Calmodulin Interacts with the Third Intracellular Loop of the Serotonin 5-Hydroxytryptamine$_{1A}$ Receptor at Two Distinct Sites

PUTATIVE ROLE IN RECEPTOR PHOSPHORYLATION BY PROTEIN KINASE C$^*$

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The serotonin 5-HT$_{1A}$ receptor couples to heterotrimeric G proteins and intracellular second messengers, yet no studies have investigated the possible role of additional receptor-interacting proteins in 5-HT$_{1A}$ receptor signaling. We have found that the ubiquitous Ca$^{2+}$-sensor calmodulin (CaM) co-immunoprecipitates with the 5-HT$_{1A}$ receptor in Chinese hamster ovary fibroblasts. The human 5-HT$_{1A}$ receptor contains two putative CaM binding motifs, located in the N- and C-terminal juxtamembrane regions of the third intracellular loop of the receptor. Peptides encompassing both the N-terminal (i3N) and C-terminal (i3C) CaM-binding domains were tested for CaM binding. Using in vitro binding assays in combination with gel shift analysis, we demonstrated Ca$^{2+}$-dependent formation of complexes between CaM and both peptides. We determined kinetic data using a combination of BIAcore surface plasmon resonance (SPR) and dansyl-CaM fluorescence. SPR analysis gave an apparent $K_D$ of $-110 \text{ nM}$ for the i3N peptide and $-700 \text{ nM}$ for the i3C peptide. Both peptides also caused characteristic shifts in the fluorescence emission spectrum of dansyl-CaM, with apparent affinities of $87 \pm 23 \text{ nM}$ and $1.70 \pm 0.16 \mu\text{M}$. We used bioluminescence resonance energy transfer to show that CaM interacts with the 5-HT$_{1A}$ receptor in living cells, representing the first in vivo evidence of a G protein-coupled receptor interacting with CaM. Finally, we showed that CaM binding and phosphorylation of the 5-HT$_{1A}$ receptor i3 loop peptides by protein kinase C are antagonistic in vitro, suggesting a possible role for CaM in the regulation of 5-HT$_{1A}$ receptor phosphorylation and desensitization. These data suggest that the 5-HT$_{1A}$ receptor contains high and moderate affinity CaM binding regions that may play important roles in receptor signaling and function.

The 5-HT$_{1A}$ receptor is arguably the most well characterized of the 5-HT receptor subtypes, having been cloned over a decade ago. It has been implicated in numerous physiological and pathological processes, including thermoregulation (1, 2), sexual behavior (3), memory (4), immune function (5), depression (6, 7), and anxiety (8, 9). As a prototypical G protein-coupled receptor (GPCR), the 5-HT$_{1A}$ receptor couples to a broad array of second messengers, including adenyl cyclase (10, 11), phospholipase C (12), PKC (13), K$^+$ channels (14–16), mitogen-activated protein kinases (MAPKs) (17, 18), and Na$^+/H^+$ exchange (19, 18). Virtually all of these cellular effects are averted by pretreatment with pertussis toxin, indicating that 5-HT$_{1A}$ receptors couple specifically and/or preferentially to Gi$_o$ proteins.

Although previous studies have thoroughly detailed the coupling of the 5-HT$_{1A}$ receptor to heterotrimeric G proteins and intracellular second messengers, no studies have investigated the possible role of additional receptor-interacting proteins in 5-HT$_{1A}$ receptor signaling. Growing evidence suggests that GPCRs can bind a variety of proteins, which can subsequently modify receptor signaling, internalization, and/or interaction with G protein subunits. For example, several GPCRs contain C-terminal PDZ motifs that permit them to interact with multiple intracellular proteins, including the Na$^+/H^+$ exchanger regulatory factor and post-synaptic density protein PSD-95 (20). The i3 isoform of 14-3-3 protein has been shown to bind and regulate the surface expression of different α$_2$-adrenergic receptor subtypes (21). Other reported GPCR-interacting proteins include endothelial nitric-oxide synthase (22), the small nonheterotrimeric G proteins ARF and RhoA (23), and the tyrosine phosphatases SHP-1 and SHP-2 (24).

