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Feature Article

Calmodulin Binding to Peptides Derived from the i3 Loop of Muscarinic Receptors

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Purpose. This study was conducted to identify and characterize the structural requirements of a calmodulin-binding motif identified in the third intracellular (i3) loop of muscarinic acetylcholine receptors (M1–M5), a region important for G protein coupling.
Methods. GST fusion proteins and synthetic peptides derived from the hM1 i3 loop were tested for binding to CaM using a cross-linking gel shift assay and a dansyl-CaM fluorescence assay. Mutagenesis studies further characterized the structural requirements for the interaction and identified critical residues.
Results. 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 5-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.
Conclusion. Calmodulin can bind to an M1 region implicated in G protein coupling. This indicates an important role for CaM in the regulation of muscarinic signal transduction.

KEY WORDS: calcium; calmodulin; G-protein; G-protein coupled receptor; muscarinic acetylcholine receptors.

INTRODUCTION

Muscarinic acetylcholine receptors (mAchR) are seven transmembrane spanning proteins, members of the G-protein coupled receptor (GPCR) superfamily. There are five subtypes in humans (hM1–5). The hM1, hM3, and hM5 receptors couple to heterotrimeric G protein $G_{\alpha q/11}$ to activate phospholipase C and increase 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 (1).

The muscarinic receptors have a broad and overlapping distribution in the body. The hM1 receptor, expressed in hippocampal and cortical regions of the brain and in parasympathetic ganglia (2–4), is involved in the initiation of seizures, learning and memory, regulation of the force and rate of heart contractions, and more (5,6). Furthermore, hM1 is a drug target for treating Alzheimer's disease and other neurological and psychiatric disorders (5).

Calmodulin is a key mediator of calcium-activated cell signaling. It has been shown to regulate 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 D_2 dopamine receptors to inhibit G protein coupling (7,8). It can also inhibit phosphorylation by protein kinase C (PKC) and regulate receptor desensitization by binding to the C terminus of the metabotropic glutamate receptors types 5 and 7 or the i3 loop of the 5HT1A serotonin receptor (9–11). We have found a putative CaM binding motif on the i3 loop of muscarinic receptors (12). Previous studies in our laboratory used an S-tag labeling assay to identify peptides derived from several GPCRs, including hM1–3, capable of binding CaM (13). To better understand the role CaM is playing in GPCR signal transduction, the current study characterizes the structural requirements for CaM–muscarinic receptor interactions, to enable functional characterization of the interaction in intact cells. We show that peptides derived from this region of M1–3 bind CaM in a Ca^{2+}-dependent manner. In hM1, we identify 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. By replacing key residues involved in

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ABBREVIATIONS: CAm, calmodulin; GPCR, G-protein coupled receptor; hM1–3, human muscarinic acetylcholine receptor, types 1–3; mAchR, muscarinic acetycholine receptor; mGluR, metabotrophic glutamate receptor; MOR, μ opioid receptor; PKC, protein kinase C.

