regions of the brain as well as in the parasympathetic ganglia (Dorje et al., 1991; Levey et al., 1991; Felder et al., 2001). The M1 receptor is involved in many processes, including the initiation of seizures, learning and memory, and regulation of the force and rate

of heart contractions (Hamilton et al., 1998; Hamilton et al., 2001). Because of its involvement in these processes, the M1 receptor is a compelling drug target for Alzheimer's disease and other neurological and psychiatric disorders (Hamilton et al., 1998). Indeed, for the past 20 years, the cholinergic hypothesis has proposed that loss of cholinergic function is responsible for the cognitive symptoms of Alzheimer's (Bartus, 2000). It is thought that stimulation of the M1 receptor would alleviate some of the symptoms of Alzheimer's by several pathways, including increased secretion of the nontoxic α-amyloid peptide and decreased secretion of the toxic ß-amyloids generated from the amyloid precursor protein (Fisher et al., 1998; Fisher et al., 2000).

G-protein coupling domain. Muscarinic receptors bind to the heterotrimeric G-protein through the intracellular loops. Extensive mutagenesis studies have been done with the M1 muscarinic receptor to pinpoint the residues most important for G-protein binding. These residues are located on the i2 loop and near the transmembrane domain junctions of the i3 loop. On the i2 loop, a hydrophobic residue, L131, is critical for G-protein coupling. Mutation of this residue will decrease PI stimulation, a measure of a G_q-coupled receptors ability to activate its G-protein, by 80% (Moro et al., 1993a). The i3 loop is another region crucial for G-protein coupling. The i3 loop of M1 is quite large, 158 residues, but Δ232-358, a deletion mutant which contains only the N and C-tails of i3, is able to couple normally to G-proteins (Arden et al., 1992). This indicates that only the N and C terminal ends of the i3 loop are involved in G-protein coupling. A triple mutant of three charged residues on the N-terminal end of i3, E214A/E216K/E221K did not affect G-protein coupling as measured by stimulation of PI hydrolysis (Arden et al., 1992). However, a second triple mutant of

hydrophobic residues W209A/I211A/Y212A decreased carbachol-stimulated PI release by 65% (Moro et al., 1993a). The single mutants I211A and Y212A were also defective in coupling (Hogger et al., 1995b). Together, these studies show that the hydrophobic residues on the N-terminal end of i3 are involved in G-protein coupling.

The C-terminal end of the i3 loop is crucial for G-protein coupling. Moro et al. showed that a triple mutant, E360A/K362A/T366A had a 60% decrease in carbachol-stimulated [^{3}H] phosphoinositol release as compared to control (1993a). The single mutant E360A has been shown to constitutively activate M1 (Hogger et al., 1995b). K362 is part of a BBXXB motif (B is a basic residue, either lysine or arginine, and X is any other amino acid), which has been proposed as a universal G-protein coupling motif. In M1 this motif is 361-KKAAR-365, and it has been shown to be important in G-protein coupling. A triple mutant in which the basic residues are changed to alanine has virtually no ability to stimulate PI (Lee et al., 1996b). Studies with the single mutants show that K361A, which has 5 times decreased potency but can still maximally stimulate PI, plays a minor role in G-protein coupling (Lee et al., 1996b). However, in another study, a double mutant K359A/K361A did not affect G-protein coupling (Arden et al). K362 and R365 play more important roles. R365A caused a 50% decrease in the maximal PI stimulation but did not affect potency. K362A caused both a 50% decrease in maximal PI stimulation and decreased the potency 13 times (Hogger et al., 1995b; Lee et al., 1996b). Certain residues on the i3 loop are therefore crucial for G-protein coupling. Altogether, these studies illustrate the importance of the intracellular loops, especially i3, in the interaction with the G-protein.

Regulation Muscarinic receptor signaling is regulated by phosphorylation at serine and threonine residues. Phosphorylation effectively uncouples the receptor from its G-protein and desensitizes the receptor's response to agonist stimulation. Protein kinase C (PKC) phosphorylation on M1 is agonist-independent and is therefore thought to be involved in heterologous desensitization. A putative PKC phosphorylation site is located on the C-terminal end of the i3 loop, at T354 or S356 (Haga et al., 1996).

Continued stimulation of a muscarinic agonist, such as carbachol, results in phosphorylation by G protein coupled receptor kinase (GRK) on the activated M1 receptor. M1 is phosphorylated by GRK2 on 4-5 sites, most likely on a serine/threonine rich domain located in the center of the i3 loop (Haga et al., 1996). This internalization domain is located between residues 284-292, as determined by mutagenesis studies (Lameh et al., 1992; Moro et al., 1993b). In HEK-293 cells, phosphorylation of the M1 receptor by GRK is followed by the binding of arrestin-2 to the phosphorylated domain, leading to the internalization of the receptor. Internalization of the M1 muscarinic receptor is via clathrin-coated vesicles (Tolbert and Lameh, 1996), and is dependent on the small GTPase dynamin (Vogler et al., 1998). In general there are two pathways that internalized GPCRs follow: they are either recycled back to the membrane or degraded in lysosomes, resulting in the down-regulation of receptor expression on the membrane. Whether recycling or down-regulation occurs is cell-type specific; M1 is down-regulated in CHO cells (Shockley et al., 1997) but recycled in HEK-293 cells (Vogler et al., 1998).

Signal Transduction. The M1 muscarinic receptor couples primarily to the $G_{\alpha q/11}$ heterotrimeric G-protein. Activation of $G_{\alpha q/11}$ results in activation of phospholipase C (PLC),

which hydrolyzes phosphoinositol-4, 5-bisphosphate to produce the second messengers IP_3 (inositol-1, 4, 5-triphosphate) and DAG (diacylglygerol). IP_3 binds to its receptor on the endoplasmic reticulum to release intracellular calcium stores (Felder, 1995). DAG activates protein kinase C (PKC) resulting, among other things, in the activation of calcium channels on the plasma membrane, causing an influx of extracellular calcium (Felder, 1995). Calcium influx activates a variety of pathways. Muscarinic receptors have been shown to stimulate Ca^{2+}-activated chloride current (Janssen and Sims, 1992; Pacaud et al., 1992; Janssen, 1996; Liu and Farley, 1996; Wayman et al., 1997), and to inhibit Na^+ channels in a PKC-dependent manner (Cantrell et al., 1996). The M1 muscarinic receptor has been shown to be able to increase the cyclic AMP concentration in the cell by activation of type I adenylyl cyclase, which is activated by the ubiquitous calcium sensor CaM and CaM kinase II (Choi et al., 1992). In addition, activation of adenylyl cyclase has been shown to regulate activation of the mitogen-activated protein kinase (MAPK) cascade (Russell et al., 1994).

M1-mediated activation of the MAPK cascade is important for memory. M1 has been shown to activate extacellular signal-regulated kinase 1 and 2 (ERK1/2), members of the MAPK family. ERK1/2 is necessary for long-term potentiation, which is an important component of learning and memory (Rosenblum et al., 2000). Knockout studies in mice have shown the M1 is the major subtype that activates ERK1/2 in mouse forebrain (Hamilton and Nathanson, 2001). Muscarinic receptors are linked to ERK1/2 activation in hippocampus and cortex and ERK1/2 activation is necessary for long-term potentiation *in vivo* (Rosenblum et al., 2000). In neurons and fibroblasts, activation of ERK1/2 is src-dependent and partially dependent on PI_3K and calcium, and not dependent on PKC (Rosenblum et al., 2000). M1 mediation of amyloid precursor protein (APP) processing in PC12M1 cells has been shown

to occur through two converging pathways: a PKC-dependent pathway and a second pathway dependent upon the small GTPase Ras and ERK (Haring et al., 1998). Activation of ERK1/2 in PC12D cells is through the second messenger DAG, which then activates the small GTPase Rap through the guanine nucleotide exchange factor (GEF) CalDAG-GEF (Guo et al., 2001).

M1 has also been shown to activate tyrosine kinases. M1 receptors can transactivate EGFR (epidermal growth fact receptor), a receptor tyrosine kinase, through a pathway involving metalloprotease cleavage of proHB-EGF (Prenzel et al., 1999). Activation of EGFR is known to trigger the MAPK cascade, showing that M1 participates in cross talk across signaling pathways. M1 can activate the potassium channel Kv1.2 through the tyrosine kinase Pyk2, which then autophosphorylates and binds c-src and Grb-2 to activate the channel. The exact mechanism by which M1 activates Pyk2 is unclear (Felsch et al., 1997). M1 receptors have been shown to activate Bruton's tyrosine kinase through a direct interaction of the kinase with $G_{q\alpha}$ (Bence et al., 1997). M1-mediated activation of SRF (serum response factor) in Jurkat T cells is through a novel pathway involving the non-receptor tyrosine kinase Pyk2, CaM, calcineurin and the small GTPase RhoA (Lin et al., 2002). G_q is necessary but not sufficient for this pathway and the G proteins G_{12}, G_{13}, G_{14} and G_{15} appear not to be involved. SRF binds to the serum response element (SRE) to activate immediate early genes. Immediate early genes regulate the expression of many proteins involved in mitogenesis and neuronal differentiation (Lin et al., 2002).

M1 has also been shown to activate the immediate early gene *zif268* through a RhoA and SRE-dependent mechanism (Hirabayashi and Saffen, 2000). Induction of other immediate early genes from the *fos* and *jun* families has been shown to occur through

different pathways (Ding et al., 1998). The immediate early genes *c-fos*, *fosB* and *junB* were actived through a pathway involving PKC, whereas induction of *c-jun* and *junD* involved CaM and CaMKII (Ding et al., 1998).

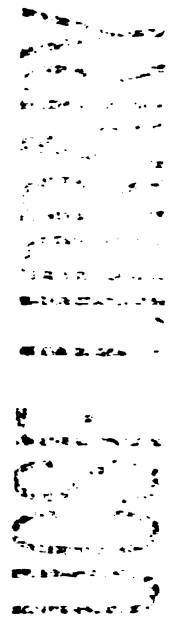
In summary M1 is a G_q-coupled GPCR, which is expressed in both the brain and parasympathetic ganglia. It binds the G-protein on the intracellular loops, especially the C-terminal end of the i3 loop. Activation of the M1 receptor with ligand results in the activation of a variety of signaling pathways including Ca^{2+} mobilization, ion channel regulation, the activation of the MAPK cascade and the regulation of gene expression, especially the immediate early genes. The ability of M1 to initiate signal transduction is regulated by phosphorylation and internalization of the receptor, although other mechanisms could exist. In this study, I investigate the novel interaction of M1 with the calcium sensor calmodulin.

CALMODULIN

Structure and binding to target proteins. Calmodulin (CaM) is a 16 kD calcium sensor. It is ubiquitous in eukaryotic cells and all mammals have identical protein sequences. There are three CaM genes in humans, all of which produce the same protein. CaM is highly expressed in both the developing and adult rat brain, as determined by *in situ* hybridization studies (Palfi et al., 1999; Kortvely et al., 2002). Calmodulin changes its conformation and is usually activated upon an influx of calcium into the cell, but calcium-independent pathways also exist. Calmodulin contains four EF-hand calcium-binding domains, of which at least two must be occupied for it to be activated (Vogel, 1994). CaM binds calcium in a positively

cooperative manner, allowing it to be very sensitive to changes in cytosolic calcium concentrations (Vogel, 1994).

Although CaM binds to a wide variety of target proteins, one common feature of many CaM binding sites is that they are predicted to form an amphipathic α-helix. In this structure, one side of the helix contains basic residues and the other side contains hydrophobic residues(Rhoads and Freidberg, 1997; O'Day and Myrc, 2004). This has given rise to the description of a series of related motifs, such as the 1-8-14, the 1-14, the 1-10 and the 1-16 motif, where the numbers describe the location of hydrophobic residues phenylalanine, isoleucine, leucine, valine or tryptophan (FILVW) (Rhoads and Freidberg, 1997; O'Day and Myrc, 2004). Another well-known motif is the IQ motif, which is found in many proteins that bind CaM in a calcium-independent manner (although this motif has been found in proteins that bind CaM in the presence of calcium as well) (Rhoads and Freidberg, 1997; O'Day and Myrc, 2004). These motifs are useful in identifying potential candidates for CaM interaction.



Understanding how CaM binds to other well-characterized targets gives insight into how it may interact with the hM1 receptor. The structure of CaM is intrinsically flexible, allowing it to bind to a wide array of targets (Rhoads and Freidberg, 1997; Zhang and Yuan, 1998). It is a dumbbell shaped molecule with two calcium-binding sites on each end, connected by a linker. The linker is an α-helix and is able to wind and unwind as necessary to accommodate different sequence lengths (Zhang and Yuan, 1998). The calcium-binding lobes of CaM are also flexible. When calcium is bound, they can change conformation to reveal hydrophobic methionine patches that interact with the target (Gellman, 1991; Vogel, 1994). Furthermore, the nature of the methionine side chain allows even more versatility, as

it can accommodate multiple residues in the binding pocket (Gellman, 1991; Vogel, 1994). The lobes without calcium bound have also been found to interact with some targets (Schumacher et al., 2001). CaM can also not only wrap itself lengthwise around a single strand of amino acid residues, it can stretch to accommodate multiple strands. Recently, a crystal structure was solved in which two CaM each bound three α-helical strands of the Ca^{2+} activated K^+ channel (SK channel) (Schumacher et al., 2001). In this structure, one lobe of CaM had calcium bound and interacted with one α-helix, and the other lobe had no calcium bound and interacted with two α-helices.

Signal Transduction. CaM mediates a wide variety of calcium-activated processes. CaM plays an inhibitory role in the regulation of L-type Ca^{2+} channels, thereby providing a negative feedback mechanism to control Ca^{2+} entry and downstream signaling (Romanin et al., 2000; Pitt et al., 2001b). CaM can bind and activate type 1 adenylyl cyclase (Mons et al., 1999), which M1 has also been shown to activate. Type I adenylyl cyclase has been shown to have a role in memory formation (Mons et al., 1999; Wang and Storm, 2003). CaM has been shown to bind to and stimulate the activity of the small GTPase Ral-A (Wang and Roufogalis, 1999). CaM can also bind the βγ subunits of the heterotrimeric G protein. This interaction interfered with the formation of the $G_{\alpha\beta\gamma}$ trimer, but not with PLC-β2 activity (Liu et al). CaM can bind and inhibit type-1 inositol trisphosphate (IP_3) receptors in a Ca^{2+}-independent manner (Cardy and Taylor, 1998).

CaM binds to several kinases, including to skeletal muscle phosphorylase kinase as an integral subunit, and regulates the calcium-dependent activity of this kinase in its role in glycogen metabolism (Dasgupta et al., 1989). CaM can bind to two sites on GRKs1, 2 and 5,

and inhibits the kinases ability to phosphorylate GPCRs (Chuang et al., 1996; Levay et al., 1998). It binds and activates a family of CaM kinases, including CaM kinase II, which is enriched in the brain and has an important role in memory (Hedou et al). CaM binds and activates the phosphatase calcineurin (Klee et al., 1998). Since calcineurin has as it targets some phosphoproteins that are activated by CaM, this is another negative feedback mechanism that regulates cell signaling (Klee et al., 1998). CaM also binds directly to several GPCRs to regulate signal transduction, as discussed in more detail later on.

G PROTEIN-INDEPENDENT SIGNALING

It is now well accepted that GPCRs interact with a variety of proteins besides the G protein, and often these interactions regulate signal transduction. Many GPCRs, for example, contain PDZ-binding domains on their C-terminus. The β2 adrenergic receptor binds to NHERF/EBP50 on a PDZ binding motif located on the last four amino acids of the C-tail (Hall et al., 1998; Cao et al., 1999). After agonist activation, NHERF/EBP50 binds to this region, and the β2AR internalized and recycled back to the membrane. Mutation of the receptor resulted in degradation in lysosomes (Cao et al., 1999). Another GPCR, the rat somatostain receptor type2 interacts with the cortactin-binding protein, type 1 (CBP1) through its PDZ domain in an agonist-dependent manner. Since CBP1 is part of the cytoskeleton, it is thought that this interaction shows that GPCRs are involved in structural rearrangements that take place after activation (Zitzer et al., 1999). The 5HT2C receptor interacts with a novel protein containing 13 PDZ domains and no obvious catalytic domain, termed MUPP1. The function of the interaction is not known (Ullmer et al., 1998). The

5HT2B receptor contains a PDZ domain in its C-terminus, which is functionally coupled to c-NOS activation (Manivet et al., 2000). The exact mechanism is unknown although it was shown to be independent of $G_{\alpha 13}$.

GPCRs have been shown to bind to other proteins on the C-tail as well. Homer, an immediate early gene, binds to group 1 metabotropic glutamate receptors (subtypes 1 and 5), and acts as an adaptor linking these receptors with inositol trisphosphate receptors to modulate intracellular calcium release (Tu et al., 1998; Xiao et al., 1998). This is one of the best examples of G protein-independent signaling. The metabotropic glutamate receptor, type 1α has been shown to directly interact with tubulin (Ciruela et al., 1999), perhaps to stabilize the receptor. The $GABA_B$ receptor has been shown to bind two related transcription facrors, CREB2 and ATFx, through a leucine zipper-binding motif on its C-tail. This a mechanism by which the receptor can directly regulate gene transcription (Nehring et al., 2000; White et al., 2000). In addition, several adrenergic receptors have been shown to bind eIF-2B, a guanine nucleotide exchange protein involved in the regulation of translation, on their C-terminal tails (Klein et al., 1997). This interaction also enhances β2 adrenergic receptor signaling, but the mechanism is unclear (Klein et al., 1997). Binding of the β2 adrenergic receptor to NSF (N-ethylmaleimide-sensitive factor) has been shown to be required for efficient internalization and recycling of the receptor to the cell surface (Cong et al., 2001). The M3 muscarinic receptor has been shown to be able to activate phospholipase D in a G-protein independent manner. This activation is dependent on the small GTPases ARF and RhoA. A region of M3 located in the seventh transmenbrane domain is dependent on PLD activation and is necessary for coimmunoprecipitation with ARF and Rho (Mitchell et al., 1998).

GRKs, as discussed above for the M1 receptor, play a role in the regulation of GPCR signal transduction. After receptor activation, GRKs phosphorylate many GPCRs, effectively uncoupling the receptor from its G protein. After GRK phosphorylation arrestin binds, which then leads to internalization of the receptor via clathrin-coated pits. Recently, it has been suggested that internalization is required for binding of the protein src and activation of the MAP kinase cascade of the β2 adrenergic receptor (Luttrell et al., 1999). Internalization, then, may play a role in the activation of certain pathways. GPCRs are therefore able to activate signal transduction pathways via multiple mechanisms, not all of which are through a heterotrimeric G protein. This work focuses on the interaction of GPCRs with the calcium sensor calmodulin.

KNOWN INTERACTIONS OF CALMODULIN WITH GPCRS

Calmodulin has been previously found to be able to bind to five GPCRs: the metabotropic glutamate receptor (mGluR) types 5 and 7, the serotonin 5-HT1A receptor, the D2 dopamine receptor and the μ opioid receptor (MOR). Calmodulin bound to the C-terminal tail of mGluR5 and mGluR7 (Minakami et al., 1997; Nakajima et al., 1999). In both receptors, CaM bound to a phosphorylation site and inhibited phosphorylation by protein kinase C (PKC). CaM also inhibited phosphorylation by PKA and PKG on mGlu5R (Sorensen et al., 2002). Conversely, phosphorylation by these kinases prevented CaM binding. There is evidence that shows that CaM binding to the C-tail of these receptors inhibits binding of the G-protein βγ subunit to regulate GIRK currents (Dev et al, O'Connor et al). However, these finding have been disputed (Sorensen et al., 2002). Although the

exact role of CaM binding to the mGluR remains unclear, these studies do show that CaM does regulate the function of these receptors.

The MOR, D2 and 5HT1A receptors are especially interesting as they bind CaM on the i3 loop. The 5HT1A receptor bound CaM on two non-adjacent sites located at the amino and carboxy ends of the i3 loop (Turner et al., 2004b). The site located on the amino end of the loop had higher affinity for CaM than the other site. Phosphorylation of peptides from these regions by PKC inhibited CaM binding (Turner et al., 2004b). CaM was found to inhibit G-protein binding and regulate receptor activity of the D2 and MOR receptors. The D2 dopamine receptor binds CaM on the $-NH_2$ end of the i3 loop. This binding does not directly interfere with G-protein coupling, but rather noncompetitively inhibits it (Bofill-Cardona et al., 2000). However, the binding of CaM and the G-protein to MOR are mutually exclusive (Wang et al., 1999). CaM was able to regulate the basal activity of MOR. In addition, CaM bound to the MOR i3 loop has a biological signaling role, as evidenced by two studies. The release of CaM from MOR results in the translocation of CaM to the cell nucleus to regulate the phosphorylation of the transcription factor CREB (Wang et al., 2000). In addition, CaM is implicated in MOR-mediated transactivation of the EGF receptor (Belcheva et al., 2001). Activation of MOR resulted in a CaM-dependent activation of ERK, through the transactivation of EGF. This pathway was activated through a direct CaM-MOR interaction; a MOR mutant that could couple to G-protein but was deficient in CaM binding could not tranactivate EGF. These studies demonstrate that CaM, through its direct i3 loop interaction, could play a role as a GPCR second messenger.