Calmodulin (CaM) is a ubiquitous intracellular Ca$^{2+}$-sensor that plays an important role in several downstream GPCR

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$^$The abbreviations used are: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino)tetrinal; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethylester); BRET, bioluminescence resonance energy transfer; CaM, calmodulin; CHO, Chinese hamster ovary; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GPCR, G protein-coupled receptor; i3C, 23-amino acid peptide fragment from the C-terminal end of the third intracellular loop of the human serotonin 5-HT$_{1A}$ receptor; i3N, 23-amino acid peptide fragment from the N-terminal end of the third intracellular loop of the human serotonin 5-HT$_{1A}$ receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MLCK, myosin light chain kinase; MOPS, 3-[N-morpholino]propanesulfonic acid; PBS, phosphate-buffered saline; PDZ, post synaptic density/disk large/ZO-1; PKC, protein kinase C; PVDF, polyvinylidene fluoride; RLuc, Renilla luciferase; SPR, surface plasmon resonance; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; YFP, yellow fluorescent protein; MUSC, Medical University of South Carolina; α$_2$, α$_2$-adrenergic receptor; mGlur5, metabotropic glutamate subtype 5 receptor.

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signaling pathways. CaM can bind to and modulate a diverse array of cellular proteins, including enzymes, ion channels, transcription factors, and cytoskeletal proteins. Recently, CaM has been shown to bind to the epidermal growth factor receptor (25), to platelet glycoprotein VI (26), and to some GPCRs (27). The first GPCR that was shown to interact with CaM was the metabotropic glutamate subtype 5 receptor (mGlur5), which contains a CaM-binding site in a region of the extended C terminus of the receptor that is also known to bind G protein βγ subunits and to contain a PKC phosphorylation site (28). Wang et al. (27) showed that the µ-opioid receptor interacts with CaM at a C-terminal juxtamembrane region in the third intracellular loop. They concluded that CaM binding reduces G protein coupling, probably through a competitive mechanism. Similarly, Boffill-Cardona et al. (29) showed that CaM interacts with an N-terminal juxtamembrane region of the D2-dopamine receptor third intracellular loop, resulting in a blockade of the receptor-operated G protein activation switch. These examples indicate that CaM bindings may play important and diverse roles in GPCR signaling, although those roles remain largely undefined.

Our group has previously reported a role for CaM in 5-HT1A receptor signaling to extracellular signal-regulated protein kinase (ERK) MAPKs (18, 30). 5-HT1A receptor-mediated ERK activation is inhibited by chelation of intracellular Ca²⁺, CaM inhibitors, and expression of the CaM-sequestering protein calsperrin. CaM appears to play a role in ERK activation by modulating receptor endocytosis, a step required for the activation of the mitogen-activated protein kinases kinase, MEK, which phosphorylates and activates ERK. The fact that CaM plays a vital role at such a proximal step in the 5-HT1A receptor signaling pathway suggests that perhaps CaM exerts its effects by interacting with the receptor itself.

In this work, we report that CaM co-immunoprecipitates with the 5-HT1A receptor in CHO fibroblasts. A search of the primary sequence revealed that the human 5-HT1A receptor contains two potential CaM binding motifs, located in the N- and C-terminal juxtamembrane regions of the third intracellular loop of the receptor. Both motifs contain consensus PKC phosphorylation sites and are important for Gia protein coupling, indicating that these regions of the receptor likely play an important regulatory role in receptor function. The purpose of this work was to determine whether the two putative CaM binding sites could bind to CaM, and if so, whether this would alter some parameter of receptor function.

EXPERIMENTAL PROCEDURES

Materials—Purified bovine brain calmodulin, biotinylated calmodulin, and purified rat brain PKC were purchased from Calbiochem (La Jolla, CA). Mouse anti-CaM antibodies were from Upstate Biotechnology (Charlottesville, VA). Coelenterazine h and dansyl chloride were purchased from Molecular Probes (Eugene, OR). CM5 carboxymethylated sensor chips were purchased from BIAcore AB (Piscataway, NJ). Anti-5-HT1A receptor antibodies were raised as previously described (13, 31). [γ-32P]ATP was purchased from PerkinElmer Life Sciences (Boston, MA).

Synthesis of 5-HT1A Receptor i3 Loop Peptides—Peptides derived from the primary sequence of the N-terminal (3N, aa 215–237, YGRIFRAFRRIKRINKVKVEGT) and C-terminal (3C, aa 328–350, EAKKKMALAREKTVKTGLIGM) regions of the 5-HT1A receptor third intracellular loop were synthesized on a Rainin PS3 automated peptide synthesizer by the MUSC Peptide Synthesis Facility, using standard solid-phase methods. Peptide size and purity were verified using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. N-Terminal 32P-labeled peptides were purified on a Waters Delta Prep 3000 chromatography system using a C-18 silica column, and elution was carried out across a linear gradient of acetonitrile in water containing 0.1% (w/v) trifluoroacetic acid (Emory University, Microchemical Facility, Atlanta, GA).