CaM binding, we identified residues required for CaM binding but dispensable for G protein coupling. 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, USA). 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 USA). The μ opioid receptor peptide (sequence containing residues 258–286), used as a positive control, was synthesized as previously described (8). The FLAG peptide, used as a negative control, was obtained from Sigma-Aldrich (St. Louis, MO, USA). 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, USA). The pGEX-5X-2 vector, Factor Xa, and glutathione sepharose beads were obtained from Amersham Pharmacia (Piscataway, NJ, USA). Restriction enzymes were from New England Biolabs (Beverly, MA, USA). 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-CGGGATCCCCCGCATCTACCGG GAG; S237r-GGAATTCCTCAGCTGCCACCCCCTTT GCC; A313f-CGGGATCCCCGCCCCCACCAAGCAG; K339f-CGGGATCCCCAAGGGCCAGAAGCCC; K342r-GGAATTCCTCACTTCTGGCCCTTGCC; S368r-GGAATTCCTCAACTCAGGGTCCGAGC) and cloned into the pGEX-5X-2 vector (Amersham Pharmacia) using *Eco*R1 and *Bam*HI sites. The vector was transformed into the DH5α strain of *E. coli* (Stratagene, La Jolla, CA, USA). Protein purification was performed as previously described (14). Briefly, 5 mL of the transformed bacteria was grown overnight in YTA media (20 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl, 100 μg/mL carbanicillin). The next day, the cultures were transferred to 200 mL of the same media and grown for 5 h at 37°C, induced with isopropylβ-D-thiogalactopyranoside (IPTG), and grown at 30°C for 1–2 h more. Bacteria are spun down, washed with 15 mL STE buffer (200 mM Tris–HCl, pH 8.0, 1 M NaCl, 0.1 M EDTA), spun again, and resuspended in STE buffer plus lysozyme and protease inhibitors. This was incubated on ice for 15 min, and then 5 mM DTT (dithiothreitol), 1 mM DMF (*N*, *N*-dimethylformamide), 1.5% sarkosyl, and 10 mL B-PER bacterial protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) are added. This was spun down, and 2% Triton-X 100 is added to the supernatant before incubation with the Glutathione Sepharose beads (Amersham-Pharmacia) to segregate the fusion protein. After incubation, the beads are washed three times with phosphate-buffered saline (PBS; 1 mM 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 the Bradford assay.

Mutagenesis

Mutagenesis was performed 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.

Cross-Linking Gel Shift Assay

The cross-linking gel-shift assay was performed essentially as previously described (7). Briefly, CaM and peptide in a molar ratio of either 1:1 or 1:10 (CaM/peptide) were incubated in 50 μl of 10 mM HEPES, pH 7.5, plus 1 mM

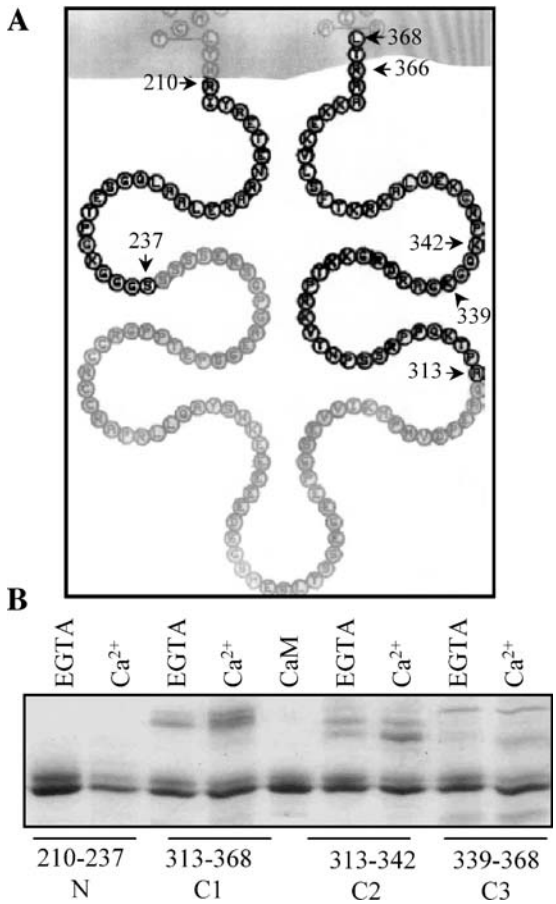

Fig. 1. Studies with peptides derived from GST fusion proteins. (A) Diagram of the hM1 i3 loop. (B) Cross-linking gel shift assay using hM1 peptides representing different regions of the i3 loop. Assay was done in the presence of calcium or the calcium chelator EGTA. The ability of a peptide to bind CaM is indicated by a mobility shift consistent with the increased molecular weight of the peptide over free calmodulin.