Binding of both CaM and G protein to the GPCR i3 loop raises the possibility that the two binding sites are similar and may overlap. This is further supported by the ability of

melittin and mastoparan, two peptides found in wasp venom, to bind both G_α and CaM (Malencik and Anderson, 1983; Kataoka et al., 1989; Higashijima et al., 1990b). Thus, CaM binding to the i3 loop could potentially be a way to regulate the G-protein signaling of many GPCRs.

OVERVIEW OF THE PRESENT STUDY

The M1 muscarinic receptor has a putative CaM binding domain on the C terminal end of the i3 loop, as discovered by a motif search. The motif search was modified by our lab and then applied to identify MOR and muscarinic receptors (Quillan and Sadee, 1996). The motif located on the hM1 muscarinic receptor is the 1-14 motif, as described by O'Day and Myrc (2004). In this work, the functional role of a CaM interaction with the hM1 i3 loop will be investigated. Peptide studies demonstrate that CaM is capable of binding to the hM1 i3 loop. These studies combined with mutagenesis highlight the regions of the hM1 i3 loop most important for binding. Two mutants deficient in CaM binding but active in G protein coupling are also identified: E360A, a point mutation, and Δ232-358, a large deletion mutant missing most of the i3 loop. Studies done with cells lines stably expressing wild type receptors suggest that CaM regulates hM1 G-protein coupling. The effect of this interaction on gene expression is also investigated. Genotyping of the hM1 muscarinic receptor of 245 individuals in the Corriell collection was also done, in an attempt to identify polymorphisms that could affect M1 function and specifically CaM binding.

RESPECTIVE CONTRIBUTIONS

The hypothesis for this work was contributed by Wolfgang Sadée and Danxin Wang (Wang et al., 1999). The motif search was developed by Mark Quillan and adapted for CaM (Quillan and Sadee, 1996; Wang et al., 1999). The custom-designed microarray used in Chapter 3 was designed by Pascale Anderle and Vera Rakhmanova (Anderle et al, 2003). The sequencing of the individuals in the Corriell collection (Chapter 4) was done by Joseph DeYoung, but I did all of the subsequent data anaylsis. All of the other work was done by me, although none of it would have been possible without many conversations, suggestions and advice from the Sadée lab and others at UCSF and Ohio State.

Chapter 2

Calmodulin binding to peptides derived from the i3 loop of Muscarinic Acetylcholine Receptors

SUMMARY

A calmodulin-binding motif had been identified in the third intracellular (i3) loop of muscarinic acetylcholine receptors (M1-M5), a region important for G-protein coupling. Synthetic peptide derived from the hM1 i3 loop, and containing a CaM binding motif, were tested for binding to CaM using a crosslinking gel shift assay and a dansyl-CaM fluorescence assay. 28-mer peptides from the C-terminus of i3, representing the putative calmodulin-domains of M1, M2, and M3 were found capable of interacting with CaM. In addition, smaller peptides defined a 10-amino acid sequence essential for calmodulin binding. Studies performed with M1-peptides derived from GST fusion proteins, representing larger portions of the i3 C-terminus, suggested the presence of a second adjacent, CaM binding site. Mutagenesis studies identified two mutants that are unable to bind CaM: a point mutation, E360A, and a deletion mutant, Δ232-358. The results suggest that calmodulin can bind to an M1 region implicated in G protein coupling.

INTRODUCTION

Muscarinic acetylcholine receptors (mAchR) are seven transmembrane spanning proteins, members of the G-protein coupled receptor (GPCR) superfamily. There are 5 subtypes in humans (hM1-M5). The hM1, hM3 and hM5 receptors couple to the heterotrimeric G-protein $G_{\alpha q/11}$ to activate phospholipase C and increase the intracellular calcium concentration. hM2 and hM4 couple to $G_{i/o}$ to inhibit adenylyl cyclase, thereby decreasing the cAMP concentration of the cell, among other signaling pathways (van Koppen and Kaiser, 2003).

The muscarinic receptors have a broad and overlapping distribution in the body. The hM1 receptor, expressed in hippocampal and cortical regions of the brain as well as in the parasympathetic ganglia (Dorje et al., 1991; Levey et al., 1991; Felder et al., 2001) is involved the initiation of seizures, learning and memory, regulation of the force and rate of heart contractions, and more (Hamilton et al., 1998; Hamilton et al., 2001). Furthermore, hM1 receptor is a compelling drug target for treating Alzheimer's disease and other neurological and psychiatric disorders (Hamilton et al., 1998).

Calmodulin is a key mediator of calcium-activated cell signaling. It has been shown to regulate a wide variety of proteins, including ion channels, kinases, phosphatases, and small GTPases. Moreover, CaM is emerging as an important regulator of GPCR signaling at the receptor level. CaM has been found to bind to the i3 loops of the μ opioid and D2 dopamine and 5HT1A receptors to inhibit G-protein coupling (Wang et al., 1999; Bofill-Cardona et al., 2000). It also binds to the C-terminus of the metabotropic glutamate receptors types 5 and 7, thereby inhibiting phosphorylation by protein kinase C (PKC) and regulating

receptor desensitization (Minakami et al., 1997; Nakajima et al., 1999; Turner et al., 2004). Using a motif search, a putative CaM binding motif on the i3 loop of muscarinic receptors has been identified (Wang et al., 1999; Quillan and Sadee, 1996). In this study, peptides derived from this region of M1-3 are shown to bind CaM in a Ca^{2+}-dependent manner. In hM1, two binding sites for CaM derived from adjacent domains representing the C-terminal portion of the i3 loop known to be involved in G protein coupling were identified. By replacing key residues involved in CaM-binding, residues required for CaM-binding but dispensable for G protein coupling have been identified. CaM binding to the C-terminal region of the hM1 i3 loop has the potential to regulate hM1 G-protein coupling. Moreover, this region is conserved in many GPCRs, raising the hypothesis that CaM can bind to a large number of GPCRs in their coupling domains.

MATERIALS AND METHODS

Materials. Bovine brain calmodulin was obtained from Calbiochem (La Jolla, CA). All muscarinic peptides, except for M1-C3-B and M1-C3-C, were from the Biomolecular Resource Center, University of California San Francisco. M1-C3-B and M1-C3-C were from Alpha Diagnostic International (San Antonio, TX). The μ opioid receptor peptide (sequence containing residues 258-286), used as a positive control, was sythnesized as described previously (Wang et al., 1999). The FLAG peptide, used as a negative control, was obtained from Sigma-Aldrich. Primers, except for those used for mutagenesis, were from the Biomolecular Resource Center, University of California San Francisco. Mutagenesis primers were from Integrated DNA Technologies (Coralville, IA). The pGEX-

5X-2 vector, Factor Xa and glutathione sepharose beads were from Amersham Pharmacia. Restriction enzymes were from New England Biolabs. All other reagents were from Sigma-Aldrich, unless otherwise stated.

Construction and purification of GST fusion proteins. The desired regions of the hM1 i3 loop were amplified using PCR (R210f-CGGGATCCCCCGCATCTACCGGGAG; S237r-GGAATTCCTCAGCTGCCACCCCCTTTGCC; A313f-CGGGATCCCCGCCCCCACCAAGCAG; K339f-CGGGATCCCCAAGGGCCAGAAGCCC; K342r-GGAATTCCTCACTTCTGGCCCTTGCC; S368r-GGAATTCCTCAACTCAGGGTCCGAGC) and cloned into the pGEX-5X-2 vector (Amersham Pharmacia) using *EcoR1* and *BamHI* sites. The vector was transformed into the DH5α strain of *E. Coli* (Stratagene). To purify the fusion protein, 5 ml of the transformed bacteria were grown overnight in YTA media (20g/L tryptone, 10g/L yeast extract, 10g/L NaCl, 100μg/ml carbenicillin). The next day, the cultures were transferred to 200 ml of the same media and grown for 5 hrs at 37°C, induced with IPTG (isopropylβ-D-1-thiogalactopyranoside) and grown at 30°C for an additional1-2 hrs. Bacteria are spun down, washed with 15 mL STE buffer (200mM Tris-HCl, pH 8.0, 1M NaCl, 0.1M EDTA), spun again and resuspended in STE buffer plus lysozyme and protease inhibitors. This is incubated on ice for 15 minutes, and then 5mM DTT (dithiothreitol), 1mM DMF (*N,N*-dimethylformamide), 1.5% sarkosyl and 10 ml B-PER bacterial protein extraction reagent (Pierce Biotechnology, Rockford, IL) are added. The mixture is centrifuged, and 2% Triton-X 100 is added to the supernatant before incubation with the Glutathione Sepharose beads

(Amersham-Pharmacia) to separate out the fusion protein. After the incubation, the beads are washed 3 times with phosphate-buffered saline (PBS; 1mM KH_2PO_4, 10 mM $NaHPO_4$, 137 mM NaCl, 2.7 mM KCl). Factor Xa (Amersham Pharmacia) is then added to the beads to cleave off the peptide. Peptide concentrations are calculated using a Bradford assay.

Mutagenesis. Mutagenesis was done using the Quik-Change site directed mutagenesis kit (Stratagene). Primers containing the desired nucleotide change plus 12-19 bases on either side of the change were annealed to the desired vector, and cycled for 12-15 times, purified and transformed into DH5α. All mutants were sequenced to verify that they contained the correct sequence.

Crosslinking gel shift assay. The crosslinking gel-shift assay was done essentially as described (Bofill-Cardona et al., 2000). Briefly, CaM and peptide in a molar ratio of either 1:1 or 1:10 (CaM: peptide) were incubated in 50 µl of 10mM Hepes, pH 7.5, plus 1mM DSS (disuccinimidyl suberate, Pierce Biotechnology, Rockford, IL), in the presence of 200µM $CaCl_2$ or 200µM of the calcium chelator EGTA, for 30 minutes at room temperature. The sample was loaded onto a 15% SDS PAGE gel and stained with Coomassie Blue.

Dansyl-CaM fluorescence assay. Dansyl-CaM was first prepared as described previously (Kincaid et al., 1982). The fluorescence of 50µg dansyl-CaM in 1ml of 10mM Hepes, pH 7.5 and 200 µM EGTA was measured with an excitation at 340 nm and emission from 400-600 nm. If necessary, $CaCl_2$ was added to attain a total concentration of 200µM (since 200µM EGTA was already present in the solution, 400 µM $CaCl_2$ was added), and the

sample was measured again. Peptide was then added so that there was a molar ratio of CaM: peptide of 1:1, and the sample was measured again.

Overlay assay. This is done as described previously (Warr and Kelly, 1995). Purified whole GST fusion proteins are run on a 10% SDS-PAGE gel. The gel is then blotted onto a PVDF membrane (Biorad). The membrane is incubated with biotinylated CaM (Calbiochem), washed, and treated with avidin-horse radish peroxidase (Amersham Pharmacia). This is developed using ECL chemiluminescent detection reagents (Amersham Pharmacia). The presence of protein was confirmed using a Coomassie stain.

RESULTS

CaM binding to peptides derived from hM1-GST fusion proteins. Four GST fusion proteins representing different regions of the hM1 i3 loop were constructed (Figure 2-1A). These were: N, which is on the N-terminal end of the i3 loop and contained residues 210-237; C3, on the C terminal end of the i3 loop and containing the putative CaM binding motif (339-368); C2 representing part of the i3 loop adjacent to C3 (313-342); and C1, which encompassed both C3 and C2 (313-368). The GST fusion proteins were purified from bacteria and peptides cleaved using a Factor Xa cleavage site. The ability of these peptides to bind CaM was tested using a crosslinking gel shift assay (Figure 2-1B). In this assay, the ability of a peptide to bind CaM is indicated by a mobility shift consistent with the increased molecular weight of the fusion protein over free calmodulin. The N-peptide yielded no detectable CaM binding. The C3 peptide, containing the putative CaM binding motif,

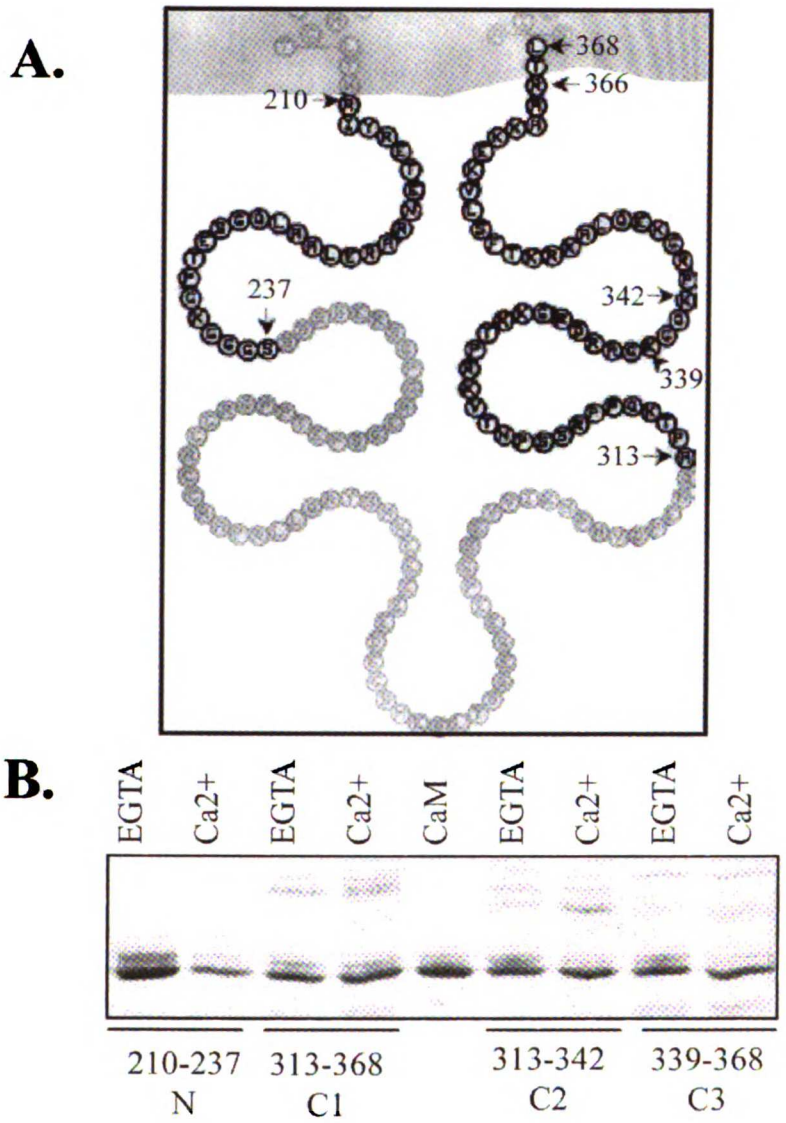
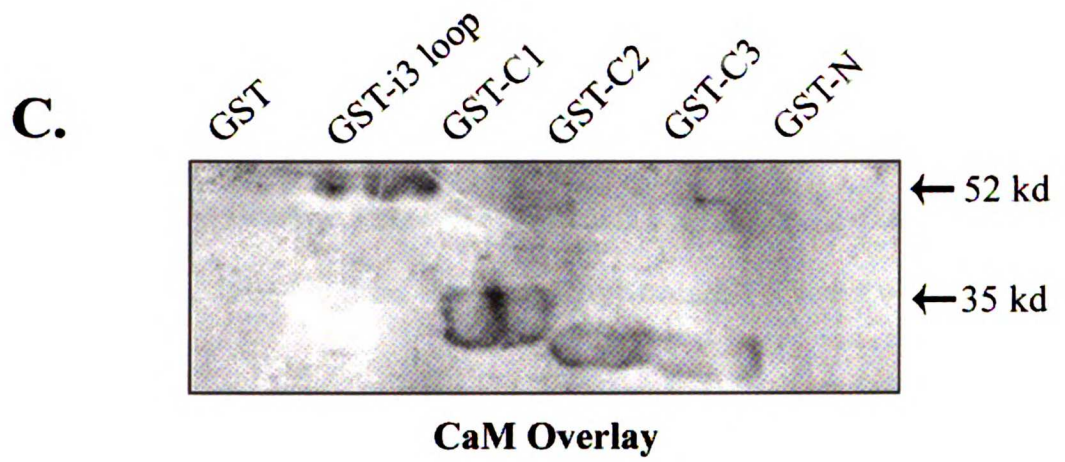


Figure 2-1. Studies with peptides derived from GST fusion proteins. **A.** Diagram of the hM1 i3 loop. **B.** Crosslinking gel shift assay using hM1 peptides representing different regions of the i3 loop. The three C-terminal peptides shifted the CaM band at 1:1 peptide: CaM ratio in the absence and presence of calcium. Under the same conditions, the N-terminal peptide (210-237) did not shift.



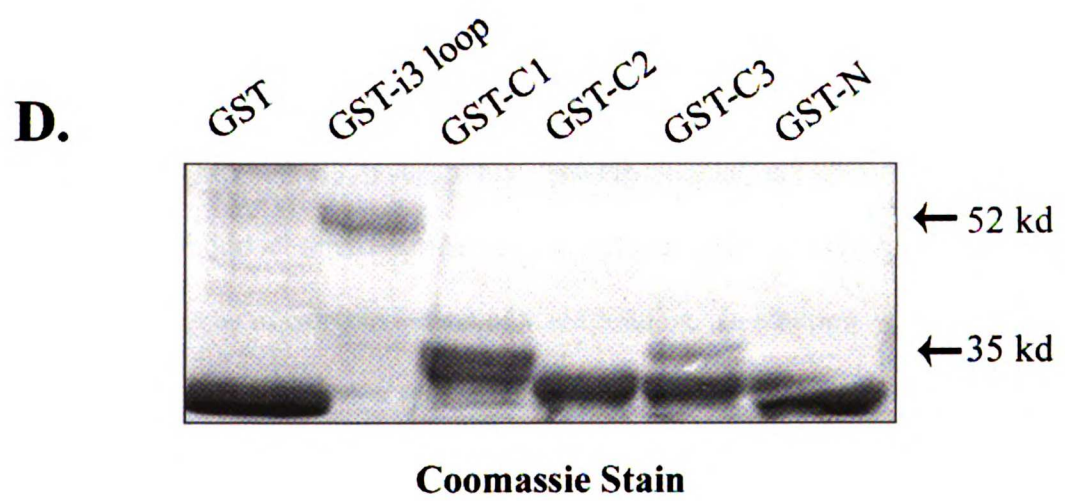


Figure 2-1. Studies done with GST fusion proteins. **C.** CaM overlay assay showing that only the GST fusion proteins containing the entire i3 loop, C1, C2 or C3 constructs bind CaM; N and GST alone do not. **D.** Coomassie stain of the above gel showing that protein was present in all samples.

yielded a shifted band consistent with a peptide-CaM adduct. Interestingly, the C2 peptide, which is adjacent to the putative CaM binding domain, also appeared to bind CaM. C1, which contains both C2 and C3, similarly bound to CaM. All peptides preferred binding CaM in the presence of calcium. These results suggest that there may be two binding sites for CaM on the hM1 i3 loop. These results were confirmed using a CaM overlay assay (Figure 2-1C and D). However, the overlay assay was less reproducible being sensitive to incubation conditions, and therefore, subsequent experiments relied on the gel shift assay.

CaM binding to synthesized peptides. Synthesized peptides representing the putative CaM binding region of hM1-3 were made (Fig 2-2A) and tested for ability to bind CaM (Fig 2B). The peptide from hM1 is designated M1-C3-A, since its sequence is similar to the C3 peptide made from the GST fusion protein. The peptides from the hM2 and hM3 receptors are called M2 and M3, respectively. The crosslinking gel shift assay revealed that CaM was able to bind to all three peptides (Fig 2-2B) in molar ratios of CaM:peptide of 1:1 and 1:10. The presence of calcium enhanced CaM binding. In addition, when peptide is added in molar excess over CaM, a second, higher molecular weight band appears, with a molecular weight equivalent of two peptides binding to one CaM. For a positive control, a peptide derived from the i3 loop of the MOR was used. This peptide has been shown previously to bind to CaM (Wang et al., 1999). A FLAG peptide was used as a negative control (Wang et al., 1999).