Plasmids and Transfections—The yellow fluorescent protein expression vector eYFP-N1 was obtained from Clontech (San Jose, CA). The Renilla Luciferase protein expression vectors RLuc-N1 and RLuc-C1 were kindly provided by J. Yordy (MUSC, Charleston, SC). The calmodulin coding sequence without its stop codon was amplified from HeLa cell cDNA using sense and antisense primers containing unique XmnI and SacII restriction sites. The fragment was then subcloned in-frame to the C-terminal Luc-N1 and RLuc-C1 vectors. The 5-HT1A receptor coding sequence was amplified from pcDNA3.1 containing the entire 5-HT1A receptor coding sequence and 3′ untranslated region using sense and antisense primers containing unique BaMHI and Xhol restriction sites (19). The fragment was then subcloned in-frame into the eYFP-N1 vector. All vector constructs were verified by DNA sequencing.

Cell Culture and Transfection—CHO-K1 (CHO) cells expressing the 5-HT1A receptor were maintained in F-12/Ham’s medium supplemented with 10% fetal calf serum, streptomycin (100 µg/ml), penicillin (100 units/ml), and gentamicin (400 µg/ml) at 37 °C in a 5% CO2-enriched, humidified atmosphere. 24–48 h before each experiment, cells were switched to serum-free medium containing 0.5% fatty acid-free bovine serum albumin (Sigma). For transient transfections, CHO cells were plated in 6-well plates at 1 × 10⁶ cells per well, and cultured for 24 h in F-12/Ham’s medium supplemented with 10% fetal calf serum. Cells were then transferred to antibiotic- and serum-free F-12/Ham’s medium and transfected using LipofectAMINE 2000 reagent according to the manufacturer’s instructions (Invitrogen). The final amount of transfected DNA for a single well was ~2 µg. After transfection, cells were cultured in F-12/Ham’s medium supplemented with 10% fetal calf serum and 10% fetal bovine serum for 48 h. Cells were then transferred to antibiotic- and serum-free F-12/Ham’s medium supplemented with 10% fetal calf serum and 400 µg/ml Geneticin. Cells were cultured for 1–2 weeks to allow for the selection of stable clones.

Gel Shift Assays—Gel shift analysis of CaM-peptide complexes was performed using urea-polyacrylamide gel electrophoresis, as described by Erickson-Vitanken and Delgrado (33). Reactions (30–100 µl total volume) containing 300 pmol of CaM (~5 µg) and increasing amounts of peptide (0–500 pmol) were incubated in 100 mM Tris-HCl, pH 7.5, 4% (v/v) urea, and either 0.1 mM CaCl2 or 1 mM EGTA at 22 °C for 30 min. 15 µl of a 50% glycerol/0.1% bromphenol blue loading buffer was added to each reaction, and the samples were resolved on 14% polyacrylamide gels containing 4% urea and either 0.1 mM CaCl2 or 1 mM EGTA in the running buffer. Protein was visualized by staining with Gel-code blue (BioRad) staining reagent.

Blot Overlay Assays—Peptides (1–100 nmol) were immobilized to PVDF membranes by slot-blot and washed twice with 100 mM Tris-HCl, pH 7.5. The membranes were blocked with 5% bovine serum albumin in 0.1% Tween 20 for 1 h at room temperature, and then were incubated with 0.5 µg/ml biotinylated CaM in the presence of either 0.1 mM CaCl2 or 1 mM EGTA overnight at 4 °C. The PVDF membranes were then washed 3× in the same buffer without CaM, followed by incubation with alkaline phosphatase-conjugated avidin for 1 h at room temperature. Detection was with a chemiluminescent reagent.