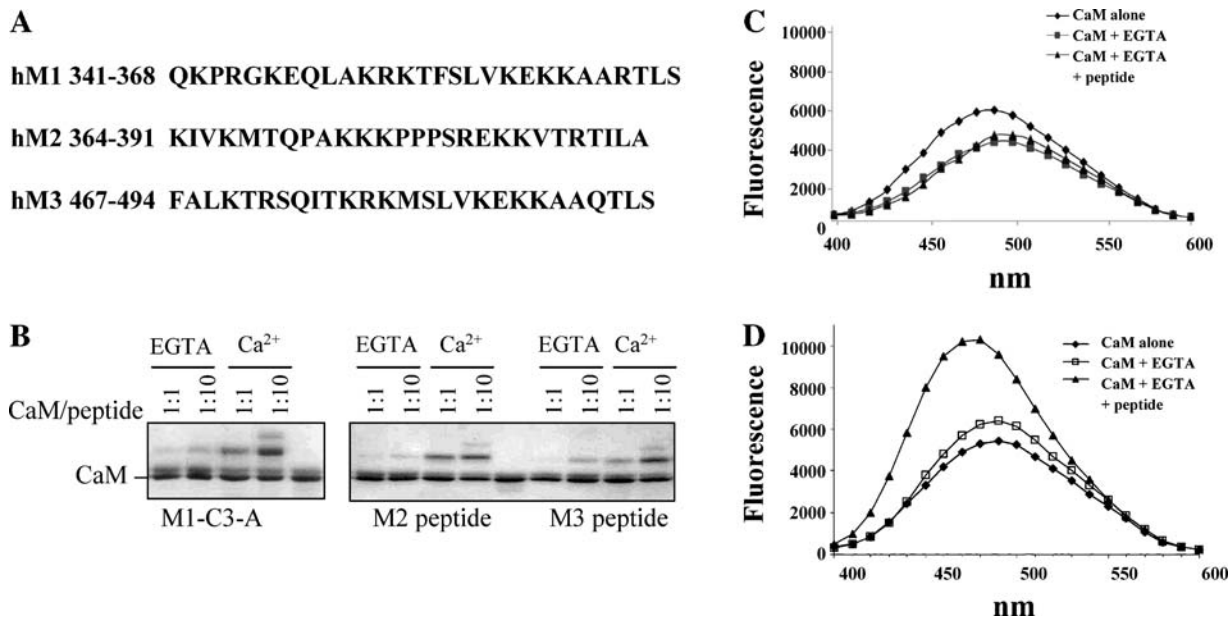

Fig. 2. Binding of peptides derived from the i3 loop of muscarinic receptors hM1–3 to CaM. (A) Sequences of hM1 (designated M1-C3-A), hM2, and hM3 peptides. (B) Cross-linking gel shift assay of CaM binding to peptides derived from the i3 loops of hM1, hM2, and hM3. Assay was done in the presence of calcium or the calcium chelator EGTA. The ability of a peptide to bind CaM is indicated by a mobility shift consistent with the increased molecular weight of the peptide over free calmodulin. (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.