These results were confirmed using the dansyl-CaM fluorescence assay (Fig 2-2C, D; results for hM2 and hM3 were very similar to M1-C3-A and therefore not shown). CaM undergoes a large conformational change when binding a peptide. This is reflected in this

A.

hM1 341-368 QKPRGKEQLAKRKTFSLVKEKKAARTLS

hM2 364-391 KIVKMTKQPAKKKPPPSREKKVTRTILA

hM3 467-494 FALKTRSQITKRKMSLVKEKKAAQTLS

B.

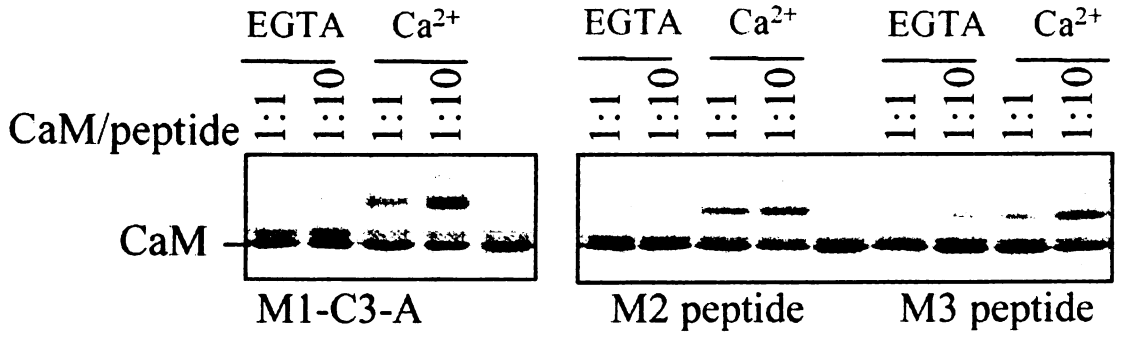


Figure 2-2. A. Sequences of hM1 (designated M1-C3-A), hM2 and hM3 peptides. **B.** Crosslinking gel shift assay of CaM binding to peptides derived from the i3 loops of hM1, hM2 and hM3. The band was shifted at both 1:1 and 1:10 CaM: peptide molar ratios. All three peptides bound to CaM, with the strongest binding in the presence of calcium. The larger band in the 1:10 molar ratio is the MW equivalent of two peptides binding to one CaM.

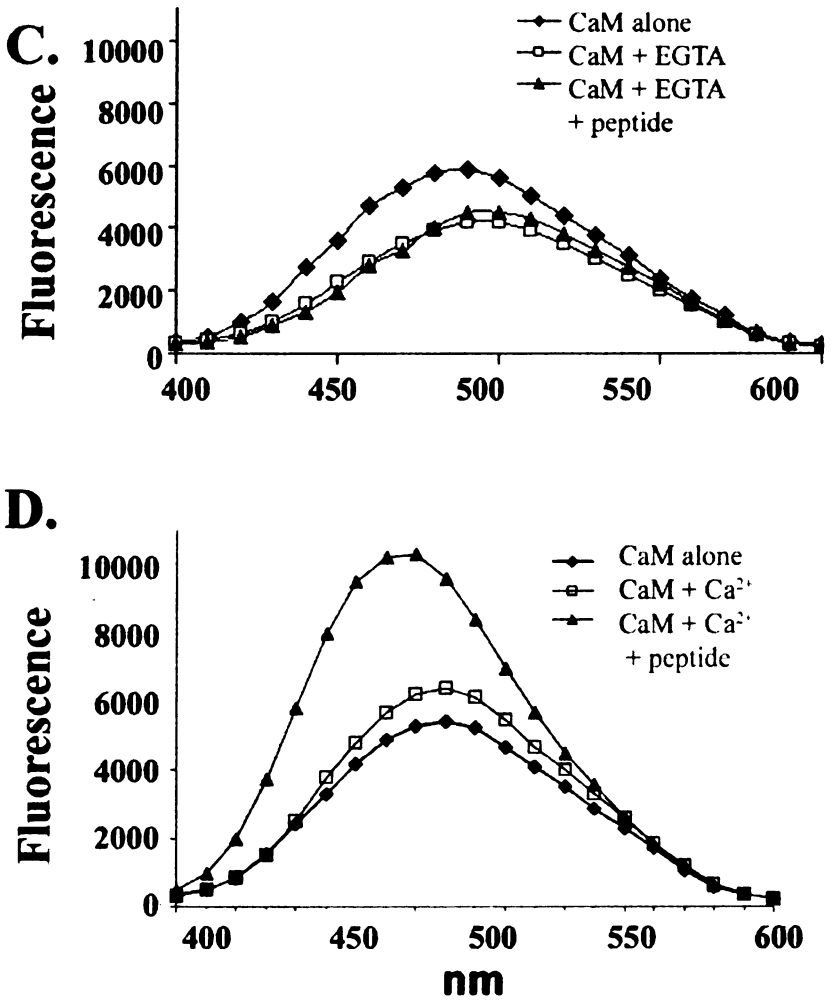


Figure 2-2. C-D. Dansyl-CaM fluorescence assay of CaM with the hM1 peptide. An increase in fluorescence and shift of the curve to the left is indicative of binding. As in the crosslinking gel shift assay, the peptide preferred to bind CaM in the presence of calcium (D). M2 and M3 peptides had similar results (data not shown).

A.

KEKKAARTLS	**M1-C3-D**
TFSLVKEKKAARTLS	**M1-C3-C**
QLAKRKTFSLVKEKKAARTLS	**M1-C3-B**
QKPRKEQLAKRKTFSLVKEKKAARTLS	**M1-C3-A**

Figure 2-3. A. Sequences of synthesized peptides. M1-C3-A is derived from residues 342-368, M1-C3-B from 348-368, M1-C3-C from 354-368, and M1-C3-D from 359-368.

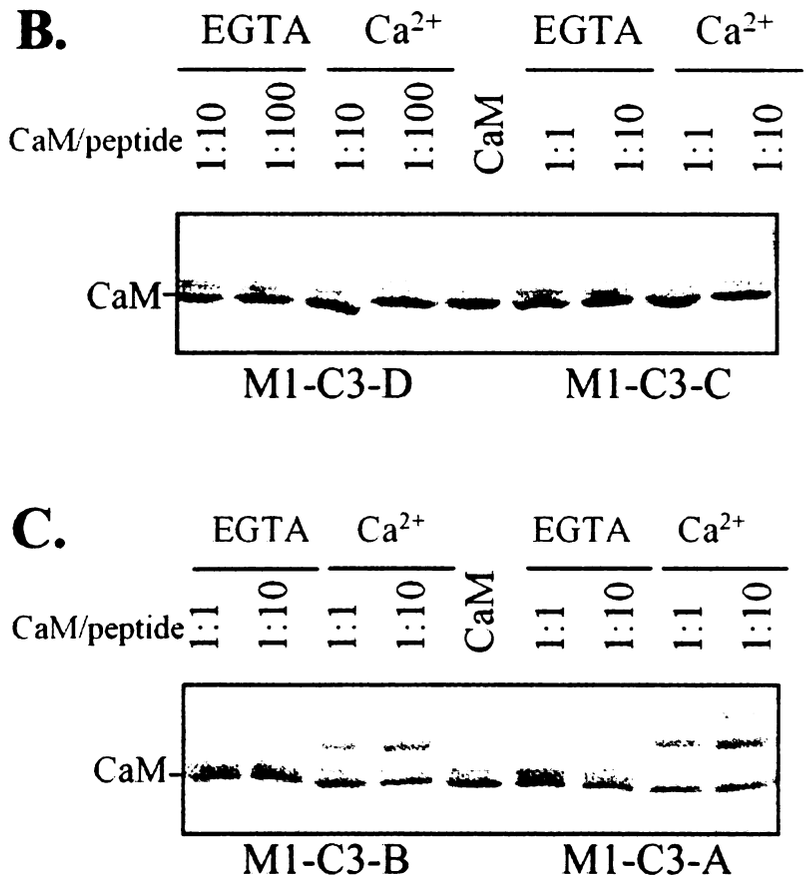


Figure 2-3. B-C. Crosslinking gel shift assay of peptides with CaM. There is binding both in the presence and absence of calcium, with preference for calcium. M1-C3-D failed to bind CaM while M1-C3-A, M1-C3-B and M1-C3-D bound. M1-C3-A and M1-C3-B also showed two bands when peptide was in excess (1:10 peptide:CaM molar ratio), suggestive of a 2:1 stoichiometry.

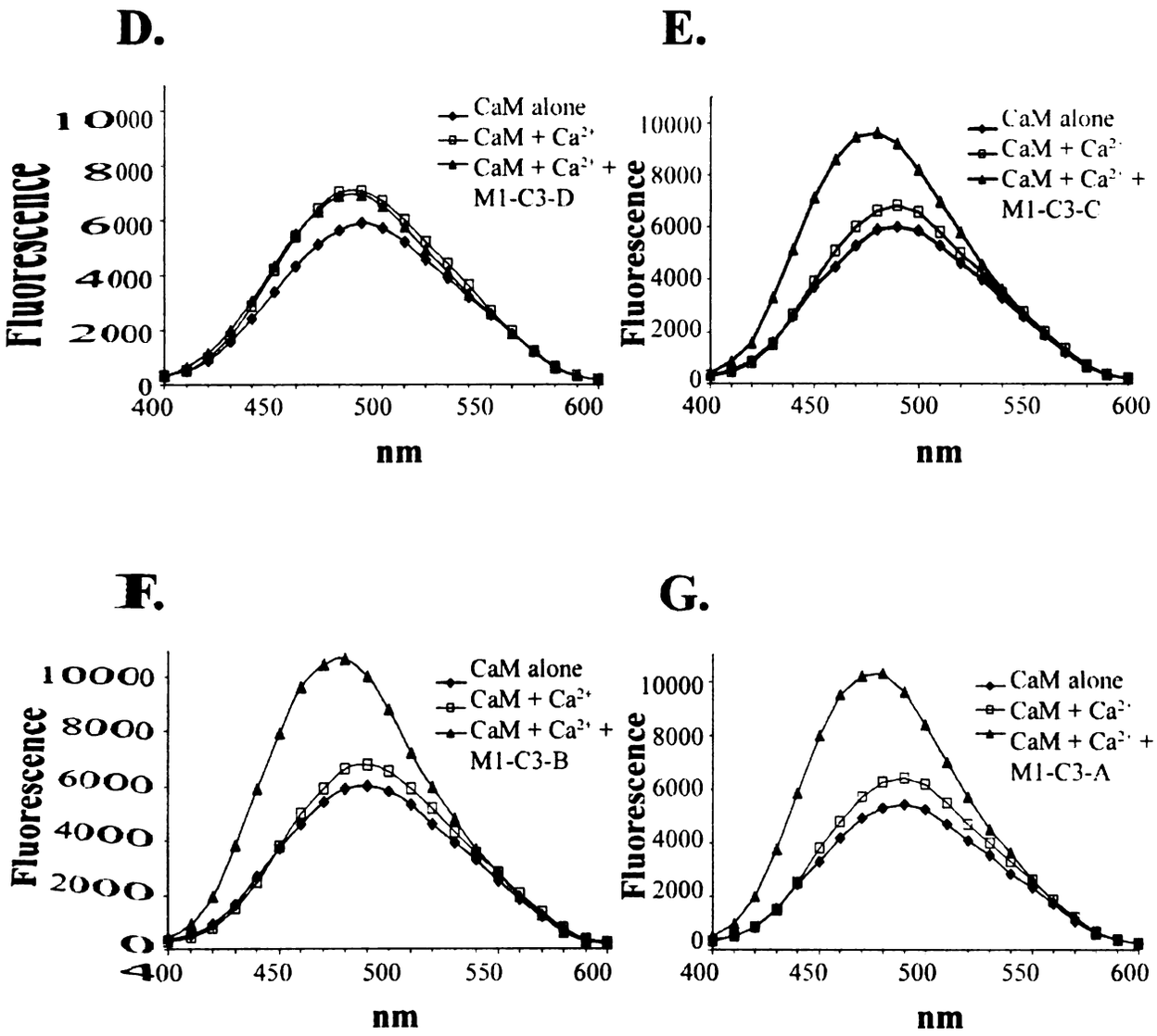


Figure 2-3. D-G. Fluorescent spectra of peptides with dansyl-CaM. Only spectra done in the presence of calcium is shown. None of the peptides bound dansyl-CaM in the absence of calcium, which was done in 200□M EGTA. M1-C3-C (E), M1-C3-B (F) and M1-C3-A (G) all bound CaM while the 10-mer, M1-C3-D (D), did not.

say by an increase in the fluorescence of dansylated CaM and a shift of the peak to the left. e increase in fluorescence and shift in the peak occurred only in the presence of calcium, ain confirming the hM1 peptide is able to bind CaM preferentially in the presence of cium. Similar results were obtained for the hM2 and hM3 peptides (not shown). The sitive and negative control peptides were also tested (data not shown). In order to specify region of hM1 required for CaM binding, a series of sequentially smaller peptides was nthesized; a 21-mer (M1-C3-B), a 15-mer (M1-C3-C) and a 10-mer (M1-C3-D) (Fig 2-). Also shown, for comparison, in Fig 3 are results with M1-C3-A, the 28-mer capable of ding to CaM in Fig 2-2. Each of the shorter peptides was tested for their ability to bind M using the crosslinking gel shift assay (Fig 2-3B and 2-3C) and the dansyl-CaM orescence assay (Fig 2-3D-G). M1-C3-B and M1-C3-C, which contain 21 and 15 amino ds, respectively, were both able to bind CaM, again with a preference for calcium. M1--D, which contains 10 amino acids, was unable to bind CaM. This suggests that residues 9-368, from which this peptide is derived, are insufficient for CaM binding. Furthermore, e or more of the residues 354-358 (from M1-C3-C) are important for CaM binding.

Amino acid substitutions and mutagenesis studies. The purpose of the tagenesis studies was two fold: first, to identify the regions of the hM1 i3 loop important CaM binding; second, to identify a mutant that either lacks CaM binding but still couples G-proteins, or *vice versa*, for subsequent use in functional studies. A series of point tations was made in the C3 GST fusion protein, the resulting peptide cleaved and used in rosslinking gel shift assay to screen for ability to bind CaM. Table 2-1 lists the mutants de, the peptide sequence, and the peptide's ability to bind CaM. Mutations of the

Mutation	Peptide Sequence	Ability to Bind CaM
S322A	APTKQPPKSAPNTVKRPTKKGRDRAGKGQK	+
K327A	APTKQPPKSSPNTVARPTKKGRDRAGKGQK	+
K346A	KGQKPRGAEQLAKRKTFSLVKEKKAARTLS	+
R352A	KGQKPRGKEQLAKAKTFSLVKEKKAARTLS	+
K359T	KGQKPRGKEQLAKRKTFSLVTEKKAARTLS	+
E360A	KGQKPRGKEQLAKRKTFSLVKAKKAARTLS	-
K361A	KGQKPRGKEQLAKRKTFSLVKEAKAARTLS	+
Δ351-353	KGQKPRGKEQLA TFSLVKEKKAARTLS	+
Δ363	QLAKRKTFSLVKEKKARTLS	+
Δ232-358	RIYRETENRARELAALQGSETP KEKKAARTLS	-

Table 2-1. HM1 mutants, their sequences and the effect of the mutation on CaM binding. Mutations were made in the C3-GST fusion protein (see Fig 2-1), and the crosslinking gel shift assay was done as a screen for CaM binding. Two mutants were unable to bind CaM: E360A and □232-358.

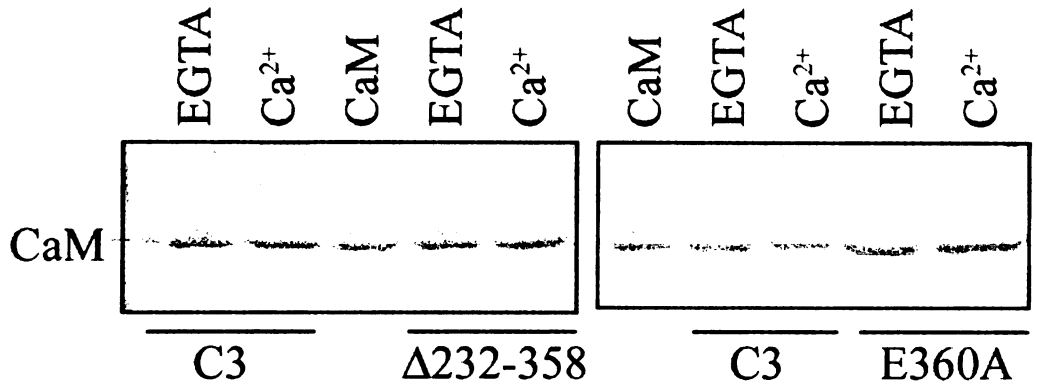


Figure 2-4. Crosslinking gel shift assay of hM1 mutants deficienct in CaM binding. The wild-type (C3) peptide was able to bind CaM , while the two mutants E360A and □232-358 were unable to cause a shift in CaM mobility. Assay was done as described in Figs 2 & 3.

positively charged residues of hM1 had no effect on CaM binding. Two mutants, however, were deficient in CaM binding: E360A, a point mutation, and a large deletion mutant, Δ232-358, missing most of the hM1 i3 loop and all but the last 10 residues of the putative CaM binding domain (Fig 2-4). Both mutants have been previously described as being able to couple to G-proteins (Maeda et al., 1990a; Hogger et al., 1995a). Moreover, E360A has also been described as being constitutively active (Hogger et al., 1995a).

DISCUSSION

In this study, we have shown that CaM is able to bind to peptides representing the C-terminal region of the hM1 i3 loop in a Ca^{2+}-dependent manner. When peptide is in excess,

CaM is capable of binding two hM1 peptides. This may indicate a unique binding property of the CaM-hM1 interaction. CaM has been shown to be able to stretch to accommodate multiple α-helical peptide strands. A crystal structure of the Ca^{2+} activated K^+ channel (SK channel) revealed that two CaM molecules each bind three α-helical strands of different portion of the channel protein. In this structure, one lobe of CaM had calcium bound and interacted with one α-helix, and the other lobe had no calcium bound and interacted with two α-helices (Schumacher et al., 2001). This crystal structure illustrates that CaM has the potential to act as a crosslinker. With hM1, CaM could be involved in receptor homodimerization, in which one CaM molecule crosslinks two hM1 mAchR molecules. Muscarinic receptors, especially hM3, have been shown to dimerize (Zeng and Wess, 1999). Further studies need to be done to establish what role, if any, CaM plays in dimerization.

M1-C3-C, a 15-mer located at the C-terminal region of i3, was the smallest hM1 muscarinic receptor peptide capable of binding to CaM, whereas M1-C3-D, the 10-mer corresponding to residues 359-368 on hM1, was insufficient for CaM binding. This result is consistent with lack of CaM biding of the large i3-loop deletion mutant, Δ232-358, which also contains only the last 10 amino acids of the hM1 putative CaM binding domain. One or more of the residues 354-358 are also important for CaM binding, since the addition of these residues enabled M1-C3-C to bind CaM.

CaM binding was also tested for peptides with single amino acid substitutions. A longer peptide containing a substitution equivalent to the E360A mutation in M1 and located within these 10 C-terminal residues (Fig 2-5), also did not display detectable CaM binding. Replacement of the positively charged basic residues with uncharged amino acids failed to affect CaM binding, whereas two of these positively charged residues, K362 and R365, had

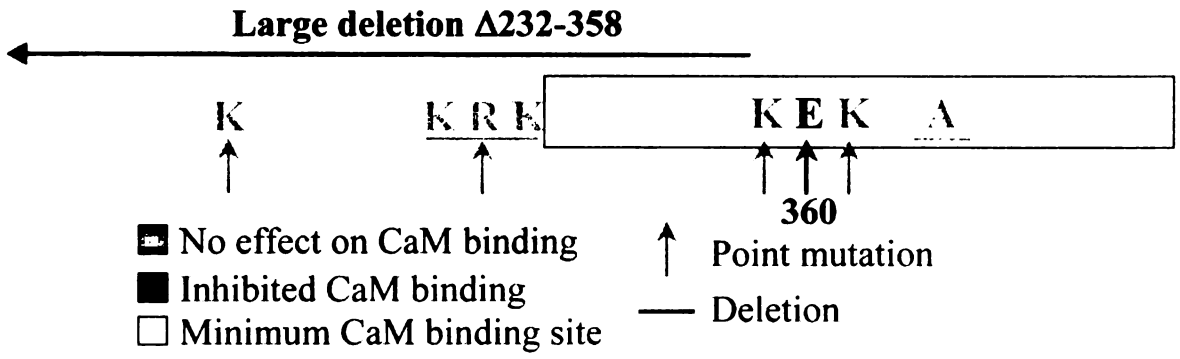


Figure 2-5. Summary of results from mutagenesis and peptide studies. Positively charged amino acids do not seem important for CaM binding. E360 is necessary but not sufficient for binding. One or more of the five residues TFSLV are important for CaM binding.

been found critical for G-protein coupling. Previous mutagenesis studies indicate that R365A caused a 50% decrease in the maximal PI stimulation by M1 but did not affect potency. The mutation of K362 to alanine caused both a 50% decrease in maximal PI stimulation and decreased the potency 13 times(Hogger et al., 1995a; Lee et al., 1996a). These results suggest that one will be able to construct M1 mutant that are deficient in either G protein coupling or in CaM-binding. Moreover, CaM has the potential to bind to a region of M1 important for G-protein coupling. One likely function of the CaM-hM1 mAchR interaction is that CaM could regulate the G-protein coupling of the hM1 muscarinic receptor.