Surface Plasma Resonance—Real-time binding and kinetic analyses were performed at the Molecular Biotechnology Institute at the BiAcore 3000 biosensor system (Pharmacia Biosensor AB) using surface plasmon resonance (SPR) measurements. Carboxymethylated sensor chips (type CM5) were activated with a 1:1 mixture of 0.2 M N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide in water. Synthetic peptides (100 µg/ml, 31.5 ng in 10 mM sodium acetate at pH 4.8) were then immobilized on the sensor chips using a 1:1 mixture of N,E-carboxyanhydride and 0.1 M ethanolamine. Unreacted sites were blocked with 1 M ethanolamine (pH 8.5). The SPR signals from the immobilized peptides generated BiAcore response units ranging from 400 to 720. Control flow cells were activated and blocked in the absence of protein. Binding was evaluated over a range of CaM concentrations (15.6 nm to 1 µM) in 150 mM NaCl, 100 mM HEPES (pH 7.4), and 400 µg/ml CaCl2, at a continuous flow of 5 µl/min at 25 °C. 10 µl of CaM-containing solutions was pulsed over the surface of the chip for 2 min using the kinjet command. Binding of CaM to peptide-immobilized flow cells was corrected for binding to control flow cells. Flow cells were regenerated by passing over running buffer without CaCl2. Binding data were fitted to a 1:1 Langmuir binding model.
under steady-state conditions using BIAevaluation version 3.1 software (BIAcore).

**Fluorometric Measurements with Dansyl-CaM—Dansyl-CaM was synthesized according to the method of Bertrand et al. (34). Briefly, 10 μg of CaM was incubated with ~1 μg dansyl-chloride for 1 h at 4 °C. Dansyl-CaM was purified from unincorporated dye using a Centricon™ concentrator with a molecular mass cutoff of 10,000 Da. Measurement of absorbance at 340 nm (molar extinction coefficient: 3,400 M⁻¹ cm⁻¹) gave an incorporation of ~1.3 dansyl units per CaM molecule. Fluorescence emission spectra of dansyl-CaM were measured from 400 to 600 nm using a SLM 8000™ spectrophotometer (Aminco-Bowman) with an excitation wavelength of 340 nm. Test peptides (0–8 μM) were incubated with dansyl-CaM in 100 mM Tris-HCl, pH 7.5, supplemented with 0.1 mM CaCl₂ for 2 h at room temperature. The concentration of dansyl-CaM (0.1–0.5 μM) was varied, and concentration-response curves were generated for fluorescence enhancement at each dansyl-CaM concentration. The apparent Kᵦ values for each concentration of dansyl-CaM were fit to the Hill equation by linear regression to calculate true affinities.

**Bioluminescence Resonance Energy Transfer—**To determine whether CaM and the 5-HT₁A receptor come into close proximity in intact cells, we used bioluminescence resonance energy transfer (BRET), a technique that detects close proximity of proteins using energy transfer between luminescent and fluorescent tags. A bioluminescent donor source (Renilla reniformis luciferase, RLuc) can transfer energy to an acceptor fluorophore (yellow variant of Aequorea green fluorescent protein, YFP) within a radius of ~50 Å, and this transfer is virtually undetectable at distances greater than 100 Å (35). CHO-5-HT₁AR cells were transfected as described above and cultured in F-2/Ham's media for 48 h to allow for protein expression. Cells were detached with PBS/1 mM EDTA and distributed into a 96-well plate at 1 × 10⁵ cells/well. Fluorescence measurements were acquired using a Victor® multilabel plate reader (PerkinElmer Life Sciences). In some cases, cells were incubated in the presence of inhibitors for 20 min, followed by the addition of 1 μM 8-OH-DPAT for 5 min. Coelenterazine h was then added to a final concentration of 5 μM, and sequential measurements were made with filters at 460 ± 25 nm and 525 ± 25 nm. The BRET ratio was calculated as the ratio of light emitted at 525 nm (YFP) over the light emitted at 460 nm (Luciferase).

**In Vitro Kinase Assays—**Thirty-five nanograms (~0.06 unit) of purified rat brain PKC was incubated with increasing concentrations of i3N (0–5 μM) and i3C (0–10 μM) peptides in kinase buffer (20 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM CaCl₂, 0.1 mM MgCl₂, 0.01 mM dicyclohexylcarbodimide, 100 μM DMAP, 10 μM NaF, 100 μM Na₂VO₄) in a total volume of 50 μl. In some cases, purified bovine brain calmodulin (0–120 μM) was added to reactions. Assays were started by the addition of 5 μCi of [γ⁻³²P]ATP and incubated at 30 °C for 1 h. Reactions were then transferred to nitrocellulose squares, washed several times with 0.75% phosphoric acid, followed by a final wash with 100% acetone. Bound radioactivity was measured using liquid scintillation counting.