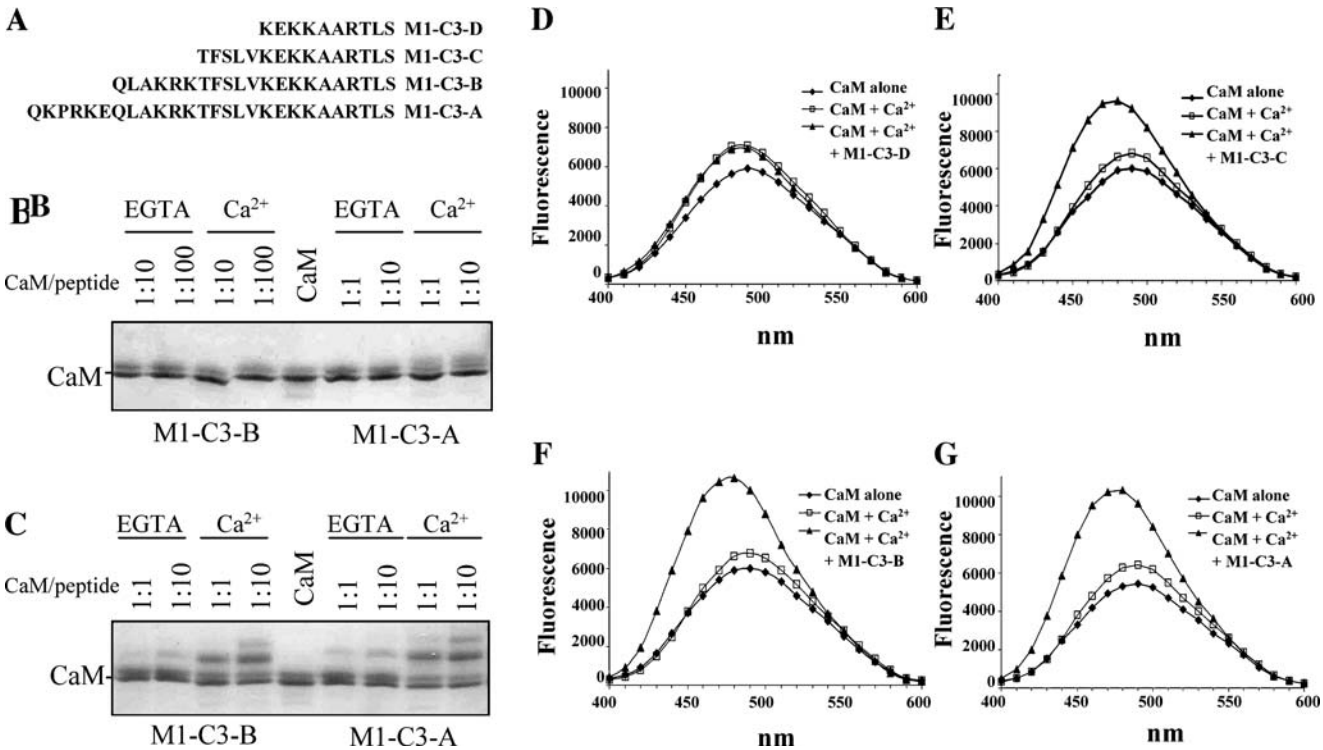

Fig. 3. Binding of peptides derived from the i3 loop of hM1 to CaM. (A) Sequences of synthesized peptides. M1-C3-A is derived from residues 342 to 368, M1-C3-B from 348 to 368, M1-C3-C from 354 to 368, and M1-C3-D from 359 to 368. (B–C) Cross-linking gel shift assay of peptides with CaM in the presence of calcium or the calcium chelator EGTA. The ability of a peptide to bind CaM is indicated by a mobility shift consistent with the increased molecular weight of the peptide over free calmodulin. (D–G) Fluorescent spectra of peptides with dansyl-CaM. Only spectra done in the presence of calcium are shown. None of the peptides bound dansyl-CaM in the absence of calcium, which was measured in 200 μM EGTA. An increase in fluorescence and shift of the curve to the left is indicative of binding.

Table I. hM1 Mutants, their Sequences and the Effect of the Mutation on CaM Binding

Mutation	Peptide sequence	Ability to bind CaM
S322A	APTKQPPKS**A**PNTVKRPTKKGRDRAGKGQK	+
K327A	APTKQPPKSSPNTV**A**RPTKKGRDRAGKGQK	+
K346A	KGQKPRG**A**EQLAKRKTFSLVKEKKAARTLS	+
R352A	KGQKPRGKEQLAK**A**KTFSLVKEKKAARTLS	+
K359T	KGQKPRGKEQLAKRKTFSLV**T**EKKAARTLS	+
E360A	KGQKPRGKEQLAKRKTFSLVK**A**KKAARTLS	−
K361A	KGQKPRGKEQLAKRKTFSLVKE**A**KAARTLS	+
Δ351–353	KGQKPRGKEQLA TFSLVKEKKAARTLS	+
Δ363	QLAKRKTFSLVKEKKARTLS	+
Δ232–358	RIYRETENRARELAALQGSETP KEKKAARTLS	−