CaM regulation of GPCR G-protein coupling may extend beyond muscarinic receptors. Two tetradecapeptides found in wasp venom, melittin and mastoporan, can both bind CaM and activate G-proteins (Malencik and Anderson, 1983; Higashijima et al., 1988a;

Kataoka et al., 1989; Higashijima et al., 1990a). This indicates that CaM and G-protein binding sites are similar and overlap. In addition, BBXXB, which has been proposed as a universal G-protein coupling motif (Lee et al., 1996a), resembles a CaM binding motif, but our current results indicate that not all basic residues are needed for CaM-binding. The CaM-binding domain of many proteins that bind CaM in a Ca^{2+}-dependent manner contain hydrophobic and basic residues with a propensity to form an amphipathic α-helix (O'Day and Myrc, 2004). The α-helix of transmembrane domain 6 appears to extend into the C-terminus of the i3 loop in the cytoplasm, generating a putative CaM-binding site(Lu et al., 2002) predicted by a motif search (Quillan et al.). That CaM binding requirements may be similar to those for G-proteins also allows for the hypothesis that CaM binding to the i3 loop may be common among GPCRs. Since CaM is a ubiquitous molecule and there are over six hundred GPCRs in humans, CaM has the potential to be an important regulator of GPCR signaling.

Indeed, CaM-binding to the i3 loop is already known to be able to regulate the G-protein coupling of two GPCRs, the μ opioid receptor (MOR) and the D2 dopamine receptor (Wang et al., 1999; Bofill-Cardona et al., 2000). For the μ opioid receptor we have proposed that CaM binding suppresses basal receptor – G protein coupling; upon receptor activation by an agonist, CaM is released and G protein coupling ensues (Wang et al.). Similarly, the E360A mutant also has been found to display increased basal activity (Hogger et al., 1995a). If CaM were to regulate basal M1-G protein coupling, one would expect a mutant deficient in CaM-binding to have an increased basal coupling activity. These mutants will be used in future studies to ascertain the role of CaM in the regulation of hM1 mAchR G-protein coupling.

Peptides derived from GST fusion proteins suggest the existence of two adjacent CaM binding sites on the hM1 mAchR i3 loop. CaM regulates other proteins using two sites; it can control ion channel gating, including inactivation of L-type Ca^{2+} channels and Trpl, and regulate adenylate cyclase (Ladant, 1988; Warr and Kelly, 1995; Pitt et al., 2001a). Two GPCRs known to bind CaM, mGluR7 and 5HT1A receptors, both have two CaM binding sites. Binding of CaM to both sites can inhibit phosphorylation by PKC. The 5HT1A receptor binds CaM on the i3 loop, and metabotropic glutamate receptors, types 7, on the C-terminus (Nakajima et al., 1999; Turner et al., 2004). Another GPCR, the metabotropic glutamate receptor, type 5, has one CaM binding sites on the C-terminus (Minakami et al., 1997). CaM binding to this site interferes with PKC phosphorylation. CaM may therefore play a role in the regulation of GPCR desensitization. The hM1 receptor has two putative PKC phosphorylation sites on the C-terminal end of the i3 loop, at T354 and S356. PKC has been shown to be able to phosphorylate a peptide containing these residues, indicating a role for PKC in heterologous desensitization (Haga et al., 1996). CaM could regulate PKC phosphorylation at these sites. The requirements for a CaM binding site and a PKC site also overlap, since a PKC site requires basic residues close to a serine or threonine (Haga et al., 1996). Since many GPCRs are regulated by PKC phosphorylation, CaM has the potential to be an important regulator of GPCR desensitization.

In summary, we have shown that peptides derived from the hM1 i3 loop C-terminal tail, a region of the protein critical for G-protein binding, are able to bind CaM. These findings suggest a direct interaction of CaM with muscarinic receptors. Our results show that there are two potential, adjacent CaM binding sites on the hM1 mAchR i3 loop. Furthermore, we have identified altered peptides representing two mutants that are deficient

in CaM binding but are still able to couple to G-proteins, while another mutant is defective in G protein coupling with intact CaM-binding. Future studies will investigate the functional role of this interaction in muscarinic cell signaling. Since the region in which CaM binds the hM1 muscarinic receptor is crucial for G-protein binding and contains two putative PKC phosphorylation sites, possible functional roles for the CaM-hM1 i3 loop interaction include regulation of G-protein signaling and PKC phosphorylation; however, CaM may also serves as a second messenger *per se* as reported for the μ opioid receptor (Wang et al., Belcheva et al.). This study supports a broader trend suggesting that CaM is a key regulator of GPCR signaling at the receptor level.

Acknowledgement. This work was supported by NIH Research Grant DA04166, from the National Insititute on Drug Abuse.

Chapter 3

Regulation of hM1 Muscarinic Acetylcholine Receptor signaling by Calmodulin

SUMMARY

In the previous chapter, the interaction of calmodulin (CaM) with peptides derived from two adjacent sites on the i3 loop of the hM1 muscarinic acetylcholine receptor was described. Two peptides derived from mutant M1 sequences that are unable to bind CaM were also identified: E360A-hM1, a point mutation, and Δ232-358-hM1, a deletion mutant missing most of the i3 loop. In this study, the role of the CaM-hM1 interaction in regulating signal transduction was studied. Microarray studies reveal that there are differences in the ability of HEK-293 cells stably expressing either wild type or mutant hM1 mAchR to regulate gene expression. Furthermore, addition of CaM to membranes from cells expressing hM1 inhibits [^{35}S] GTPγS incorporation, a measurement of G-protein coupling. This data suggests that CaM regulates hM1 G-protein coupling. The results of this study support the hypothesis that CaM, through its direct interaction with hM1, regulates signal transduction.

INTRODUCTION

Muscarinic acetylcholine receptors, members of the G-protein coupled receptor (GPCR) superfamily, are seven transmembrane-spanning signal transduction molecules. There are five subtypes in humans (hM1-hM5). Muscarinic acetylcholine receptors are located throughout the body in the parasympathetic ganglia and in the brain. They regulate fundamental processes such as smooth muscle contraction and learning and memory (van Koppen and Kaiser, 2003). They have been shown to be involved in neurological disorders such as schizophrenia, Parkinson's and Alzheimer's disease (Kasa et al., 1997; Bartus, 2000). Destruction of the cholinergic system is thought to be responsible for the memory loss in Alzheimer's, and hM1 is a compelling drug target for treatment of this disorder (Kasa et al., 1997; Fisher et al., 1998; Fisher et al., 2000).

CaM, as a calcium-activated regulator of cell signaling, has a wide variety of targets, including kinases, phosphatases, ion channels, adenylyl cyclases and many others (Zhang and Yuan, 1998). CaM is emerging as a regulator of GPCR signal transduction at the receptor level. CaM binding to the C-terminus of the metabotropic glutamate receptor, types 5 and 7, has been found to inhibit phosphorylation by protein kinase C (PKC) (Minakami et al., 1997; Nakajima et al., 1999). Similarly, CaM binds to the i3 loop of the serotonin 5HT1A receptor to inhibit PKC phosophorylation (Turner et al., 2004b). CaM binds to the i3 loops of the μ opioid receptor (MOR) and the D2 dopamine receptor to inhibit G-protein coupling (Wang et al., 1999; Bofill-Cardona et al., 2000). In addition, mastoporan and melittin, two tetradecapaptides from wasp venom, can bind both CaM and G-proteins, suggesting that the requirements for binding to these two proteins may be similar (Malencik and Anderson,

1983; Higashijima et al., 1988b; Kataoka et al., 1989; Higashijima et al., 1990b). It is therefore possible that many other GPCRs other than the ones listed above bind to CaM.

In addition, CaM bound to the MOR i3 loop has a biological signaling role: the release of CaM from MOR results in the translocation of CaM to the cell nucleus to regulate the phosphorylation of the transcription factor CREB (Wang et al., 2000). In addition, CaM can cause the transactivation of the EGF receptor (Belcheva et al., 2001). Activation of MOR resulted in a CaM-dependent activation of ERK, through the transactivation of EGF. This pathway was activated through a direct CaM-MOR interaction; a MOR mutant that could couple to G-protein but was deficient in CaM binding could not transactivate EGF. These studies with MOR demonstrate that CaM, through its direct i3 loop interaction, could play a role as a GPCR second messenger.

Two adjacent regions located on the C-terminal end of the hM1 muscarinic receptor i3 loop bind calmodulin (CaM) in a calcium-dependent manner *in vitro* (Chapter 2). The C-terminal end of the hM1 i3 loop is crucial for G-protein coupling and contains two putative protein kinase C phosphorylation sites. CaM may function as a regulator of hM1 G-protein coupling or PKC phosphorylation. Peptides derived from the sequence of two mutants, E360A and Δ232-358, are unable to bind CaM, but have been previously shown to couple to G-proteins. The purpose of this study is to investigate the function of the CaM–hM1 mAchR i3 loop interaction. To this end, the gene expression patterns in cells expressing the two mutants or wild-type hM1 mAchR in basal and carbachol-treated cells were evaluated for differences in the ability of wild type and mutant mAchR to activate signaling pathways. The effect of CaM on G-protein coupling and a co-immunoprecipitation to determine whether this interaction occurs in cells were also studied.

MATERIALS AND METHODS

Cell Culture. HEK-293 cells were maintained at 37°C and under 5% CO_2 in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100μg/ml streptomycin and 100 IU/ml penicillin. PC12D cells, a rapidly differentiating subline of rat pheochromocytoma-derived PC12 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% fetal bovine serum, 5% horse serum, 0.16% sodium bicarbonate, 3.6 mM glutamine, 10 units/ml penicillin, and 45 ng/ml streptomycin at 37 °C under 5% CO_2 as described previously (Guo et al., 2001).

Construction of stable cell lines. cDNAs containing the hM1 mAchR wild-type or mutant sequence were subcloned into a pcDNA3 vector (Invitrogen) using *BamHI* and *XhoI* restriction sites (wild-type and E360A-hM1) or *EcoRI* and *NotI* sites (Δ232-358-hM1). The resulting construct, or empty vector (mock-transfected), was then transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells expressing the desired plasmid were selected by treatment with geneticin (Invitrogen). Expression levels were measured using [^{3}H] N-methylscopolamine, a cell-impermeable muscarinic antagonist (Lin et al., 2002).

Mutagenesis. The Quik-Change site-directed mutagenesis kit (Stratagene) was used to make the E360A mutant. Primers containing the desired nucleotide change plus 12-19 bases on either side of the change (E360As-

GACCTTCTCGCTGGTCAAGGCGAAGAAGGCGGCTCG; E360Aas-GGTCCGAGCCGCCTTCTTCGCCTTGACCAGCGAGAAGG) were annealed to the hM1/pcDNA3 vector, and cycled for 12-15 times, purified and transformed into DH5α. The mutant was then sequenced to verify that it contained the correct sequence. Δ232-358 was made previously (Maeda et al., 1990b), but was subcloned into pcDNA3 for this study.

RNA Extraction. Cells were grown in a 60 cm^2 dish until 90% confluent. Trizol (5 ml; Invitrogen) was added to each 60 cm^2 dish, and lysates were transferred into a 50 ml falcon tube and frozen at -80°C until needed. Samples were thawed completely in a 37°C water bath before addition of 0.2 ml chloroform per ml Trizol. The samples were vigorously shaken by hand for 15 seconds, incubated at room temperature for 2-3 min, and centrifuged at 4°C for 15 min (Sorvall super T21, SL-250T with adaptor), at 12000x*g*. RNA separates into the colorless upper aqueous phase, and DNA and other components in the interphase or below. The aqueous phase containing RNA (600 μl or less per ml Trizol) was transferred to a new 50 ml tube, and precipitated with 0.5 ml isopropyl alcohol per ml Trizol. Samples were incubated at 15 to 30°C for 10 minutes, centrifuged at 4° C for 10 min (Sorvall super T21, SL-250T with adaptor) at 9500 RPM, and the supernatant was removed and discarded. RNase free water (100 μl) was added to dissolve the RNA pellet, followed by RNA purification with a Rneasy Mini prep kit (Qiagen). RNA was stored at –80°C until used for microarray analysis.

Microarray

A custom designed oligonucleotide microarray, containing approximately 1000 human genes in quadruplicate (mainly transporters and ion channels) was used in this study (Anderle et al., 2003).

Reverse Transcription. This is derived from a protocol developed at Rosetta Inpharmatics, Kirkland, WA. Each slide has two samples, an experimental and a control sample. For each sample, 7.5 μg oligo dT and 3.75 μg pdN6 (random hexamers) were added to 12.5 μg RNA so that the total volume was 16 μl in a 0.2 ml PCR tube. The samples were incubated at 70°C for 10 min, followed by 10 min on ice. A dNTP cocktail (Invitrogen) (0.6 μl of 25 mM dA, 25 mM dG, 25 mM dC, 15 mM aa-dUTP and 10 mM dT), 6 μl of 5x First Strand Buffer (Invitrogen), 3 μl of 0.1M DTT (dithiothreitol), 1.9 μl Superscript Reverse Transcriptase (Invitrogen) and 3 μl H_2O were added to each tube and the samples were incubated at 42°C for 2 hrs.

Hydrolysis. Samples were hydrolyzed by the addition of 10 μl each of 1N NaOH and 0.5M EDTA and incubated at 65°C for 15 minutes. Hydrolyzed samples were neutralized with 25 μl 1M Tris, pH 7.4 and stored at 4°C overnight.

Cleanup. On the following day, the Tris was removed from the reaction by washing 3 times with water in a Micron 30 concentrator (10,000 rpm for 10 min in a microcentrifuge). Samples were eluted by turning the Micron 30 concentrator upside down,

and spinning 2min at 10,000 rpm. The solution was then dry-eluted in a speed vacuum. Samples were stored at –20°C until needed.

Coupling of cDNA to fluorescent dyes. Cy3 was used as the green fluorescent dye (control cDNA sample) and Cy5 was used as the red fluorescent dye (experimental sample). For the transfected vs. untranfected slides, the untransfected cDNA was the control (green) and the cDNA from transfected cells was the experimental sample (red). For the untreated vs. carbachol-treated slides, the cDNA from untreated cells was the control (green) and the cDNA from carbachol-treated cells was the experimental sample (red). Dye swaps were done for all experiments. The monofunctional NHS-ester Cy3 and Cy5 dye powder (one vial is used for eight slides) was resuspended in 74 µl 0.05 M sodium bicarbonate buffer, pH 9. Either Cy3 or Cy5 (9 µl) was added to the cDNA pellet and the sample was vortexed and centrifuged. The samples were incubated 1 hour in the dark at room temperature. Samples were then purified with a Qiagen PCR purification kit to remove unincorporated Cy dyes.

Array Hybridization. For each slide, the Cy3-labeled and the Cy5-labeled samples were combined and concentrated to 25 µl with a Micron 30 concentrator. Samples were eluted by flipping the concentrator upside down, and spinning in a microcentrifuge for 1 min at 10,000 rpm. The following were added to the combined samples: 3 µl 20xSSC, 1.5 µl 10 mg/ml poly A oligo, 0.5 µl 1M HEPES pH 7.0 and 1µl 10mg/ml tRNA. Samples were then filtered with a Millipore 0.45 µm membrane, 0.5 µl 10% SDS was added to each sample, and they were incubated at 99°C for 2 min, followed by 37°C for 20-30 min.

Samples were then carefully added to the microarray slides. In this study, a custom-designed oligonucleotide array containing probes for approximately 1000 human genes was used. The slides were first secured to the hybridization chamber with 5µl water. The sample containing the probe was then dropped onto to the array area, and a Lifterslip coverslip was flipped down so that no air bubbles were present. SSC buffer (10 µl of 3x) was added to the end of slide at the top of the label. The hybridization chamber lid was then tightly screwed on and placed inside a plastic bag containing wet paper towels. The slides were incubated in a 63°C water bath for 8-12 hours.

Array Washing. The next day, the arrays were carefully washed as follows. They were removed from the hybridization chamber, and individually submerged in washing solution I (340 ml Milli-Q water, 10 ml 20X SSC, 1 ml of 10% SDS) to gently remove the coverslip. The slides were then transferred to a second container, which holds a slide rack submerged in washing solution I. Once all microarrays are present, the slides were washed by plunging the rack up and down 20 times. The slide rack containing the microarray chips was then transferred to another container, which held washing solution II (350 ml Milli-Q water, 1 ml 20X SSC). Slides were washed again by plunging the rack several times. The rack was transferred to a second container that held fresh washing solution II and the plunging was repeated. The slides were dried by placing the rack in a Beck-Coulter tabletop centrifuge at room temperature for 2 min at 600 rpm. Arrays were scanned within 2 hrs of washing. They were analyzed using GenePix 3.0 and normalized as described (Yang et al., 2002).

[^{35}S]GTPγS binding assay. The HEK-293 cell clones were grown to confluence and harvested using 10 mM Hepes, 500 μM EDTA and 150 mM NaCl, pH 7.4. Cells were then pelleted and resuspended in ice-cold buffer consisting of 10 mM Hepes, 10 mM EDTA, pH 7.4. Cells were homogenized using a polytron tissue disrupter (level 6, 6 x 3 s bursts). Crude membranes were pelleted in a refrigerated centrifuge at 40,000*g* for 10 min, at 4°C. Membranes were finally resuspended in binding buffer consisting of 10 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4, at a final protein concentration of 1 μg/μl (50μg/reaction). Fresh membranes were prepared prior to each experiment. Incubations were conducted in a final assay volume of 100 μl, for 1 min, at 37°C. Incubations were conducted in the presence of 3 μM GDP and approximately 70 pM [^{35}S]GTPγS, in the presence and absence of various concentrations of carbachol. Incubations were terminated by the addition of 0.5 ml ice-cold assay buffer. Samples were spun down at 40,000*g* for 10 min and the pellet washed with 1ml ice-cold phosphate-buffered saline. Radioactivity in the samples was assessed by liquid scintillation spectrometry (Burford et al., 1995).

Co-Immunoprecipitation. Cultures of PC12D cells (95% confluent) were used for all of the experiments. Cells were rinsed once with cold phosphate-buffered saline and then lysed by addition of 500 μl of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40 (nonylphenoxypolyethoxyethanol), 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 2 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin). Lysed cells were collected in 1.5 ml microcentrifuge tubes and sonicated at setting 2 (Fisher Model 100 Sonic Dismembrator) 3 times for 30 sec with 30 sec intervals on ice. Cell lysates were cleared by centrifugation at 5,000 rpm for 10 min and supernatant fractions were incubated overnight at

4°C with 1:100 dilution of anti-M1 receptor antibodies, (Santa Cruz Biotechnology). The following day 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added to each sample and samples were incubated for an additional 1 hr incubation at 4°C. The immune complexes were washed three times with lysis buffer and resuspended in 2X SDS sample buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and boiled for 5 min. A Western blot was performed as described below. This procedure was developed by JuYoung Kim and David Saffen, Ohio State University, Columbus, OH.

Western Blot. Proteins were separated by standard SDS-polyacrylamide gel electrophoresis (10% acrylamide resolving gel) and were electrophoretically transferred to polyvinylidene difluoride membranes (0.2 µm, Biorad). After the transfer, membranes were blocked for 2 hr at room temperature with TNT buffer (20 mM Tris, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) containing 5% powdered skim milk. The membranes were then exposed to anti-CaM antibody (1:1000 dilution, Amersham-Pharmacia) in blocking buffer overnight at 4 °C. The membranes were washed three-times with TNT buffer and incubated in buffer containing anti-mouse IgG antibodies cross-linked with horseradish peroxidase (Promega; 1:2500 dilution) for 2 hrs at room temperature. Membranes were washed three-times, and proteins were visualized by enhanced chemiluminescence (ECL, Pierce Biotechnology).