**Statistical Analysis—**Results shown represent the means ± S.E. of the number of experiments indicated in each case. Statistical analysis was performed by student's t test.

**RESULTS**

**Interaction of CaM with the Serotonin 5-HT₁A Receptor—**We have previously shown that CaM plays roles in numerous 5-HT₁A receptor signaling pathways, including stimulation of MAPKs (18, 30), activation of Na⁺/H⁺ exchange (19), and receptor internalization (30). We wondered if, in addition to its distinct roles in downstream signaling pathways, CaM might interact directly with the receptor through one of its intracellular domains. To that end, we immunoprecipitated the 5-HT₁A receptor from CHO fibroblasts that were transfected to stably express this receptor at physiological levels (36). As shown in Fig. 1, a CaM-specific antibody detected a distinct band at ~19 kDa, which corresponds to the Ca²⁺-bound mobility of CaM under denaturing conditions. This band was not present when immunoprecipitation was performed with non-specific-IgG. The density of the CaM band was similar to or slightly decreased in samples treated with the 5-HT₁A receptor-selective agonist 8-OH-DPAT. These results suggest that in CHO cells CaM is constitutively complexed with the 5-HT₁A receptor.

**Identification of Putative CaM Binding Regions in the Third Intracellular Loop of the 5-HT₁A Receptor—**Using a computer search program that identifies putative CaM-binding sites based on evaluation criteria such as hydrophathy, α-helical propensity, residue charge, helical class, residue weight, and hydrophobic residue content (37), we identified two putative CaM binding regions in the protein sequence of the 5-HT₁A receptor. Both putative CaM-binding domains were localized to the third intracellular loop of the receptor, at the N- and C-terminal juxtamembrane regions (Fig. 2A). As a general rule, CaM binding regions are characterized by the presence of several hydrophobic residues interspersed with several positively charged residues, often forming amphipathic α-helices (38, 39). CaM binding regions described to date have been divided into several motifs based on the distance between key hydrophobic residues. The N-terminal CaM binding region was identified as a 1-12 motif, with key hydrophobic residues at positions 1 and 12, whereas the C-terminal CaM binding region in the 5-HT₁A receptor was classified as a 1-8-14 motif, characterized by hydrophobic residues at positions 1, 8, and 14 (Fig. 2B). Further analysis of the 5-HT₁A receptor CaM binding regions revealed that they could be aligned with other well defined CaM binding motifs from other proteins. Some examples are provided in Fig. 2B. Using computer modeling (37), we created a helical wheel representation of the putative N-terminal and C-terminal 5-HT₁A receptor CaM-binding sequences (data not shown). Both helical wheel diagrams showed clusters of positively charged amino acids on one side of the α-helix, with mostly hydrophobic amino acids concentrated on the opposite side, typical of the amphipatic nature of CaM-binding sites.

**CaM Binds to Peptides Derived from the N- and C-terminal Ends of the Third Intracellular Loop of the 5-HT₁A Receptor—**We synthesized peptides encompassing amino acids 215–237 (i3N) and 328–350 (i3C) of the 5-HT₁A receptor. To test if either or both regions are capable of interacting with CaM, we slot-blotted increasing amounts of each peptide (1–100 nmol) to PVDF membranes and incubated them with biotinylated CaM. We also tested a negative control, 17-amino acid peptide corresponding to the CaM binding region of myosin light chain kinase (MLCK), which contains an amino acid change that disrupts CaM binding. Both the i3N and i3C peptides bound biotinylated CaM in the presence of 0.1 mM Ca²⁺, with the N-terminal peptide binding with apparent greater affinity.
whereas no binding was observed for the MLCK control peptide (Fig. 3). Binding was inhibited by removing Ca$^{2+}$ from the incubation mixture and replacing it with 1 mM EGTA, although moderate binding to the i3N peptide in the presence of EGTA was still observed.