Mutations were made in the C3-GST fusion protein (**shown in bold; see Fig. 1**) and the cross-linking gel shift assay was done as a screen for CaM binding. Two mutants were unable to bind CaM: E360A and Δ232–358.

disuccinimidyl substrate (DSS; Pierce Biotechnology) in the presence of 200 μM $CaCl_2$ or 200 μM of the calcium chelator, ethyleneglycotetraacetic acid (EGTA), for 30 min 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 previously described (15). The fluorescence of 50 μg dansyl-CaM in 1 mL of 10 mM HEPES, pH 7.5, and 200 μM EGTA was measured with an excitation at 340 nm and emission from 400 to 600 nm. When necessary, $CaCl_2$ was added so that a total concentration of 200 μM is reached (400 μM total added), and the sample was measured again. Peptide was then added so that there was a CaM/peptide molar ratio of 1:1, and the sample was measured again.

Overlay Assay

This is done as described previously (16). Purified whole GST fusion proteins are run on a 10% SDS-PAGE gel. The gel is then blotted onto a polyvinylidene difluoride (PVDF) membrane (Biorad, Hercules, CA, USA). 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 (Fig. 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 cross-linking gel shift assay (Fig. 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

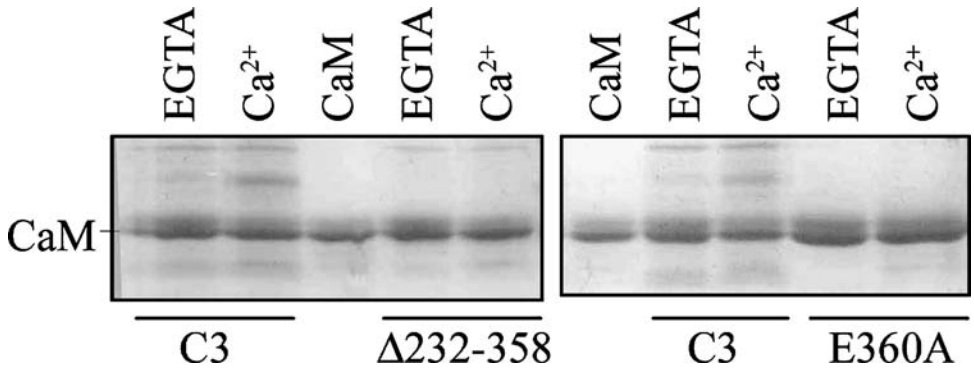

Fig. 4. Cross-linking gel shift assay of hM1 mutants deficient in CaM binding. The ability of a peptide to bind CaM is indicated by a mobility shift consistent with the increased molecular weight of the peptide over free calmodulin. Assay was carried out as described in Figs. 2 and 3.

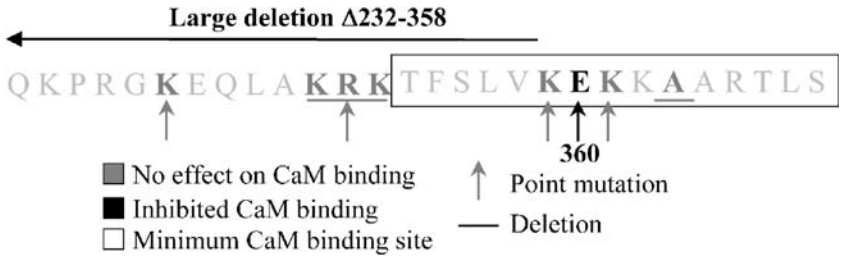

Fig. 5. Summary of results from mutagenesis and peptide studies.

detectable CaM binding. The C3 peptide, containing the CaM binding motif, yielded a shifted band consistent with a peptide–CaM adduct. Interestingly, the C2 peptide, which is adjacent to the putative CaM binding domain, also seemed to bind CaM. C1, which contains both C2 and C3, similarly bound to CaM. All peptides preferably bound 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 (16) (data not shown). 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 (Fig. 2A) were tested for ability to bind CaM (Fig. 2B). The peptide from hM1 is designated M1-C3-A, because 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 cross-linking gel shift assay revealed that CaM was able to bind to all three peptides (Fig. 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 MOR was used. This peptide was previously shown to bind to CaM (8). A FLAG peptide was used as a negative control (data not shown).