RESULTS AND DISCUSSION

Effect on gene expression of basal (unstimulated) activity of hM1 wild-type and mutant receptors carrying mutated sequences defective in CaM binding. To determine if there are differences in the ability of wild type and mutants deficient in CaM binding to transduce signal at basal levels, the gene expression profile of HEK-293 cells expressing wild type or mutant hM1 mAchR was examined. RNA was extracted from cells, reverse transcribed to cDNA, coupled to either green (control) or red (experiment) dye, and hybridized onto a custom-designed microarray chip. This chip contains mostly ion channels and transporters (Anderle et al., 2003). For each microarray chip, the control is from untransfected HEK-293 cells and the experiment was from wild type, Δ232-358-hM1 or E360A-hM1 expressing cells. Dye swaps were done, and only genes with complimentary changes in both regular and dye-swapped chips are shown (Table 3-1).

CHRM1, since it was transfected into HEK-293 cells, was expected to be up regulated in all three cell lines. This was observed with wild type and E360A-transfected cells. However, significant up-regulation was not seen in cells expressing Δ232-358. Radioligand binding studies with [^{3}H] N-methylscopolamine, a cell-impermeable muscarinic antagonist, show all three cell lines express receptor at similar levels. It is possible that the Δ232-358-hM1 cDNA does not bind well to the oligo on the microarray, perhaps because it is missing a large piece of the i3 loop.

Three genes were down regulated in both mutants, but not in wild-type (hM1) expressing cells. These genes are *SCN3A, CLCA4* and *T1A-2*. *T1A-2* encodes the lung type-1 membrane associated glycoprotein, which is not well characterized. It has been proposed as

Gene name	Protein Name	Change in Expression (fold over non-transfected cells)		
		M1	Δ232-358	E360A
CHRM1	Muscarinic acetylcholine receptor, type 1	**6.0±1.19**	1.4±0.22	**3.7±0.80**
SCN3A	Sodium channel, voltage-gated, type 3α	1.0±0.05	**-1.7±0.36**	**-3.2±0.74**
CLCA4	Calcium-activated chloride channel, 4	1.1±0.09	**-1.5±0.06**	**-1.9±0.12**
AQP9	Aquaporin 9	1.1±0.06	1.1±0.10	**2.0±0.35**
T1A-2	Lung cell membrane-associated glycoprotein, type 1	1.1±0.06	**-1.7±0.11**	**-1.8±0.21**
SLC21A14	Solute carrier family 21 (organic anion transporter), member 14	1.0±0.06	-1.2±0.12	**1.8±0.30**
SCN11A	Sodium channel, voltage-gated, type 11α	1.3±0.17	-1.2±0.12	**-1.6±0.30**

Table 3-1. Changes in gene expression of cells transfected with either hM1, E360A-M1 or Δ232-358-M1 mAchR vs. nontransfected HEK-293 cells. A custom oligonucleotide microarray, containing approximately 1000 human genes, most of whch are transporters and ion channels, and in which every gene was printed four times per slide, was used. Dye swaps were done for all experiments. Shown are the median values plus or minus SD.

a marker of lung injury and has been shown to induce platelet aggregation (Zimmer et al., 1999; Kato et al., 2003). No association of this gene with mAchR had been reported previously.

The proteins that are encoded by the other two genes, *SCN3A* and *CLCA4*, are regulated by both CaM and hM1 mAchR. *CLCA4*, which encodes the Ca^{2+} activated Cl^- channel, type 4, is the only calcium-activated chloride channel to be expressed in the brain (Agnel et al., 1999; Pauli et al., 2000). CLCA channel inactivation is mediated by phosphorylation via CaM and CaMKII (Wang and Kotlikoff, 1997; Kotlikoff and Wang, 1998). Muscarinic acetylcholine receptors activate Ca^{2+} activated Cl^- current ($I_{Cl(Ca)}$). Carbachol-induced muscarinic activation of $I_{Cl(Ca)}$ has been shown in a wide variety of cell types but the mechanism, including possible CaM involvement, behind the $I_{Cl(Ca)}$ activation is unclear (Janssen and Sims, 1992; Pacaud et al., 1992; Janssen, 1996; Liu and Farley, 1996; Wayman et al., 1997).

SCN3A encodes the α subunit of the voltage-gated sodium channel, type III. Another gene, *SCN11A*, which encodes the α subunit of the type XI voltage-gated sodium channel, was down regulated in the E360A cells but not in either wild type or Δ232-358 expressing cells. Voltage-gated sodium channels are involved in the generation of action potentials in excitable cells. The α subunit is the pore-forming component and is responsible for voltage-sensitive gating and ion channel permeation (Mori et al., 2000; Deschenes et al., 2002). Activation of M1 mAchR modulates Na^+ channel activity by activation of PKC. PKC phosphorylates the channel to inactivate it (Cantrell et al., 1996). Like other voltage-gated sodium channels, both type III and type XI contain an IQ CaM-binding motif (Mori et al., 2000). CaM has been shown to bind to voltage-gated sodium channels in a Ca^{2+}-dependent

manner. This results in a shift of the steady-state inactivation curve in the hyperpolarizing direction (Mori et al., 2000).

Two genes were up regulated in the E360A expressing cells, but not in wild type or Δ232-358 cells. These genes are *AQP9,* which encodes aquaporin 9, a neutral solute channel, and *SLC21A14*, which is a member of the organic anion transporter family (Tsukaguchi et al., 1999; Pizzagalli et al., 2002). Muscarinic receptors have not been previously shown to regulate either gene.

Altogether, these results show that there are differences in the ability of mutant and wild-type muscarinic receptor to regulate gene expression at basal levels. The wild-type receptor did not cause changes in gene expression. E360A-hM1, which has been reported as having basal activity, had the most changes in gene expression. If CaM regulates the basal activity of the M1 mAchR receptor, mutants deficient in CaM binding would be expected to have increased basal signaling. The microarray results are consistent with this hypothesis, since gene expression changes were observed only in the mutant mAchRs, which are deficient in CaM binding. Two of these genes, *CLCA4* and *SCN3A*, which are down regulated in both mutants but not in wild type, encode ion channels that are regulated by both M1 mAchR and CaM. Further studies need to be done to determine if these are signaling pathways activated through a direct hM1-CaM interaction..

Effect of potential CaM-hM1 interaction on carbachol-activated gene expression. Cells expressing wild type or mutant hM1 were also analyzed to determine if they had different gene expression patterns when activated by the agonist carbachol. For each chip, the control (green-labeled) was cDNA from cells expressing wild type, Δ232-358-

hM1 or E360A-hM1 and the experiment (red-labeled) was cDNA from the same cell line as control, but treated with 100 μM carbachol for 24 hrs prior to RNA extraction. As above, dye-swaps were done for all experiments. Results are shown in Table 3-2. Several gene changes were observed; these are discussed in further detail below.

CHRM1 was up regulated in carbachol-treated wild type and Δ232-358 cells. Carbachol-induced stimulation of mAchR mRNA has been previously reported, and has been tied to mAchR induction of nitric oxide synthase through calcium/CaM and PKC (Chou et al., 1993; Sterin-Borda et al., 2003).

CYR61 was up regulated in all three cell lines. *CYR61* is an immediate early gene, which encodes a secretory growth regulatory protein. Carbachol stimulated induction of *CYR61* has been previously reported (Albrecht et al., 2000). M1 induction of *CYR61* gene expression is through a pathway involving PKC and an increase in intracellular calcium levels. Muscarinic acetylcholine receptors are known to be involved in learning and memory processes through the activation of immediate early genes and promote the generation of long-term potentiation (Albrecht et al., 2000; Lin et al., 2002).

ADAMTS1 was up regulated in both wild type and Δ232-358 cells, but not in E360A expressing cells. It is a disintegrin and metalloproteinase (ADAM) family member, with three thrombospondin type I motifs at its C-terminus, which permits it to be incorporated into the extracellular matrix. Studies with *ADAMTS1* null mice demonstrate that ADAMTS1 is essential for normal growth, fertility and organ morphology and function (Shindo et al., 2000). Although no studies have shown an involvement of hM1 mAchR or CaM with ADAMTS1, mAchR have been shown to regulate other ADAM family members. This

Gene name	Protein Name	Change in Expression (fold over untreated cells)		
		M1	Δ232-358	E360A
CHRM1	Muscarinic acetylcholine receptor, type 1	**1.7±0.19**	**2.8±0.28**	1.1±0.09
CYR61	Cysteine-rich heparin-binding protein, 61	**1.7±0.19**	**2.4±0.28**	**1.5±0.16**
ADAMTS1	A disintegrin and metalloprotease with thrombospondin type 1 motif, 1	**1.6±0.19**	**2.0±0.06**	1.0±0.06
NRP1	Neuropilin-1	-1.3±0.09	**-1.8±0.15**	-1.1±0.12
ATP1B3	ATPase, N+/K+ transportor, β3 subunit	1.3±0.12	**1.6±0.07**	1.1±0.09
CTGF	Connective tissue growth factor	1.4±0.23	**1.5±0.23**	1.1±0.20

Table 3-2. Changes in gene expression of carbachol-treated hM1, E360A-M1 or Δ232-358-M1 mAchR transfected cells vs. untreated cells expressing the same receptor. Cells were treated with 100μM carbachol 24 hrs prior to mRNA extraction. As in Table 1, a custom oligonucleotide microarray was used. Dye swaps were done for all experiments. Shown are the median values plus or minus SD.

includes the carbachol-stimulated activation of ADAM17, ADAM10 and ADAM9 (Slack et al., 2001; Webster et al., 2002; Allinson et al., 2003).

One gene, *NRP1*, was down regulated in Δ232-358 cells but not in wild type or E360A cells. *NRP1* encodes for neuropilin-1, which has been shown to be involved in many cell activities: a receptor for VEGF-induced angiogenesis, a semaphorin receptor, essential for initiating the primary immune response and its overexpression is associated with several types of cancer, including melanoma, glioma, breast, and colon adenocarcinoma (Bagri and Tessier-Lavigne, 2002; Klagsbrun et al., 2002; Nakamura and Goshima, 2002; Neufeld et al., 2002; Puschel, 2002; Romeo et al., 2002). Regulation of this gene by mAchR has not been previously reported.

Two genes, *ATP1B3* and *CTGF*, were up regulated in Δ232-358 cells but not in either E360A or wild type. *ATP1B3* encodes the β-3 subunit of a Na^+/K^+ ATPase, a class of proteins responsible for establishing and maintaining the electrochemical gradient of K^+ and Na^+ ions across the plasma membrane (Malik et al., 1996). Regulation of this gene by mAchR has not been previously reported. *CTGF* encodes a connective tissue growth factor, which belongs to the same family as *CYR61*. Previous studies have shown that wild-type hM1 mAchR does not up-regulate *CTGF* (Albrecht et al., 2000), which is consistent with our results.

The results from the untreated vs. carbachol-treated microarray confirm previous studies that showed that *CHRM1* and *CYR61* are up regulated by carbachol in wild-type hM1 mAchR-expressing cells. One or both of these genes were also up-regulated in cells expressing one of the mutant mAchR, indicating that the mutants are able to activate at least some of the same signaling pathways as wild-type. In addition, *ADAMTS1* has been

identified as a new gene whose activity is regulated by carbachol-stimulated hM1 mAchR. Several gene changes were also observed in cells expressing Δ232-358, but not in cells expressing either wild type or E360A. Δ232-358 is known to be deficient in internalization (Maeda et al., 1990b), in contrast to the other constructs. This could cause downstream changes, and result in an augmented ability to regulate carbachol-induced gene transcription.

[^{35}S]GTPγS binding experiments. Since the location of the CaM binding site on the hM1 i3 loop is in a region known to be critical for G-protein coupling, the effect of CaM on [^{35}S]GTPγS binding, a measurement of G-protein coupling was examined. Membranes from HEK-293 cells stably expressing hM1 or empty vector (mock-transfected) were used for these studies. [^{35}S]GTPγS incorporation was measured at basal or increasing concentration of the agonist carbachol. This was compared to the [^{35}S]GTPγS incorporation in membranes in which CaM was added. As can be seen in Figure 3-1, addition of CaM significantly inhibits [^{35}S]GTPγS binding in membranes expressing the hM1 receptor. This is true for basal and sub-maximal doses of carbachol. CaM has no effect on high does of carbachol yielding a maximal [^{35}S]GTPγS response. CaM is able to inhibit the [^{35}S]GTPγS binding of hM1 mAchR down to the same level as background (as seen by the mock-transfected [^{35}S]GTPγS incorporation).

When the membranes are treated with the muscarinic antagonist atropine, the ability of CaM to inhibt [^{35}S]GTPγS incorporation is eliminated, indicating that this is an effect specific to hM1 (Figure 3-2a). Atropine has been previously reported to be an inverse agonist. However, that was not observed under the conditions used in this study (this is difficult to understand). In addition, when membranes from cells stably expressing the hM1

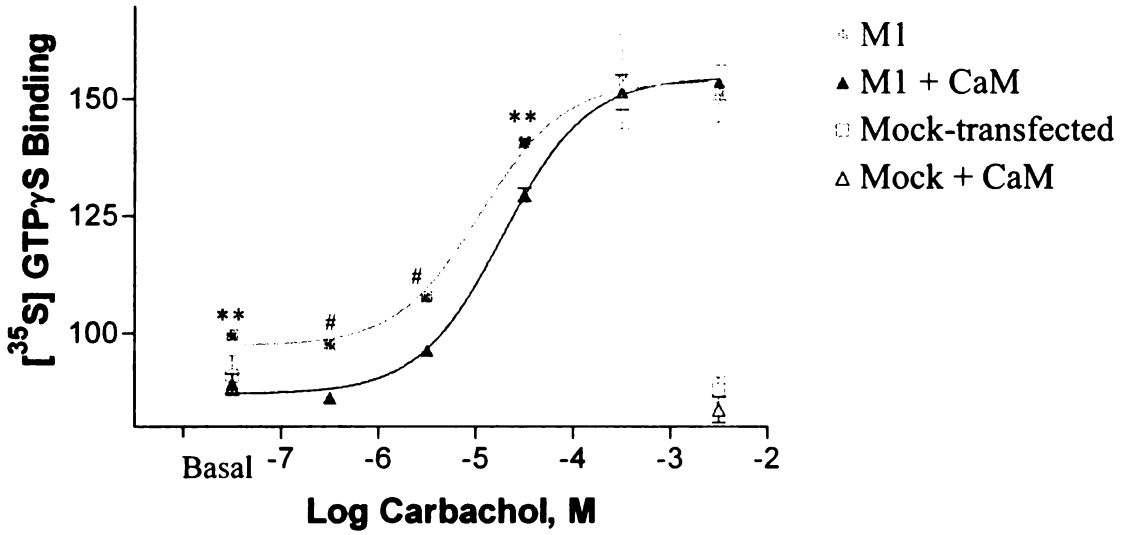


Figure 3-1. CaM inhibits hM1 mAchR [^{35}S]GTPγS binding. Membranes from cells expressing the hM1 receptor or vector only (mock-transfected) were treated with various concentrations of carbachol with and without CaM. CaM was able to inhibit hM1 mAchR [^{35}S]GTPγS binding down to background (mock-transfected) levels at basal and sub-maximal concentrations of carbachol. Values are the mean of 3-4 experiments done in triplicate ± SD. **$p < 0.01$, #$p < 0.001$, versus M1 + CaM of the same drug concentration (unpaired two-tailed Student's t-test). Values are expressed as percent of M1 [^{35}S]GTPγS incorporation.

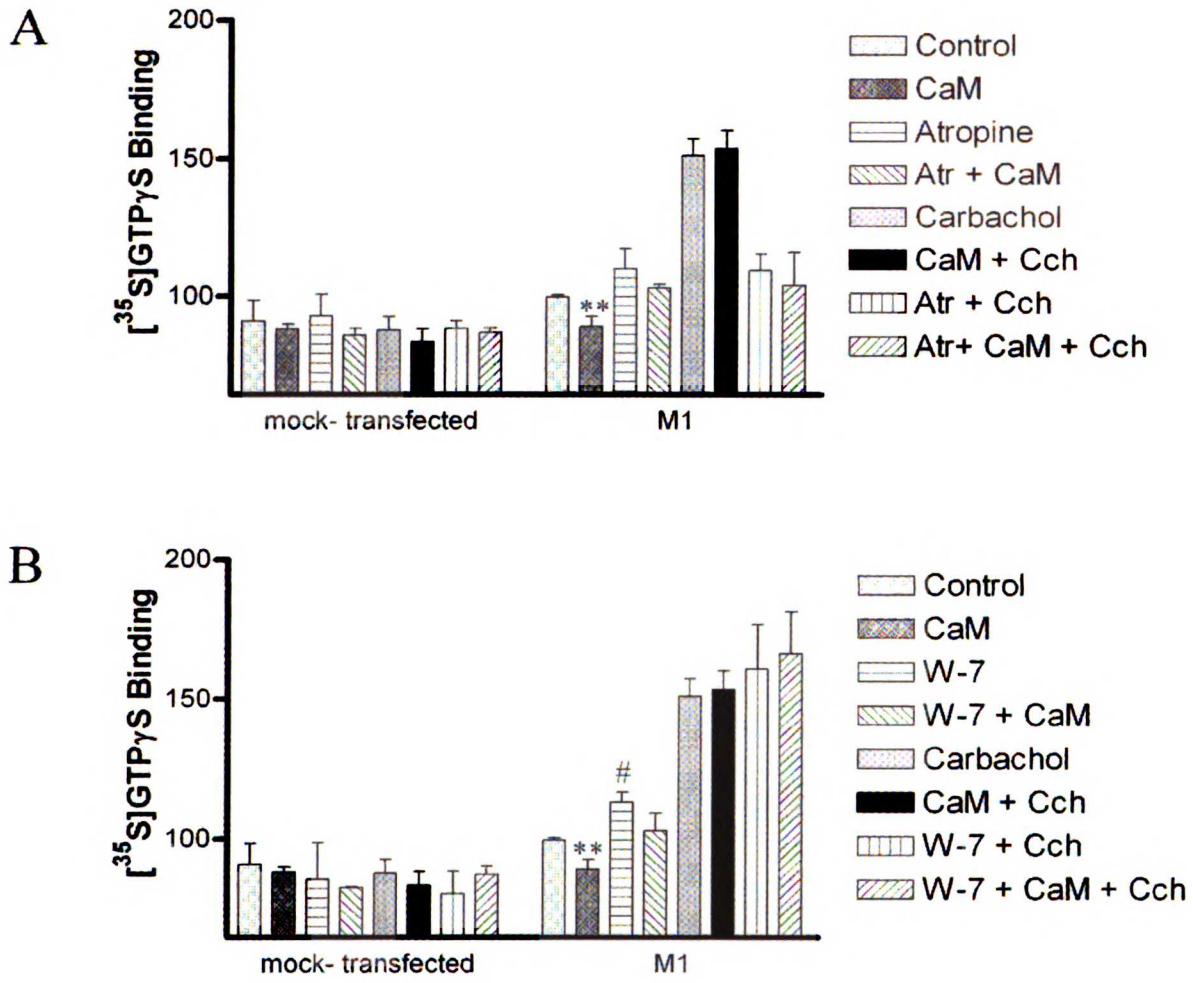


Figure 3-2. A. Effect of the muscarinic inhibitor atropine on hM1 mAchR [^{35}S]GTPγS binding. Concurrent treatment of membranes with atropine prevents CaM from inhibiting hM1 mAchR [^{35}S]GTPγS binding. **B.** Effect of the CaM inhibitor W-7 on hM1 mAchR [^{35}S]GTPγS binding. Treatment of membranes with W-7 alone slightly but significantly increases basal hM1 mAchR [^{35}S]GTPγS binding. In addition, W-7 reverses CaM ability to inhibit hM1 mAchR [^{35}S]GTPγS binding. Values are the mean of 2-4 experiments done in triplicate ± SD. **$p < 0.01$, #$p < 0.005$, versus M1 control membranes (unpaired two-tailed Student's t-test). Values are expressed as percent of M1 [^{35}S]GTPγS incorporation.

mAchR are treated with the CaM antagonist W-7, CaM no longer inhibits [^{35}S]GTPγS binding (Figure 3-2b). Also, treatment of membranes with W-7 alone yields a modest but significant increase in [^{35}S]GTPγS incorporation. Therefore, CaM appears to regulates M1 receptor G-protein coupling as measured by [^{35}S]GTPγS binding.

CaM binding to the i3 loop inhibits G-protein coupling of the μ opioid receptor and the D2 dopamine receptor (Wang et al., 1999; Bofill-Cardona et al., 2000). The current studies suggest that CaM is able to regulate hM1 G-protein coupling. Further studies need to be done in order to understand the role that CaM plays in the regulation of hM1 G-protein coupling. These include determining the effect that CaM has on the [^{35}S]GTPγS incorporation of E360A and Δ232-358, the two mutants deficient in CaM binding. Nevertheless, this result supports the hypothesis that CaM and G-protein binding sites are similar and that CaM therefore inhibits the G-protein coupling of many GPCRs.