To determine if the peptides can form high affinity complexes with CaM, we performed polyacrylamide gel electrophoresis in the presence of 4 M urea. The presence of 4 M urea dissociates lower affinity and nonspecific protein-protein interactions ($K_D > 100$ nM). A constant amount of CaM (300 pmol) was incubated with increasing amounts (75–3000 pmol) of the peptides and subsequently analyzed by nondenaturing gel electrophoresis (Fig. 4). In the presence of Ca$^{2+}$, the addition of the 5-HT$_{1A}$ i3N (Fig. 4B), but not the i3C peptide (Fig. 4C), produced an upward shift in the migration of CaM, as did a peptide corresponding to the CaM binding region of MLCK (Fig. 4A). In contrast, no peptide-CaM complexes were formed when Ca$^{2+}$ was chelated with EGTA. These data suggest that the i3N peptide binds CaM with high affinity (better than 100 nM), whereas the i3C peptide likely binds with significantly lower affinity. The mobility shift induced by the MLCK peptide was complete at a peptide:CaM molar ratio of 5:1. The 5-HT$_{1A}$ i3N peptide induced gel shifts that were incomplete up to a peptide:CaM molar ratio of 10:1. The inability of the 5HT$_{1A}$ i3N peptide and even the MLCK peptide, which is known to bind CaM with very high affinity (~6 pM), to cause complete shifts at a molar ratio of 1:1 is in line with previous reports in which peptides from the CaM binding regions of the mGluR5 (40) and D$_2$. 

![Diagram](image_url)
Fig. 3. Interaction of 5-HT1A receptor synthetic peptides with biotinylated CaM. Increasing amounts (1, 3, 10, 30, and 100 nmol) of peptides were slot-blotted to PVDF membrane and subjected to overlay with 0.5 μg/ml biotinylated CaM in the presence of either 0.1 mM CaCl2 or 1 mM EGTA as described under “Experimental Procedures.” Bound biotinylated CaM was then detected with alkaline phosphatase-conjugated avidin and a chemiluminescent reagent. Biotinylated CaM bound to both the i3N and i3C peptides but not to the MLCK control peptide, in the presence of Ca2+. CaM binding was significantly reduced in the presence of EGTA. Data are representative of three separate experiments.

Fig. 4. Complex formation between CaM and 5-HT1A receptor i3 loop synthetic peptides. CaM (300 pmol) was incubated with increasing amounts (0, 75, 150, 300, 600, 1500, and 3000 pmol) of peptide in the presence of 4 M urea and either 0.1 mM CaCl2 or 1 mM EGTA. Complexes were then resolved by nondenaturing polyacrylamide gel electrophoresis, again in the presence of 4 M urea and either CaCl2 or EGTA. Gels were stained with GelCodeBlue staining reagent. The authentic MLCK peptide (A) and i3N (B), but not the i3C (C) peptide caused a shift in the migration of CaM in the presence of Ca2+, but not in the presence of EGTA. Data are representative of three separate experiments.

dopamine receptor (29) were also unable to cause complete shifts under the same conditions.

Surface Plasmon Resonance—Interactions between CaM and the 5-HT1A receptor i3N and i3C peptides were further examined by SPR using BIAcore 2000 sensor technology. The peptides were immobilized to a carboxymethylated CM5 sensor chip using amine-coupling chemistry at typical densities of 400–720 resonance units. No significant binding was observed between CaM and the chip surface or between CaM and the negative control MLCK mutant peptide (data not shown). In contrast, CaM rapidly and reversibly interacted with both the i3N and i3C peptides in the presence of Ca2+, but not in the presence of EGTA, showing typical saturation curves (Fig. 5). Kinetic data yielded complex results with neither i3N or i3C peptides fitting accurately to a 1:1 Langmuir model. This was in part due to very high on/off rates, which were beyond the limitations of the software. Interactions were instead evaluated using steady-state analysis in which equilibrium responses (R0eq) were plotted against CaM concentration. Affinity calculations yielded KD values of ~110 nM and 700 nM for the i3N-CaM and i3C-CaM interactions, respectively. These results are consistent with our gel shift studies shown in Fig. 4.