These results were confirmed using a dansyl-CaM fluorescence assay (Fig. 2C, D; results for hM2 and hM3 not shown). CaM undergoes a large conformational change when binding a peptide. This is reflected by an increase in the fluorescence of dansylated CaM and a shift of the peak to the left. These changes occurred only in the presence of calcium, confirming the hM1 peptide binds CaM preferentially in the presence of calcium. Similar results were obtained for the hM2 and hM3 peptides (not shown).

To specify the region of hM1 required for CaM binding, a series of sequentially smaller peptides was synthesized: a 21-mer (M1-C3-B), a 15-mer (M1-C3-C), and a 10-mer (M1-C3-D) (Fig. 3A). Also shown, for comparison, in Fig. 3 are results with M1-C3-A, the 28-mer capable of binding to CaM in Fig. 2. Each of the shorter peptides was tested for their ability to bind CaM using the cross-linking gel shift assay and the dansyl-CaM fluorescence assay (Fig. 3B and C). M1-C3-B and M1-C3-C, which contain 21 and 15 amino acids, respectively, were both able to bind CaM, again with a preference for calcium. M1-C3-D, which contains ten amino acids (residues 359–368), is insufficient for binding to CaM.

Amino Acid Substitutions and Mutagenesis Studies

The purpose of the mutagenesis studies was to identify the regions of the hM1 i3 loop important for CaM binding, and to identify a mutant that either lacks CaM binding but still couples to G proteins, or *vice versa*, for subsequent use in functional studies. A series of point mutations was made in the C3 GST fusion protein, the resulting peptide cleaved, and used in a cross-linking gel shift assay to screen for ability to bind CaM. Table I lists the mutants made, the peptide sequence, and the peptide's ability to bind CaM. Mutations of the 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 ten residues of the putative CaM binding domain (Fig. 4). Both mutants were previously described as being able to couple to G proteins (17,18). Moreover, E360A has also been described as being constitutively active (18).

DISCUSSION

We have shown that CaM is able to bind to peptides representing the C-terminal region of M1–3 in a Ca^{2+}-dependent manner and have characterized the structural requirements for this interaction. Although CaM–peptide interactions observed *in vitro* must be validated in future studies with the use of intact receptors, resolving the structure of the CaM-binding motif in muscarinic receptors is essential for *in vivo* studies. Our results show that 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 portions of the channel protein (19). 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 (19), indicating that CaM has the potential to act as a cross-linker. Therefore, CaM could be involved in hM1 oligomerization. Muscarinic receptors, especially hM3, have been shown to

dimerize (20). Further studies need to be carried out 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 binding of the large i3-loop deletion mutant, Δ232–358, which also contains only the last ten amino acids of the hM1 putative CaM binding domain. One or more of the residues 354–358 is important for CaM binding, as the addition of these residues enabled peptide 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 ten C-terminal residues (Fig. 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, were 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-fold (18,21). These results indicate that one will be able to construct M1 mutants 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 (22–25). 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 (21), 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 (26). The α helix of transmembrane domain 6 seems to extend into the C terminus of the i3 loop in the cytoplasm, generating a putative CaM binding site (27) predicted by a motif search (12). That CaM binding requirements may be similar to those for G proteins supports the hypothesis that CaM binding to the i3 loop may be common among GPCRs. Because CaM is a ubiquitous molecule and there are over 600 GPCRs in humans, CaM has the potential to be an important regulator of GPCR signaling.

CONCLUSION

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. In addition, residues in this region of hM1 critical for CaM binding have been identified, including E360 and residues 354–358. These findings suggest a direct interaction of CaM with muscarinic receptors and, due to the location of the CaM binding site, point to an important role for CaM as a regulator of muscarinic receptor signal transduction. Future studies will investigate the functional role of this interaction in muscarinic cell signaling.

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