Co-immunoprecipitation. A Co-IP of M1 and CaM in PC12D cells, which natively express high levels of M1, was also carried out. Samples were immunoprecipitated with anti-M1 antibody, and blotted with anti-CaM. The results of this co-IP were negative (Figure 3-3). No bands matching that of the CaM control were present in any of the conditions. I also tried the co-IP using a crosslinker, and in the presence of calcium or EGTA, with similar results. This could mean that CaM does not bind, or that it dissociates in the process, or there was something wrong with the assay. The M1 receptor is known to be very unstable and easily denatured when extracted from cells. Further studies need to be done to confirm that CaM binds the hM1 mAchR in cells.

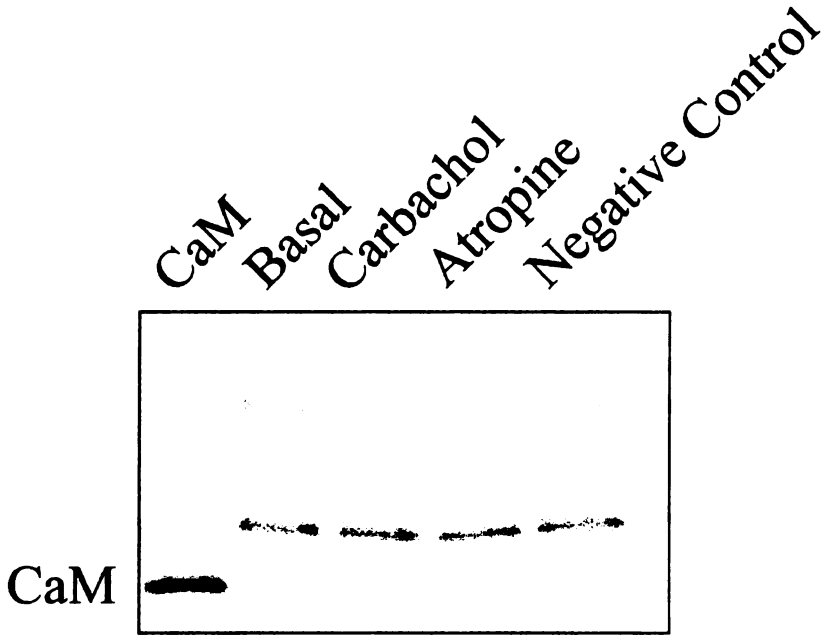


Figure 3-3. Co-immunoprecipitation. PC12D cells, which express native M1, were immunoprecipitated with anti-M1, and blotted with anti-CaM. For the negative control, I used untransfected HEK-293 cells, which express low levels of M1. The results of this co-IP were negative. No bands matching that of the CaM control were present in any of the conditions. I also tried the co-IP using a crosslinker, and in the presence of calcium or EGTA, with similar results.

CONCLUSION

In summary, evidence is presented that supports the hypothesis that CaM, through a direct interaction on the i3 loop, regulates M1 muscarinic receptor signal transduction. When no drug is present, gene expression changes are observed in mutant, but not wild type, expressing HEK-293 cells. Two of these genes, *CLCA4* and *SCN3A*, encode ion channels

that are regulated by both CaM and muscarinic receptors. The heightened ability of mutants deficient in CaM binding to regulate gene expression in the absence of agonist supports previous data reporting that E360A-hM1 is constitutively active. Previous work in this lab has established that CaM regulates the basal activity of MOR. It is possible that it also regulates the basal activity of the hM1 receptor. In this report, CaM was also shown to regulate hM1 G protein coupling, although more work needs to be done to determine if there is an effect on basal activity. Altogether, while these data are incomplete, they do suggest a role for a direct CaM interaction in regulating M1 receptor signaling, and provide a foundation for further study.

Chapter 4

Single Nucleotide Polymorphisms of the Human M1 Muscarinic Acetylcholine Receptor Gene

Previously published: Julie L. Lucas, Joseph A. DeYoung and Wolfgang Sadée. AAPS PharmSci 2001; 3(4) article 31

SUMMARY

The gene encoding the human muscarinic receptor, type 1 (*CHRM1*), was genotyped from 245 samples of the Coriell Collection (Coriell Institute for Medical Research, Camden, NJ). Fifteen single nucleotide polymorphisms (SNPs) were discovered, 9 of which are located in the coding region of the receptor. Of these, 8 represent synonymous SNPs, indicating that *CHRM1* is highly conserved in humans. Only a single allele was found to contain a nonsynonymous SNP, which encodes an amino acid change of Cys to Arg at position 417. This may have functional consequences because a C417S point mutation in rat M1 was previously shown to affect receptor binding and coupling. Furthermore, none of the four *CHRM1* SNPs previously deduced from sequencing of the human genome were found in this study despite a prediction that a majority of such inferred SNPs are accurate. The consensus sequence of *CHRM1* obtained in our study differs from the deposited reference sequence (AC NM_000738) in 2 adjacent nucleotides, leading to a V173M change, suggesting a sequencing error in the reference sequence. The extraordinary sequence

INTRODUCTION

The muscarinic receptor family, of which there are 5 subtypes (M1-M5), plays an important functional role throughout the body. Muscarinic receptors are involved in a variety of cell- type specific signaling pathways. These include the regulation of cyclic adenosine monophosphate concentration in the cell, activation of tyrosine and mitogen-activated kinases, and the regulation of ion channel function in the cell (Nathanson, 2000).

The muscarinic receptors have a broad and overlapping distribution in the body. The M1, M2, and M4 receptors are the most abundant. The M1 receptor is found in the hippocampal and cortical regions of the brain as well as in the parasympathetic ganglia (Dorje et al., 1991; Levey et al., 1991; Felder et al., 2001). The M1 receptor is involved in many processes, including the initiation of seizures, learning and memory, and regulation of the force and rate of heart contractions (Hamilton et al., 1998; Hamilton et al., 2001). Because of its involvement in these processes, the M1 receptor is a compelling drug target for Alzheimer's disease and other neurological and psychiatric disorders (Hamilton et al., 1998). Indeed, for the past 20 years, the cholinergic hypothesis has proposed that loss of cholinergic function is responsible for the cognitive symptoms of Alzheimer's (Bartus, 2000). It is thought that stimulation of the M1 receptor would alleviate some of the symptoms of Alzheimer's by several pathways, including increased secretion of the nontoxic α-amyloid peptide and decreased secretion of the toxic ß-amyloids generated from the amyloid precursor protein (Fisher et al., 1998; Fisher et al., 2000).

The M1 receptor is a member of the G-protein coupled receptor (GPCR) superfamily. Many GPCRs have been found to contain single nucleotide polymorphisms (SNPs) that are

involved in disease susceptibility or drug response (for a review, see Sadée et al., 2001). The R16G allele in the ß2 adrenergic receptor, for example, is associated with nocturnal asthma. Several of the serotonergic receptors have alleles associated with psychotic symptoms in Alzheimer's disease, whereas certain haplotypes of the μ opioid receptor (MOR) have been associated with substance abuse (Sadee et al., 2001). Although the muscarinic receptors are thought to contribute to certain neurological and psychiatric disorders, no sequence variations or alleles have been found that affect susceptibility to these diseases or response to muscarinic agonists.

Variations in the MOR sequence have also been found to disrupt CaM binding. A rare polymorphism, S268P is deficient in CaM binding but can still couple to G-proteins. Another polymorphism, R265H, is deficient in both CaM binding and G-protein coupling. Carriers of these alleles might show an altered response to analgesics (Wang et al., 2001). One objective of this study was to identify any polymorphisms on the hM1 i3 loop that could disrupt CaM binding. In this study we have genotyped the *CHRM1* gene-encoding human M1 receptor in 245 individuals of the Coriell Collection.

MATERIALS AND METHODS

Genotyping of *CHRM1* was done as described in Wang et al (2001). Briefly, the genomic DNA from 247 individuals was amplified by polymerase chain reaction (PCR) (Applied Biosystems Gene Amp PCR System 9700, Foster City, CA) using the following primers: F1 5'GAGGAAGCCCTGTAGCG; R3 5'GATCACCACTTCGGAGCC; F2 5'AGCTCTGATGATCGGCCT; R1 5'CCAAGGAATACTTAATGTTAAGCCT. They were

then sequenced on an Applied Biosystems 3700 DNA analyzer with a 3700 POP-6 polymer matrix. The resulting sequence tracings were then analyzed using Sequencher 4.0 (Gene Code Corp., Ann Arbor, MI). Haplotype analysis was done using an expectation-maximum algorithm (Fallin et al., 2001). Sequence alignments were done using the sequence alignment program Clustal W, which is freely available.

RESULTS AND DISCUSSION

The hM1 receptor gene (*CHRM1*) resides on chromosome 11. *CHRM1* consists of 1 large exon containing the entire coding region of the hM1 receptor. A promoter region and 3 noncoding exons of *CHRM1* based on similarity to the rat gene have been reported approximately 14 kilobases (kb) upstream of the hM1 coding region (Klett and Bonner, 1999). However, a Basic Local Alignment Search Tool (BLAST) search of these sequences against the human genome sequence showed these DNA segments to be 213 kb upstream of *CHRM1*. The promoter region and noncoding exons were not sequenced in this study.

CHRM1 was sequenced from 247 samples of the Coriell Collection. The samples used are ethnically diverse as follows: 100 Caucasians, 100 African Americans, 30 Asians, 10 Hispanics, and 7 Pacific Islanders. Fifteen SNPs were discovered, 9 of which are located in the coding region of the receptor (Table 4-1, Figure 4-1). Eight of these are synonymous (ie, leading to no change in the protein sequence). The single nonsynonymous SNP encodes an amino acid change of Cys to Arg at position 417, which is located in transmembrane domain VII (Figure 4-1). This SNP was found in only 1 heterozygous individual. Despite the

Figure 4-1. Hm1 Receptor SNPs

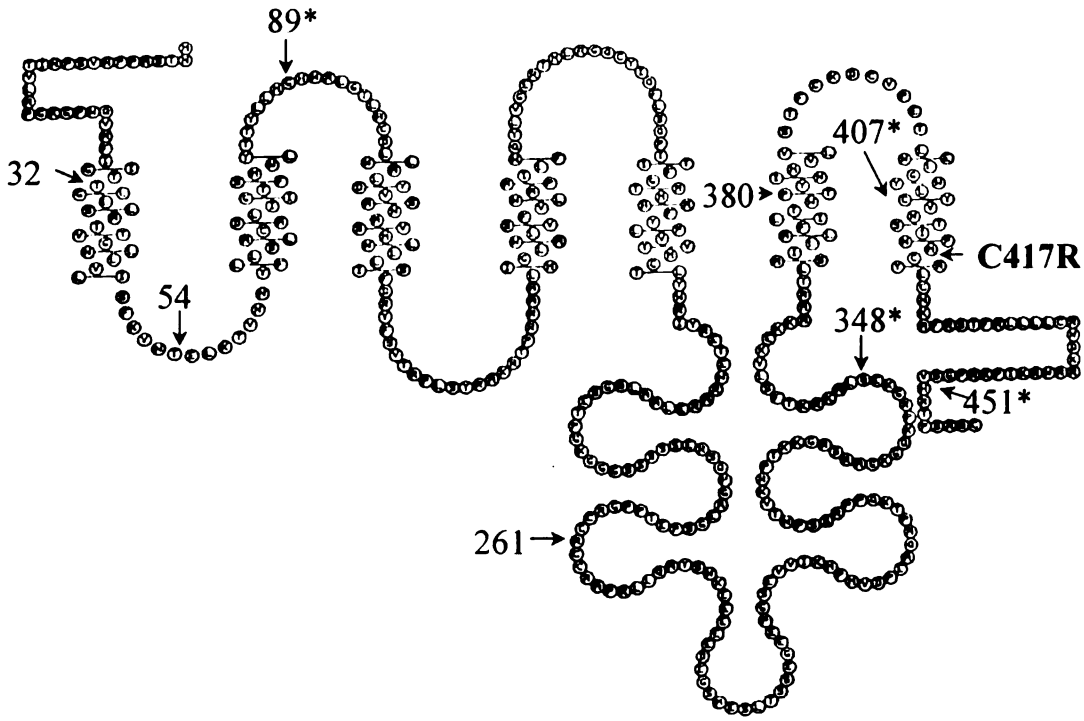


Figure 4-1. Schematic showing location of single nucleotide polymorphisms (SNPs) on the hM1 receptor. C417R, the nonsynonymous SNP, is shown in bold. SNPs with a frequency > 0.05 are marked with an asterisk (*).

SNP	Base Position	Codon	Amino Acid	Total Frequency (n=490)	CA (n = 200)	AA (n = 198)	AS (n = 58)	HIS (n = 20)	PA (n = 14)
1	96	ACG → ACA	T32	0.002	0.005	0	0	0	0
2	162	ACG → ACA	T54	0.002	0	0.005	0	0	0
3	267	GGC → GGA	G89	0.053	0.050	0.020	0.121	0.050	0.286
4	783	CGC → CGT	R261	0.004	0.005	0.005	0	0	0
5	1044	CAG → CAA	Q348	0.080	0.045	0.131	0.017	0.050	0.143
6	1140	CCG → CCA	P380	0.002	0	0.005	0	0	0
7	1221	TGC → TGT	C407	0.120	0.165	0.116	0	0.150	0
8	1249	TGC → CGC	**C417R**	0.002	0	0.005	0	0	0
9	1353	TCC → TCT	S451	0.055	0.055	0.020	0.121	0.050	0.286
10	Exon+2	A → G		0.039	0.01	0.086	0	0	0
11	Exon+13	C → A		0.035	0.005	0.081	0	0	0
12	Exon+171	C → T		0.002	0.005	0	0	0	0
13	Exon+221	C → A		0.002	0.005	0	0	0	0
14	Exon+222	T → G		0.002	0.005	0	0	0	0
15	Exon+299	T → C		0.002	0	0.005	0	0	0

Table 4-1. Single Nucleotide Polymorphisms (SNPs) of the human muscarini receptor, tyoe 1. CA indicates Caucasian; AA, African American; AS, Asian; HIS, Hispanic; PA, Pacific Islander

high amino acid sequence conservation, 43% of individuals had at least 1 variation in their DNA sequence. The results indicate that the hM1 receptor is highly conserved in humans.

The *CHRM1* -hM1 sequence conservation extends to other mammalian species as well. The DNA sequences of rhesus monkey, rat, and mouse (AC: AF026262, S7397.1, NM_007698.1, respectively) were 98.4%, 91.6%, and 91.4% identical as compared to the consensus sequence obtained from the *CHRM1* genotyping. The protein sequences (AC:

P56489, P08482, NP_031724.1, respectively) were 99.6%, 98.7%, and 98.0% identical. Most of the variations in the DNA sequence were in the "wobble" site of the codon, resulting in conservation of the amino acid residue. This indicates that there is evolutionary pressure for the protein sequences to remain conserved. Furthermore, sequence alignments with the human sequences of the other muscarinic receptors and several animal species indicate that the entire family is conserved across species, but the M1 receptor is the most highly conserved.

Although the high conservation of the M1 gene in humans and mammals emphasizes its importance, the M1 gene is not essential for survival. A knockout of the M1 receptor in mice is not lethal; the M1-deficient mouse strain displays no significant differences in body weight, longevity, fertility, or overt behavior compared with wild-type mice (Hamilton et al., 2001). There is suggestive evidence that the single nonsynonymous variation, C417R, may exhibit a change in M1 receptor function (Savarese et al., 1992). Cys 417, which is in transmembrane helix VII, is highly conserved; it is present in all 5 muscarinic subtypes in humans and all other species analyzed, including rhesus monkey, rat, mouse, chimpanzee, pig, and chicken. Cys 417 has been shown to influence receptor-ligand interactions. Previous mutagenesis studies with rat M1 have demonstrated that mutating Cys 417 to Ser resulted in increased receptor affinity to carbachol, a muscarinic agonist, and to several antagonists. The C417S mutation also caused a shift to the left in the carbachol dose response curve for phosphoinositol hydrolysis, resulting in an EC50 value 13-fold lower than wild type (Savarese et al., 1992). Cys 417 therefore has a critical role in M1 receptor function. Because a Cys to Ser change is also more conservative than the Cys to Arg change encoded by a human allele, it is likely that this SNP will affect M1 receptor function and may warrant

Haplotype*	Base Position	Overall Frequency	Chi-Square	Approximate p-value
AAAAAA	Wild-type	0.725	253.2	*p<.000001*
ABAAAA	1044	0.042	29.7	*p<.000001*
AABAAA	1221	0.105	79.2	*p<.000001*
AAAABA	Exon+2	0.006	4.7	*p<.05*
AAAAAB	Exon+13	0.028	19.0	*p<.0001*
BAABAA	267 1353	0.044	31.3	*p<.000001*
ABAABA	1044 Exon+2	0.028	19.3	*p<.0001*
AABAAB	1221 Exon+13	0.006	4.0	*p<.05*

Table 4-2. Haplotypes of the human muscarinic receptor, type 1. *Haplotypes were done using an expectation maximum algorithm. Only single nucleotide polymorphisms with a frequency greater than 1% were analyzed.

further study, despite its low frequency (1:490 alleles). Due to its location in TM7, it is unlikely that C417R will affect CaM binding to hM1.

A haplotype analysis of *CHRM1* SNPs was also done using an expectation-maximum algorithm. Haplotype analysis serves to characterize linkage disequilibrium in various populations (Reich et al., 2001; Stephens et al., 2001) and has the potential to identify disease-predisposing alleles in a population (Fallin et al., 2001). Only hM1 SNPs with a

frequency greater than 1% were analyzed. Of the 15 SNPs found in the hM1 receptor, 6 fit this category, resulting in 8 distinct haplotypes (Table 4-2). Of these, by far the predominant one was wild type; 4 contained 1 SNP, and 3 involved 2 SNPs. This result could serve as a basis for studying possible disease association and may also involve SNPs adjacent to the *CHRM1* gene.

The recent publication of the human genome sequence has brought to light many SNPs (Venter et al., 2001). There are 8 SNPs reported in the genomic sequence of *CHRM1* (AC XM_006058). Of these, 4 SNPs were found as a result of the genome sequencing project and 4 SNPs were recently added to the accession sequence on the NCBI (National Center for Biotechnology Information) website. These were obtained from samples of 100 individuals, without frequency distribution provided. None of the 4 SNPs found as a result of sequencing for the human genome project were found in either study involving multiple samples, whereas all 4 of the SNPs identified in the alternate *CHRM1* sequencing study were found. Failure to reproduce the 4 genomic SNPs is surprising because it has been previously reported that 85% of SNPs inferred from the human genome project are correct (Venter et al. 2001). The consensus sequence obtained from this study also differed from the reference sequence for hM1 (AC NM_000738). The purpose of the NCBI reference sequences is to provide sequence standards for chromosomes, mRNA and proteins for use in mutation analysis, gene expression discovery, and polymorphism discovery and therefore should reflect the wild-type sequence of a particular gene. At base pairs 516 and 517, a GA in our consensus sequence was an AG in the reference sequence. This resulted in a Val to Met amino acid change at position 173. The V173M variation was not observed in this study, so it is either a very rare allele or (more likely) a sequencing error.

CONCLUSION

The results of genotyping the coding region of *CHRM1* reveal that it is highly conserved in humans. Only a single nonsynonymous SNP, C417R, was found in a single heterozygote with potential consequences for receptor function. Even though the allele is rare, it may contribute to pathophysiology in some ethnic populations since fewer chromosomes were sequenced in these populations. The promoter region and other regulatory elements were not sequenced in this study. It is therefore possible that additional polymorphisms may be found in the regulatory elements of *CHRM1*. Polymorphisms in this region could influence receptor expression, thereby affecting drug response or disease susceptibility.

Chapter 5

Conclusions and Future Perspectives

This study describes an interaction between a G protein couple receptor (GPCR), the hM1 muscarinic receptor and the ubiquitous calcium sensor calmodulin (CaM). A binding site on the i3 loop of hM1 was found and confirmed with peptide studies, residues on hM1 essential for CaM binding have been identified, and the interaction has some functional consequences, especially as pertains to G protein coupling and gene expression. *CHRM1*, which encodes for hM1, was found to be highly conserved in humans. Many questions remain to be answered, most significantly in the context of how the CaM interaction fits into the complexities of GPCR signal transduction. CaM, as described previously, is known to bind five GPCRs besides hM1: the metabotropic glutamate receptors 5 and 7, the 5HT1A receptor, the D2 dopamine receptor and the μ opiod receptor. In three of these receptors, CaM inhibits PKC phosphorylation; in the other two it is shown to interfere with G protein coupling. HM1 is interesting because there is the possibility that CaM may regulate both PKC phosphorylation and G protein coupling.