Interaction of 5-HT1A i3 Loop Peptides with Dansyl-CaM—To evaluate the binding affinities of i3N and i3C peptides with CaM in solution, we measured changes in the fluorescence emission spectrum of dansyl-CaM. Ligand binding to dansyl-CaM is thought to shield the fluorophore from the aqueous environment, and this can be detected as an enhancement in fluorescence emission and a blue shift of the emission peak to a lower wavelength. This is clearly illustrated in Fig. 6A. In the absence of Ca2+, dansyl-CaM displays weak fluorescence, with an emission peak of ~520 nm. In the presence of Ca2+, the fluorescence emission is enhanced and shifted to a lower wavelength. Addition of i3N and i3C peptides further enhanced dansyl-CaM fluorescence and shifted the emission peak to below 500 nm, whereas the negative control MLCK mutant peptide was completely ineffective (data not shown). We then examined the concentration-dependent binding of i3N and i3C peptides to dansyl-CaM. Fig. 6 (B and C) shows the fluorescence emission of various concentrations of dansyl-CaM (0.1, 0.2, 0.3, 0.4, and 0.5 μM) incubated with increasing concentrations of i3N or i3C peptides in the presence of Ca2+. Data points were fit by nonlinear least squares analysis to the Boltzmann one-site binding equation. Notably, apparent affinity measurements were dependent on the concentration of dansyl-CaM, with higher concentrations of dansyl-CaM requiring increased concentrations of peptide to induce enhancements in fluorescence. This can be attributed to depletion of free peptide, a phenomenon that is most apparent at low concentrations of dansyl-CaM. This can be corrected by calculating affinities over a range of dansyl-CaM concentrations and extrapolating to an infinitely low dansyl-CaM concentration, at which point depletion is essentially nonexistent. Accordingly, affinity measurements derived from peptide binding curves were plotted against the concentration of dansyl-CaM (Fig. 6, D and E). These values fell onto straight lines, which when extrapolated to the y-axis, gave apparent affinities of 87 ± 23 nM and 1.70 ± 0.16 μM for the i3N and i3C peptides, respectively. These values are in line with estimated affinities that were determined using SPR.

Association of 5-HT1AR-YFP and CaM-RLuc in CHO Cells Assessed by BRET—Having shown that CaM can interact with synthetic peptides derived from the 5-HT1A receptor i3 loop, we next set out to show that this interaction occurs in vivo. This type of analysis has been lacking in previously published reports of CaM-GPCR interactions, which have relied entirely on the use of solubilized cellular extracts and in vitro binding experiments. To demonstrate close physical interaction be-
tween the 5-HT1A receptor and CaM within living cells, we used bioluminescence resonance energy transfer (BRET), a technique that detects close proximity of proteins using energy transfer between luminescent and fluorescent tags. We tagged the C terminus of the full-length human 5-HT1A receptor with YFP and stably expressed it in CHO cells. These cells were then transfected with CaM fused to either the N or C terminus of RLuc, and the BRET ratio was determined as the ratio of light emitted by YFP (525 ± 25 nm) over that emitted by RLuc (460 ± 25 nm) following the addition of the membrane-permeable RLuc substrate, coelenterazine. Before each experiment, total YFP fluorescence was measured (excitation = 485 ± 15 nm) to verify equal protein expression between individual constructs and between experiments. As shown in Fig. 7, cells transfected with RLuc alone produced an insignificant BRET signal (BRET ratio = 0.03), which can primarily be attributed to nonspecific energy transfer and bleed-through of the luminescent signal into the fluorescent filter-set. In contrast, a significant BRET signal was observed in cells transfected with CaM fused at either the N terminus (BRET ratio = 0.18) or C terminus (BRET ratio = 0.15) of RLuc. The fact that both fusions were capable of producing a significant BRET signal is not surprising, in that CaM is a relatively symmetrical protein with N- and C-terminal EF hand motifs. These data strongly support the idea that CaM and the 5-HT1A receptor interact with each other in a constitutive manner, as was suggested by the results of the co-immunoprecipitation experiments shown in Fig. 1.

As shown previously, co-immunoprecipitation of CaM with the 5-HT1A receptor in CHO cells was not dependent on agonist treatment. However, co-immunoprecipitation often does not readily detect differences in protein association, particularly when the associations are weak or transient. We used BRET to determine whether receptor activation plays a role in the CaM-5-HT1A receptor interaction. Exposure of cells to 1 μM 8-OH-DPAT for 5 min had no effect on the constitutive BRET signal (Fig. 7B). To further characterize the BRET signal, cells were incubated for 20 min in the presence of several chemical inhibitors of CaM. A significant decrease in the BRET ratio was observed in the presence of three structurally distinct CaM antagonists, W-7, ophiobolin A, and calmidazolium chloride. In addition, the cell-permeable Ca2+ chelator, BAPTA-AM also decreased the BRET ratio. These data suggest that CaM needs
to be in a Ca\(^{2+}\)-bound and active state to interact with the 5-HT\(_{1A}\) receptor.