In Chapter 3, CaM was shown to regulate G protein coupling as measured by [^{35}S]GTPγS binding, but the effect of CaM on the G protein coupling of mutants deficient in CaM binding needs to be determined. CaM-binding to the i3 loop is already known to be able to regulate the G-protein coupling of two GPCRs, the μ opioid receptor (MOR) and the D2 dopamine receptor (Wang et al., 1999; Bofill-Cardona et al., 2000). For the μ opioid

receptor it has been proposed that CaM binding suppresses basal receptor – G protein coupling; upon receptor activation by an agonist, CaM is released and G protein coupling ensues. Whether CaM regulates hM1 basal activity needs to be determined. The E360A hM1 mutant has been found to display increased basal activity (Hogger et al., 1995). If CaM were to regulate basal M1-G protein coupling, one would expect a mutant deficient in CaM-binding to have an increased basal coupling activity. Further study is necessary to establish the role that CaM plays in the regulation of hM1 G-protein coupling.

Binding of CaM to GPCRs can inhibit phosphorylation by protein kinase C (PKC). The 5HT1A receptor binds CaM on the i3 loop, and metabotropic glutamate receptors, types 5 and 7, on the C-terminus (Nakajima et al., 1999; Minakami et al., 1997; Turner et al., 2004). The hM1 receptor has two putative PKC phosphorylation sites on the C-terminal end of the i3 loop, at T354 and S356. PKC has been shown to be able to phosphorylate a peptide containing these residues, indicating a role for PKC in heterologous desensitization (Haga et al., 1996). These sites overlap with the CaM binding site on hM1. A logical next step would be to determine whether CaM is able to inhibit PKC phosphorylation. One could also investigate the possibility that CaM regulates heterologous desensitization in multiple GPCRs, since the requirements for a CaM binding site and a PKC site overlap. A PKC site requires basic residues close to a serine or threonine (Haga et al., 1996), and one CaM binding motif is to regions that have a propensity to form an amphipathixc α-helix (O'Day and Myrc, 2004). Since many GPCRs are regulated by PKC phosphorylation, CaM has the potential to be an important regulator of GPCR desensitization.

Two adjacent sites for CaM binding were identified in Chapter 2, but only one was characterized. One question that has not yet been addressed is the function of the second site,

and what role (if any) two sites have in hM1 signaling. Whether one or both of the adjacent CaM binding sites on hM1 are necessary for the functional role CaM plays in hM1 signaling needs to be determined. Other GPCRs that bind CaM have two CaM binding sites (the 5HT1A receptor and the metabotropic glutamate receptor, type 7), as mentioned in Chapter 2, but the role of each site in the CaM-GPCR interaction has not been addressed.

CaM, after its release from the GPCR, may act as a second messenger. CaM binding to the μ opioid receptor was found to have a biological signaling role: a CaM interaction was implicated in EGFR transactivation and in the translocation of CaM from the cytoplasm to the nucleus (Belcheva et al., 2001; Wang et al., 2000). If this is true for hM1, there are signaling pathways that are activated by the direct CaM interaction and it would follow that G protein coupling is not necessary for activation. To address this possibility, a microarray experiment was done to compare the gene expression profiles of HEK-293 cells expressing either wild type or CaM binding-deficient mutant. This particular study was biased, since the microarray chip used contained mostly transporter and ion channel genes. Nevertheless, gene expression differences were observed (as explained in Chapter 3). These results are a good starting point to examine potential signaling pathways. One could look at these genes to see if a direct hM1-CaM interaction does regulate their expression. Additionally, one could look at the regulation of the proteins that these genes encode. Two genes, *SCN3A* and *CLCA4*, both encode ion channels that were down-regulated in mutant but not in wild type-expressing cells. These ion channels are regulated by both CaM and hM1 (Cantrell et al., 1996; Mori et al., 2000; Wang and Kotlikoff, 1997; Janssen and Sims, 1992; Pacaud et al., 1992). It is possible that hM1 regulates the activity of these ion channels via CaM.

The sequencing of the CHRM1 gene in 245 individuals demonstrated its high conservation in humans, since only a single heterozygote nonsynonymous allele, C417R, was identified. C417R is likely to have functional consequences (Saverese et al). However, any effects this allele would have on disease susceptibility or drug response would affect only a very few individuals; investigating functional consequences of the C417R allele are less compelling than one with higher frequency. The promoter region and other regulatory elements were not sequenced in this study. It is therefore possible that additional polymorphisms may be found in the regulatory elements of *CHRM1*. Polymorphisms in this region could influence receptor expression, thereby affecting drug response or disease susceptibility, perhaps at greater frequency than the single nonsynonymous allele identified in the coding region.

In this study, a novel interaction of CaM with the i3 loop of the hM1 muscarinic receptor, and key residues involved in this interaction were identified. CaM was found to regulate G-protein coupling and mutants deficient in CaM binding had a gene expression profile different from wild type, indicating changes in signaling. Many questions remain to be answered, including better understanding of the CaM regulation of G protein coupling, a possible regulation of PKC phosphorylation, better understanding of the existence of two CaM binding sites on hM1 and using the microarray results as a foundation for the identification of CaM-dependent signaling pathways. This study supports a broader trend suggesting that CaM is a key regulator of GPCR signaling at the receptor level.

WORKS CITED

Agnel, Magali, Thierry Vermat and Jean-Michel Culouscou (1999). "Identification of novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea." FEBS Letters 455: 295-301.

Albrecht, Claudia, Heinz von der Kammer, Manuel Mayhaus, Jaroslav Klaudiny, Michaela Schweizer and Roger M. Nitsch (2000). "Muscarinic acetylcholine receptors induce the expression of the immediate early growth regulatory gene *CYR61*." Journal of Biological Chemistry 275: 28929-28936.

Allinson, Tobias M.J., Edward T. Parkin, Anthony J. Turner and Nigel M. Hooper (2003). "ADAMs Family Members As Amyloid Precursor Protein α-Secretases." Journal of Neuroscience Research 74: 342–352.

Anderle, Pascale, Vera Rakhmanova, Katie Woodford, Noa Zerangue and Wolfgang Sadee (2003). "Messenger RNA expression of transporter and ion channel genes in undifferentiated and differentiated Caco-2 cells compared to human intestines." Pharmaceutical Research 20: 3-15.

Arden, J.R., O. Nagata, Melinda S. Shockley, Mohan Phillip, Jelveh Lameh and Wolfgang Sadee (1992). "Mutational analysis of third cytoplasmic loop domains in G-protein coupling of the hm1 muscarinic receptor." Biochemical and Biophysical research communications 188: 1111-1115.

Bagri, A. and M. Tessier-Lavigne (2002). "Neuropilins as semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance." Advances in Experimental Medical Biology 515: 13-31.

Bartus, R. T. (2000). "On neurodegenerative diseases, models and treatment strategies: Lessons learned and lessons forgotten a generation following the cholinergic hypothesis." Experimental Neurology 163: 495-529.

Belcheva, Mariana M., Maria Szucs, Danxin Wang, Wolfgang Sadee and Carmine J. Coscia (2001). "μ-opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation." Journal of Biological Chemistry 276: 33847-33853.

Bence, Kendra, Wei Ma, Tohru Kozasa and Xin-Yun Huang (1997). "Direct stimulation of Bruton's tyrosine kinase by G_q-protein α-subunit." Nature 389: 296-299.

Bofill-Cardona, Elisa, Oliver Kudlacek, Qiong Yang, Horst Ahorn, Michael Friessmuth and Christine Nanoff (2000). "Binding of calmodulin to the D2-dopamine receptor reduces receptor signaling by arresting the G-protein activation switch." Journal of Biological Chemistry 275: 32672-32680.

Burford, Neil T, Andrew B Tobin and Stefan R Nahorski (1995). "Coupling of muscarinic m1, m2 and m3 acetylcholine receptors, expressed in Chinese hamster ovary cells, to pertussis toxin-sensitive/insensitive guanine nucleotide-binding proteins." European Journal of Pharmacology 289: 343-351.

Cantrell, Angela R., Jenny Y. Ma, Todd Scheuer and William A. Catterall (1996). "Muscarinic modulation of sodium current by activation of protein kinase C in rat hippocampal neurons." Neuron 16: 1019-1026.

Cao, Tracy T., Heather W. Deacon, David Reczek, Anthony Bretscher and Mark von Zastrow (1999). "A kinase-regulated PDZ domain controls endocytic sorting of the β2-adrenergic receptor." Nature 401: 286-290.

Cardy, Thomas J. A. and Colin W. Taylor (1998). "A novel role for calmodulin: Ca^{2+}-independent inhibition of type-1 inositol trisphosphate receptors." Biochemical Journal 334: 447-455.

Choi, Eui-ju, Scott T. Wong, Thomas R. Hinds and Daniel R. Storm (1992). "Calcium and muscarinic agonist stimulation of type I adenylylcyclase in whole cells." Journal of Biological Chemistry 267: 12440-12442.

Chou, Hsi-hsein, Norio Ogawa, Masato Asanuma, Hiroshi Hirata, Yoichi Kondo and Akitane Mori (1993). "Rapid response of striatal muscarinic M1-receptor mRNA to muscarinic cholinergic agents in rat brain." Molecular Brain Research 19: 211-214.

Chuang, Tsu Tshen, Lina Paolucci and Antonio De Blasi (1996). "Inhibition of G protein-coupled receptor kinase subtypes by Ca^{2+}/calmodulin." Journal of Biological Chemistry 271: 28691-28696.

Ciruela, F., M. J. Robbins, A. C. Willis and R. A. J. McIlhinney (1999). "Interactions of the C terminus of metabotropic glutamate receptor type 1α with rat brain proteins: Evidence for a direct interaction with tubulin." Journal of Neurochemistry 72: 346-354.

Cong, Mei, Stephen J. Perry, Liaoyuan A. Hu, Phyllis I. Hanson, Audrey Claing and Robert J. Lefkowitz (2001). "Binding of the β2 adrenergic receptor to *N*-ethylmaleimide-sensitive factor regulates receptor recycling." Journal of Biological Chemistry 276: 45145-45152.

Dasgupta, Maitrayee, Theresa Honeycutt and Donald K. Blumenthal (1989). "The γ-subunit of skeletal muscle phosphorylase kinase contains two noncontiguous domains that act in concert to bind calmodulin." Journal of Biological Chemistry 264: 17156-17163.

Deschenes, Isabelle, Nathalie Neyroud, Deborah DiSilvestre, Eduardo Marban, David T Yue and Gordon F Tomaselli (2002). "Isoform-specific modulation of voltage-gated Na^+ channels by calmodulin." Circulation Research 90: e49-e57.

Ding, Wei-Qun, Christer Larsson and Christer Alling (1998). "Stimulation of muscarinic receptors induces expression of individual *fos* and *jun* genes through different transduction pathways." Journal of Neurochemistry 70: 1722-1729.

Dorje, F, Allan I. Levey and Mark R Brann (1991). " Immunological detection of muscarinic receptor subtype proteins (m1-m5) in rabbit peripheral tissues." Molecular Pharmacology 40: 459-462.

Fallin, Daniele, Annick Cohen, Laurent Essioux, Ilya Chumakov, Marta Blumenfeld, Daniel Cohen and Nicholas J. Schork (2001). "Genetic analysis of case/control data using estimated haplotype frequencies: Application to APOE locus variation and Alzheimer's disease." Genome Research 11: 143-151.

Felder, Christian C. (1995). "Muscarinic acetylcholine receptors: signal transduction through multiple effectors." FASEB Journal 9: 619-625.

Felder, Christian C., Amy C. Porter, Tiffanie L. Skillman, Lu Zhang, Frank P. Bymaster, Neil M. Nathanson, Susan E. Hamilton, Jesus Gomeza, Jurgen Wess and David L. McKinzie (2001). "Elucidating the role of muscarinic receptors in psychosis." Life Sciences 68: 2605-2613.

Felsch, Jason S., Teresa G. Cachero and Ernest G. Peralta (1997). "Activation of protein tyrosine kinase PYK2 by the m1 muscarinic acetylcholine receptor." Proceedings of the National Academy of Sciences 95: 5051-5056.

Fisher, Abraham, Rachel Brandeis, Rachel Haring, Nomi Eshhar, Eliahu Heldman, Yishai Karton, Orli Eisenberg, Haim Meshulam, Daniele Marciano, Nira Bar-Ner. and Zipora Pittel (1998). "Novel m1 muscarinic agonists in treatment and delaying the progression of Alzheimer's disease: an unifying hypothesis." Journal of Physiology (Paris) 92: 337-340.

Fisher, Abraham, DM Michealson, Rachel Brandeis, Rachel Haring, S Chapman and Zipora Pittel (2000). "M1 muscarinic agonists as potential disease-modifying agents in Alzheimer's disease: Rationale and perspectives." Annals of the New York Academy of Sciencesi 920: 315-320.

Gellman, Samuel H. (1991). "On the role of methionine residues in the sequence-independent recognition of nonpolar protein surfaces." Biochemistry 30: 6636-6645.

Guo, Fei-fan, Eiko Kumahara and David Saffen (2001). "A CalDAG-GEFI/Rap1/B-Raf Cassette Couples M1 Muscarinic Acetylcholine Receptors to the Activation of ERK1/2." Journal of Biological Chemistry 276: 25568 - 25581.

Haga, Kazuko, Kimihiko Kameyama, Tatsuya Haga, Ushio Kikkawa, Kazumasa Shiozak and Haruaki Uchiyama (1996). "Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C." Journal of Biological Chemistry 271: 2776-2782.

Hall, Randy A., Lynda S. Ostedgaard, Richard T. Premont, Jeremy T. Blitzer, Nadeem Rahman, Michael J. Welsh and Robert J. Lefkowitz (1998). "A C-terminal motif found in the β2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na^+/H^+ exchanger regulatory factor family of PDZ proteins." Proceedings of the National Academy of Sciences 95: 8496-8501.

Hamilton, Susan E., Michael L. Schlador, Lise A. McKinnon, Renee S. Chmelar and Neil M. Nathanson (1998). "Molecular mechanisms for the regulation of the expression and function of muscarinic acetylcholine receptors." Journal of Physiology (Paris) 92: 275-278.

Hamilton, Susan E., Sandrine N Hardouin, Stephan G Anagnostaras, Geoffrey G. Murphy, Keith N Richmond, Alcino J. Silva, Eric O. Feigl and Neil M. Nathanson (2001). "Alteration of cardiovascular and neuronal function in m1 knockout mice." Life Sciences. 68: 2489-2493.

Hamilton, Susan E. and Neil M. Nathanson (2001). "The M1 receptor is required for muscarinic activation of mitogen-activated protein (MAP) kinase in murine cerebral cortical neurons." Journal of Biological Chemistry 276: 15850-15853.

Haring, Rachel, Abraham Fisher, Daniele Marciano, Zipora Pittel, Yoel Kloog, Avi Zuckerman, Nomi Eshar and Eliahu Heldman (1998). "Mitogen-activated protein kinase-dependent and protein kinase C-dependent pathways link the m1 muscarinic receptor to β-amyloid precursor protein secretion." Journal of Neurochemistry 71: 2094-2103.

Higashijima, Tsutomu, Sonoko Uzu, Terumi Nakajima and Elliot M. Ross (1988). "Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins)." Journal of Biological Chemistry 263: 6491-6494.

Higashijima, Tsutomu, John Burnier and Elliot M. Ross (1990). "Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines: mechanisms and structural determinants of activity." Journal of Biological Chemistry 265: 14176-14186.

Hirabayashi, Toshiyuki and David Saffen (2000). "M1 muscarinic acetylcholine receptors activate *zif268* gene expression via small G-protein Rho-dependent and λ-independent pathways in PC12D cells." European Journal of Pharmacology 267: 2525-2532.

Hogger, Petra, Melinda S. Shockley, Jelveh Lameh and Wolfgang Sadee (1995). "Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine hm1 receptors." Journal of Biological Chemistry 270: 7405-7410.

Janssen, LJ and SM Sims (1992). "Acetylcholine activates nonselective cation and chloride conductances in canine and guinea pig tracheal smooth muscle cells." Journal of Physiology 453: 197-218.

Janssen, LJ (1996). "Acetylcholine and caffeine activate Cl^- and suppress K^+ conductances in human bronchial smooth muscle." American Journal of Physiology 270: L772-L781.

Kasa, Peter, Zoltan Rakonczay and Karoly Gulya (1997). "The cholinergic system in Alzheimer's disease." Progress in Neurobioology 52: 511-535.

Kataoka, Mikio, James F. Head, Barbara A. Seaton and Donald M. Engelman (1989). "Melittin binding causes a large calcium-dependent conformational change in calmodulin." Proceedings of the National Academy of Sciences 86: 6944-6948.

Kato, Yukinari, Naoya Fujita, Akiko Kunita, Shigeo Sato, Mika Kaneko, Motoki Osawa and Takashi Tsuruo (2003). "Molecular Identification of Aggrus/T1α as a Platelet Aggregation-inducing Factor Expressed in Colorectal Tumors." Journal of Biological Chemistry 278: 51599–51605.

Kincaid, Randall L., Martha Vaughn, Vsevolod A. Tkachuk and James C. Osborne Jr. (1982). "Ca^{2+}-dependent interaction of 5-dimethylaminoaphthalene-1-sulfonyl-calmodulin with cyclic nucleotide phosphodiesterase, calcineurin, and troponin I." Journal of Biological Chemistry 257: 10638-10643.

Klagsbrun, M., S. Takashima and R. Mamluk (2002). "The role of neuropilin in vascular and tumor biology." Advances in Experimental Medical Biology 515: 33-48.

Klee, Claude B., Hao Ren and Xutong Wang (1998). "Regulation of the calmodulin-stimulated protein phosphatase, calcineurin." Journal of Biological Chemistry 273: 13367-13370.

Klein, Uwe, M. Teresa Ramirez, Brian K. Kobilka and Mark von Zastrow (1997). "A novel interaction between adrenergic receptors and the α-subunit of eukaryotic initiatian factor 2B." Journal of Biological Chemistry 272: 19099-19109.

Klett, Christoph P. R. and Tom I. Bonner (1999). "Identification and characterization of the rat m1 muscarinic receptor promoter." Journal of Neurochemistry 72: 900-909.

Kortvely, E., Arpad Palfi and Karoly Gulya (2002). "Ontegeny of calmodulin gene expression in rat brain." Neuroscience 114: 301-316.

Kotlikoff, Michael I. and Yong-Xiao Wang (1998). "Calcium release and calcium-activated chloride channels in airway smooth muscle cells." American Journal of Respiratory and Critical Care Medicine 158.: S109–S114.

Ladant, Daniel (1988). "Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin: identification of two separated calmodulin-binding domains." Journal of Biological Chemistry 263: 2612-2618.

Lameh, Jelveh, Mohan Phillip, Yogesh K. Sharma, Osamu Moro, J. Ramachandran and Wolfgang Sadee (1992). "Hm1 muscarinic cholinergic receptor internaization requires a domain in the third cytoplasmic loop." Journal of Biological Chemistry 267: 13406-13412.

Lee, Norman H., Neil S. M. Geoghagen, Emily Cheng, Robin T. Cline and Claire M. Fraser (1996b). "Alanine scanning mutagenesis of conserved arginine/lysine-arginine/lysine-x-x-arginine/lysine G protein-activating motifs on m1 muscarinic acetylcholine receptors." Molecular Pharmacology 50: 140-148.

Levay, Konstantin, Daulet K. Sapaev, Alexey N. Pronin, Jeffrey L. Benovic and Vladlen Z. Slepak (1998). "Localization of the sites for Ca^{2+}-binding proteins on G protein-coupled receptor kinases." Biochemistry 37: 13650-13659.

Levey, Allan I., Cheryl A. Kitt, William F. Simonds, Donald L. Price and Mark R Brann (1991). "Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies." Journal of Neuroscience . 11: 3218-3226.

Lin, Kedan, Danxin Wang and Wolfgang Sadee (2002). "Serum response factor activation by muscarinic receptors vis RhoA: Novel pathway specific to M1 subtype invovling

Calmodulin, Calcineurin and Pyk2." Journal of Biological Chemistry 277: 40789-40798.

Liu, X. and J. M. Farley (1996). "Acetylcholine-induced chloride current oscillations in swine tracheal smooth muscle cells." Journal of Pharmacology and Experimental Therapeutics 276: 178-186.

Lu, Zhi-liang, Jose W. Saldanha and Edward C. Hulme (2002). "Seven-transmembrane receptors: crystals clarify." Trends in pharmacological sciences 23: 140-146.

Luttrell, L. M., S. S. G. Ferguson, Y. Daaka, W. E. Miller, S. Maudsley, G. J. Della Rocca, H. Kawakatsu F.-T. Lin, K. Owada, D. K. Luttrell, M. G. Caron and R. J. Lefkowitz (1999). "β-arrestin-dependent formation of β2 adrenergic receptor-src protein kinase complexes." Science 283: 655-661.

Maeda, Sadaaki, Jelveh Lameh, William G. Mallet, Mohan Phillip, J. Ramachandran and Wolfgang Sadee (1990a). "Internalization of the hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop." FEBS Letters 269: 386-389.

Maeda, Sadaaki, Jelveh Lameh, William G. Mallet, Mohan Phillip, J. Ramachandran and Wolfgang Sadee (1990b). "Internalization of the hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop." FEBS Letters 269: 386-389.

Malencik, Dean A. and Sonia R. Anderson (1983). "High affinity binding of the mastoporans by calmodulin." Biochemical and Biophysical Research Communications 114: 50-56.

Malik, Nasir, Victor A. Canfield, Marie-Claire Beckers, Philippe Gros and Robert Levenson (1996). "Identification of the Mammalian Na^+,K^+-ATPase β3 Subunit." Journal of Biological Chemistry 271: 22754–22758.

Manivet, Philippe, Sophie Mouillet-Richard, Jacques Callebert, Canan G. Nebigil, Luc Maroteaux, Syun Hosoda, Odile Kellermann and Jean-Marie Launay (2000). "PDZ-dependent activation of nitric-oxide synthases by the serotonin 2B receptor." Journal of Biological Chemistry 275: 9324-9331.

Minakami, Reiko, Nobuyoshi Jinnai and Hiroyuki Sugiyama (1997). "Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic *in vitro*." Journal of Biological Chemistry 272: 20291-20298.

Mitchell, Rory, Derek McCulloch, Eve Lutz, Melanie Johnson, Chris MacKenzie, Mykes Fennell, George Fink, Wei Zhou and Stuart C. Sealfon (1998). "Rhodopsin-family receptors associate with small G proteins to activate phospholipase D." Nature 392: 411-414.

Mons, N., J.-L. Guillon and R. Jaffard (1999). "The role of Ca^{2+}/calmodulin-stimulable adenylyl cyclases as molecular coindence detectors in memory formation." Cellular and Molecular Life Sciences 55: 525-533.

Mori, Masayuki, Takashi Konno, Takeaki Ozawa, Masayuki Murata, Keiji Imoto and Kuniaki Nagayama (2000). "Novel Interaction of the voltage-dependent sodium channel (VDSC) with calmodulin: Does VDSC acquire calmodulin-mediated Ca^{2+}-sensitivity?" Biochemistry 39: 1316-1323.

Moro, Osamu, Jelveh Lameh, Petra Hogger and Wolfgang Sadee (1993a). "Hydrophobic amino acid in the i2 loop plays a key role in receptor-G protein coupling." Journal of Biological Chemistry 268: 22273-22276.

Moro, Osamu, Jelveh Lameh and Wolfgang Sadee (1993b). "Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors." Journal of Biological Chemistry 268: 6862-6865.

Nakajima, Yoshiaki, Tomomi Yamamoto, Tatsuo Nakayama and Shigetada Nakanishi (1999). "A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7." Journal of Biological Chemistry 274: 27573-27577.

Nakamura, F. and Y. Goshima (2002). "Structural and functional relation of neuropilins." Advances in Experimental Medical Biology. 515: 55-69.

Nathanson, Neil M. (2000). "A multiplicity of muscarinic mechanisms: Enough signaling pathways to take your breath away." Proceedings of the National Academy of Sciences 97: 6245-6247.

Nehring, Ralf B., Hiroshi P. M. Horikawa, Oussama El Far, Matthias Kneussel, Johann Helmut Brandstatter, Stefan Stamm, Erhard Wischmeyer, Heirich Betz and Andreas Karschini (2000). "The metabotropic $GABA_B$ receptor directly interacts with the activating transcription factor 4." Journal of Biological Chemistry 275: 35185-35191.

Neufeld, G., O. Kessler and Y. Herzog (2002). "The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF." Advances in Experimental Medical Biology. 515: 81-89.

O'Day, Danton H. and Michael A. Myrc (2004). "Calmodulin-binding domains in Alzheimer's disease proteins: extending the calcium hypothesis." Biochemical and Biophysical Research Communications 320: 1051-1054.

Pacaud, P., G. Loirand, G. Gregoire, C. Mironneau and J. Mironneau (1992). "Calcium-dependence of the calcium-activated chloride current in smooth muscle cells of rat portal vein." Pflugers Archives 421: 125-130.

Palfi, Arpad, Sandor Vizi and Karoly Gulya (1999). "Differential distribution and intracellular targeting of mRNAs corresponding to the three calmodulin genes in rat brain: A quantitative in situ hybridization study." The Journal of Histochemistry and Cytochemistry 47: 583-600.

Pauli, Bendicht U., Mossaad Abdel-Ghany, Hung-Chi Cheng, Achim D. Gruber, Heather A. Archibald and Randolph C. Elble (2000). "Molecular characteristic and functional diversity of CLCA family members." Clinical and Experimental Pharmacology and Physiology 27: 901-905.

Pitt, Geoffrey S., Roger D. Zuhlke, Andy Hudmon, Howard Schulman, Harald Reuter and Richard W. Tsien (2001). "Molecular basis of calmodulin tethering and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels." Journal of Biological Chemistry 276: 30794-30802.

Pizzagalli, F., B. Hagenbuch, B.Stieger, U. Klenk, G. Folkers and P. J. Meier (2002). "Identification of a Novel Human Organic Anion Transporting Polypeptide as a High Affinity Thyroxine Transporter." Molecular Endocrinology 16: 2283–2296.

Prenzel, Norbert, Esther Zwick, Henrik Daub, Michael Leserer, Relmar Abraham, Christian Wallasch and Axel Ullrich (1999). "EGF receptor transactivation by G-protein-coupled receptors requires metalloprotease cleavage of proHB-EGF." Nature 402: 884-888.

Puschel, A. W. (2002). "The function of neuropilin/plexin complexes." Advances in Experimental Medical Biology. 515: 71-80.

Quillan, J. Mark and Wolfgang Sadee (1996). "Structure-based search for peptide ligands that cross-react with melanocortin receptor." Pharmaceutical Research 13: 1624-1630.

Reich, David E., Michele Cargill, Stacey Bolk, James Ireland, Pardis C. Sabeti, Daniel J. Richter, Thomas Lavery, Rose Kouyoumjian, Shelli F. Farhadian, Ryk Ward and Eric S. Lander (2001). "Linkage disequilibrium in the human genome." Nature 411: 199-204.

Rhoads, Allen R. and Felix Freidberg (1997). "Sequence motifs for calmodulin recognition." FASEB Journal 11: 331-340.

Romanin, Christoph, Roland Gamsjaeger, Heike Kahr, Daniela Schaufler, Olga Carlson, Darrell R. Abernethy and Nikolai M. Soldatov (2000). "Ca^{2+} sensors of L-type Ca^{2+} channel." FEBS Letters 487: 301-306.

Romeo, P. H., V. Lemarchandel and R. Tordjman (2002). "Neuropilin-1 in the immune system." Advances in Experimental Medical Biology 515: 49-54.

Rosenblum, Kobi, Marie Futter, Matthew Jones, E.C. Hulme and T.V.P. Bliss (2000). "ERKI/II regulation by the muscarinic acetylcholine receptors in neurons." Journal of Neurochemistry 20: 977-985.

Russell, Marijane, Sim Winitz and Gary L. Johnson (1994). "Acetylcholine muscarinic m1 receptor regulation of cyclic AMP synthesis controls growth factor stimulation of Raf activity." Molecular and Cellular Biology 14: 2343-2351.

Sadee, Wolfgang, Ellen Hoeg, Julie L. Lucas and Danxin Wang (2001). "Genetic variation in human G protein coupled receptors: Implications for drug therapy." AAPS PharmSci 3(3): Article 22.

Savarese, Todd M., Cheng-Dian Wang and Claire M. Fraser (1992). "Site directed mutagenesis of the rat m1 muscarinic acetylcholine receptor: Role of conserved cysteines in receptor function." Journal of Biological Chemistry 267: 11439-11448.

Schumacher, Maria A., Andre F. Rivard, Hans Peter Bachinger and John Adelman (2001). "Structure of the gating domain of a Ca2+-activated K+ channel complexed with Ca2+/calmodulin." Nature 410: 1120-1124.

Shindo, Takayuki, Hiroki Kurihara, Kouji Kuno, Hitoshi Yokoyama, Takashi Wada, Yukiko Kurihara, Tomihiko Imai, Yuhui Wang, Masafumi Ogata, Hiroaki Nishimatsu, Nobuo Moriyama, Yoshio Oh-hashi, Hiroyuki Morita, Takatoshi Ishikawa, Ryozo Nagai, Yoshio Yazaki and Kouji Matsushima (2000). "ADAMTS-1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function." Journal of Clinical Investigation 105: 1345-1352.

Shockley, Melinda S., Neil T Burford, Wolfgang Sadee and Jelveh Lameh (1997). "Residues specifically involved in down-regulation but not internalization of the m1 muscarinic acetylcholine receptor." Journal of Neurochemistry 68: 601-609.

Slack, Barbara E., Leona K. Ma and Ching Ching Seah (2001). "Constitutive shedding of the amyloid precursor protein ectodomain is up-regulated by tumour necrosis factor-α converting enzyme." Biochemical Journal 357: 787-794.

Sorensen, Scott D., Thomas A Macek, Zhaohui Cai, Julie A. Saugstad and P. Jeffrey Conn (2002). "Dissociation of protein kinase-mediated regulation of metabotropic glutamate receptor 7 (mGluR7) interactions with calmodulin and regulation of mGluR7 function." Molecular Pharmacology 61: 1303-1312.

Stephens, J. Claiborne, Julie A. Schneider, Debra A. Tanguay, Julie Choi, Tara Acharya, Scott E. Stanley, Ruhong Jiang, Chad J. Messer, Anne Chew, Jin-Hua Han, Jicheng Duan, Janet L. Carr, Min Seob Lee, Beena Koshy, A. Madan Kumar, Ge Zhang, William R. Newell, Andreas Windemuth, Chuanbo Xu, Theodore S. Kalbfleisch, Sandra L. Shaner, Kevin Arnold, Vincent Schulz, Connie M. Drysdale, Krishnan Nandabalan, Richard S. Judson, Gualberto Ruaño and Gerald F. Vovis (2001). "Haplotype variation and linkage disequilibrium in 313 human genes." Science 293: 489-493.

Sterin-Borda, Leonor, Sabrina Ganzinelli, Alejandro Berra and Enri Borda (2003). "Novel insight into the mechanisms involved in the regulation of the m1 muscarinic receptor, iNOS and nNOS mRNA levels." Neuropharmacology 45: 260-269.

Tolbert, Lara M. and Jelveh Lameh (1996). "Human muscarinic cholinergic receptor hm1 internalizes via clathrin-coated vesicles." Journal of Biological Chemistry 271: 17335-17342.

Tsukaguchi, Hiroyasu, Stanislawa Weremowicz, Cynthia C. Morton and Matthias A. Hediger (1999). "Functional and molecular characterization of the human neutral solute channel aquaporin-9." American Journal of Physiology 277: F685-696.

Tu, Jian Chang, Bo Xiao, Joseph P. Yuan, Anthony A. Lanahan, Kathleen Leoffert, Min Li, David J. Linden and Paul F. Worley (1998). "Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors." Neuron 21: 717-726.

Turner, Justin H., Andrew K. Gelasco and John R. Raymond (2004a). "Calmodulin interacts with the third intracellular loop of the serotonin 5-hydroxytryptamine1A receptor at two distinct sites." Journal of Biological Chemistry 279: 17027-17037.

Turner, Justin H., Andrew K. Gelasco and John R. Raymond (2004b). "Calmodulin interacts with the third intracellular loop of the serotonin 5-hydroxytryptamine1A receptor at two distinct sites." Journal of Biological Chemistry 279: 17027-17037.

Ullmer, Christoph, Karin Schmuck, Andrea Figge and Hermann Lubbert (1998). "Cloning and characterization of MUPP1, a novel PDZ domain protein." FEBS Letters 424: 63-68.

van Koppen, Chris J. and Bjorn Kaiser (2003). "Regulation of muscarinic acetylcholine receptor signaling." Pharmacology and Therapeutics 98: 197-220.

Venter, JC, MD Adams, EW Myers, PW Li, RJ Mural, GG Sutton, HO Smith, M Yandell, CA Evans, RA Holt, JD Gocayne, P Amanatides, RM Ballew, DH Huson, JR Wortman, Q Zhang, CD Kodira, XH Zheng, L Chen, i M Skupsk, G Subramanian, PD Thomas, J Zhang, GL Gabor Miklos, C Nelson, S Broder, AG Clark, J Nadeau, VA McKusick, N Zinder, AJ Levine, s RJ Robert, M Simon, C Slayman, M Hunkapiller, R Bolanos, A Delcher, I Dew, D Fasulo, M Flanigan, L Florea, A Halpern, S Hannenhalli and et al. (2001). "The sequence of the human genome." Science 291: 1304-1351.

Vogel, Hans J. (1994). "Calmodulin: a versatile calcium mediator protein." Biochemistry and Cell Biology 72: 357-376.

Vogler, Oliver, Galina S. Bogatkewitsch, Claudia Wriske, Patrick Krummener, Karl H. Jakobs and Chris J. van Koppen (1998). "Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin." Journal of Biological Chemistry 273: 12155-12160.

Wang, Danxin, Wolfgang Sadee and J. Mark Quillan (1999). "Calmodulin binding to G protein-coupling domain of opioid receptors." Journal of Biological Chemistry 274: 22081-22088.

Wang, Danxin, Lara M. Tolbert, Kurt W. Carlson and Wolfgang Sadee (2000). "Nuclear Ca2+/calmodulin tranlocation activated by μ-opioid (OP3) receptor." Journal of Neurochemistry 74: 1418-1425.

Wang, Danxin, J. Mark Quillan, Katharine Winans, Julie L. Lucas and Wolfgang Sadee (2001). "Single nucleotide polymorphisms in the human μ opioid receptor gene alter basal G-protein coupling and calmodulin binding." Journal of Biological Chemistry 276: 34624-34630.

Wang, Hongbing and Daniel R. Storm (2003). "Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system." Molecular Pharmacology 63: 463-468.

Wang, Kai-Ling and Basil D. Roufogalis (1999). "Ca^{2+}/calmodulin stimulates GTP binding to the Ras-related protein Ral-A." Journal of Biological Chemistry 274: 14525-14528.

Wang, Yong-Xiao and Michael I. Kotlikoff (1997). "Inactivation of calcium-activated chloride chammels in smooth muscle by calcium/calmodulin-dependent protein kinase." PNAS 94: 14918-14923.

Warr, Coral G. and Leonard E. Kelly (1995). "Identification and characterization of two distinct calmodulin-binding sites in the TrpI ion-channel protein of *Drosophila melanogaster*." Biochemical Journal 314: 497-503.

Wayman, Christopher P., Ian McFadzean, Alan Gibson and John F. Tucker (1997). "Cellular mechanisms underlying carbachol-induced oscillations of calcium-dependent membrane current in smooth muscle cells from mouse anococcygeus." British Journal of Pharmacology 121: 1301-1308.

Webster, Nicola J., Kim N. Green, Chris Peers and Peter F. T. Vaughan (2002). "Altered processing of amyloid precursor protein in the human neuroblastoma SH-SY5Y by chronic hypoxia." Journal of Neurochemistry 83: 1262-1271.

White, Julia H., R. A. Jeffrey McIllhinney, Alan Wise, Francisci Ciruela, Wai-Yee Chun, Piers C. Emson, Andrew Billinton and Fiona H. Marshall (2000). "The $GABA_B$ receptor interacts directly with the related transcription factors CREB2 and ATFx." Proceedings of the National Academy of Sciences 97: 13967-13972.

Xiao, Bo, Jian Chang Tu, Ronald S. Petralia, Joseph P. Yuan, Andrew Doan, Christopher D. Breder, Alicia Ruggiero, Anthony A. Lanahan, Robert J. Lenhold and Paul F. Worley (1998). "Homer regulates the association of group 1 metabotropic gluatamate receptors with multivalent complexes of Homer-related, synaptic proteins." Neuron 21: 707-716.

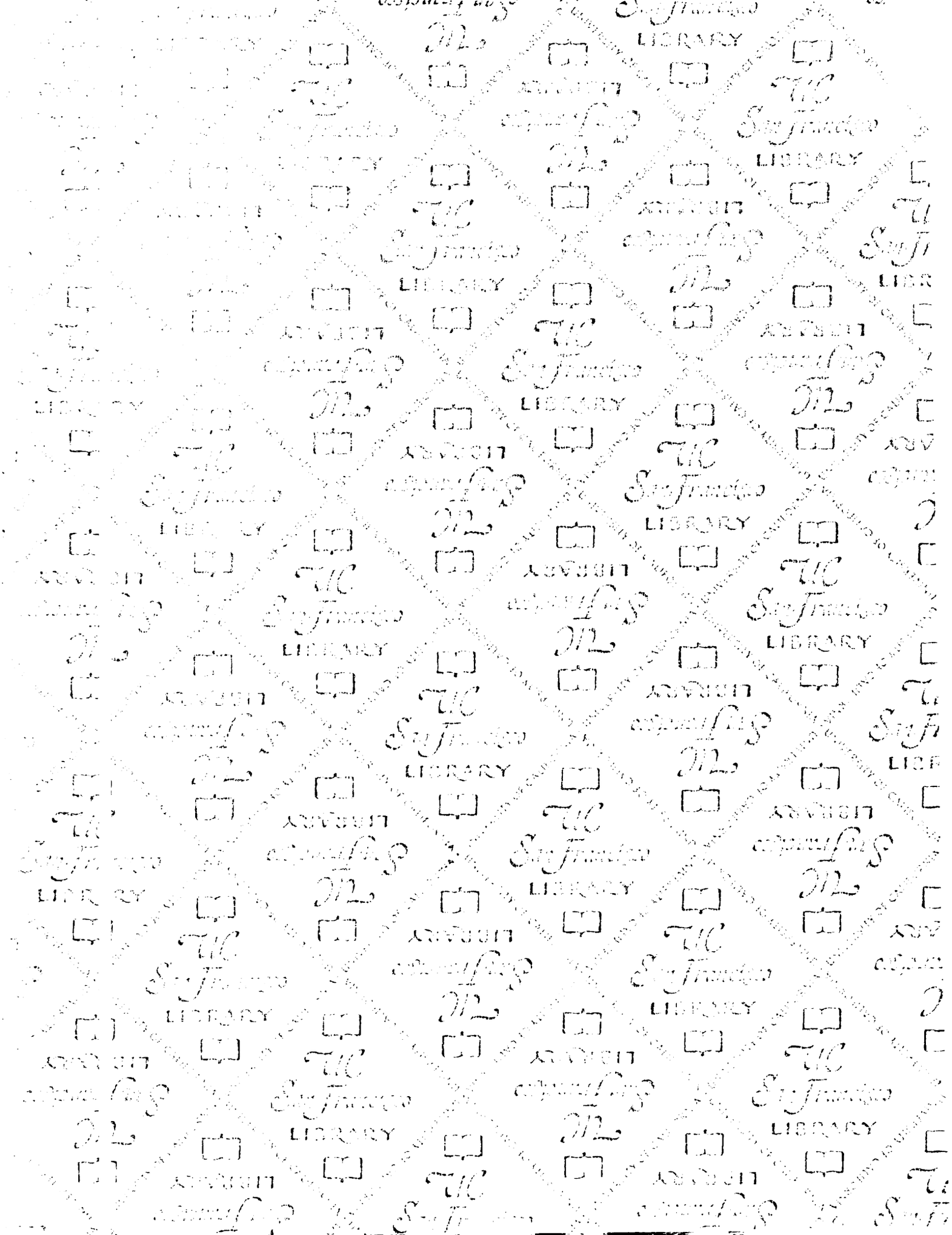
Yang, Yee Hwa, Sandrine Dudoit, Percy Luu, David M Lin, Vivian Peng, John Ngai and Terrence P Speed (2002). "Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation." Nucleic Acids Research 30: e15.

Zeng, Fu-Yue and Jurgen Wess (1999). "Identification and molecular characterization of m3 muscarinic receptor dimers." Journal of Biological Chemistry 274: 19487-19497.

Zhang, Mingjie and Tao Yuan (1998). "Molecular mechanisms of calmodulin's functional versatility." Biochemistry and Cell Biology 76: 313-323.

Zimmer, Gert, Frank Oeffner, Veronika von Messling, Thomas Tschernig, Hermann-Josef Grone, Hans-Dieter Klenk and Georg Herrler (1999). "Cloning and characterization of gp36, a human mucin type glycoprotein preferentially expressed in vascular endothelium." Biochemical Journal 341: 277-284.

Zitzer, Heike, Dietmar Richter and Hans-Jurgen Kreinkamp (1999). "Agonist-dependent interaction of the rat somatostatin receptor subtype 2 with cortactin-binding protein 1." Journal of Biological Chemistry 274: 18153-18156.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