**Effect of Phosphorylation of 5-HT\(_{1A}\) Receptor i3 Loop Peptides on CaM Binding.**—Like many G protein-coupled receptors, the 5-HT\(_{1A}\) receptor can be modulated by kinase-directed phosphorylation (36, 41). Protein kinase C (PKC), protein kinase A, and G protein-coupled receptor kinase have each been implicated in the desensitization and phosphorylation of the 5-HT\(_{1A}\) receptor. Of the four putative PKC sites identified in the 5-HT\(_{1A}\) receptor, two are located within the i3N

![Diagram of fluorescence emission spectra](image-url)
The i3N and i3C regions correspond to typical CaM-binding domains, forming amphipathic α-helices composed of positively charged and hydrophobic residues, with few or no negatively charged residues. Phosphorylation at the target threonine residues in the i3N and i3C residues (Fig. 8A) adds a negative charge and would likely influence CaM binding. We tested this hypothesis using synthetic peptides (i3N-P and i3C-P), which are identical to the previously used peptides but are phosphorylated at threonines 229 and 343, respectively. The fluorescence emission spectrum of dansyl-CaM was evaluated in the presence of each phosphorylated peptide. As shown in Fig. 8B, the i3N-P peptide produced a significantly reduced shift in the emission peak of dansyl-CaM as compared with the i3N peptide. Likewise, the i3C-P peptide also produced a reduced shift in the emission peak of dansyl-CaM (Fig. 8C). These results suggest that the phosphorylated peptides have significantly reduced affinities for CaM compared with their unphosphorylated counterparts and that the association of CaM with the i3N and i3C regions of the 5-HT1A receptor may be regulated by phosphorylation reactions.

Interaction of CaM with the i3N and i3C Peptides Antagonizes Phosphorylation by Protein Kinase C—Activation of PKC by phorbol esters induces a rapid phosphorylation of the 5-HT1A receptor (36). This phosphorylation is associated with desensitization of multiple signaling pathways, including the inhibition of adenylyl cyclase (36), K+ channel regulation (42), and hydrolysis of phosphoinositides (43). Lembo et al. (43) showed that this desensitization could be reversed by mutation of three putative PKC sites in the i3 loop of the 5-HT1A receptor, two of which correspond to the sites in the i3N and i3C peptides. We tested the i3N and i3C peptides as substrates for PKC phosphorylation using in vitro phosphorylation assays. Purified rat brain PKC (comprised primarily of PKC-α and PKC-β isozymes) readily phosphorylated the i3N peptide ($K_m = 4.21 \pm 1.11 \mu M; V_{max} = 35.35 \pm 4.89 \text{ nmol/min/mg}$) and i3C peptide ($K_m = 7.10 \pm 1.32 \mu M; V_{max} = 10.10 \pm 1.00 \text{ nmol/min/mg}$) as shown in Fig. 9. These values are similar to values described for other PKC substrates (44). To determine whether
CaM can compete with PKC for access to the i3N and i3C phosphorylation sites, we repeated the *in vitro* phosphorylation assays in the presence of increasing concentrations of CaM. Phosphorylation of both peptides was dose-dependently decreased by CaM. Not surprisingly, the peptides were differentially sensitive to CaM binding, with phosphorylation of the i3N peptide being almost completely inhibited in the presence of 20 μM CaM, whereas phosphorylation of the i3C peptide was inhibited by 75% in the presence of 120 μM CaM. These data suggest that the association of CaM with the i3N and i3C peptides can attenuate phosphorylation by PKC.

**DISCUSSION**

The purpose of this study was to characterize the interactions of CaM with two putative CaM-binding domains in the 5-HT1A receptor. We have used a variety of methods to characterize the interactions in cells and *in vitro*, resulting in several novel observations. We have reported in this work that the serotonin 5-HT1A receptor contains two putative CaM-binding sites in the juxtamembrane regions of the third intracellular loop, spanning amino acids 215–237 (i3N) and 328–350 (i3C). We used blot overlays, gel shifts, and SPR to document interactions between both peptides and CaM and calculated affinities of 100 nM and 1 μM, respectively. To determine whether interactions between the holo-5-HT1A receptor and CaM might occur in CHO cells, we used co-immunoprecipitation and BRET experiments. We showed that CaM immunoprecipitates with the 5-HT1A receptor and resides in overlapping domains of CHO cells with the 5-HT1A receptor, as determined by BRET. The presence of a strong BRET signal between CaM and the 5-HT1A receptor suggests that the two proteins reside within 50 Å of each other, and are likely to physically interact. To our knowledge, this represents the first example of a CaM-GPCR interaction shown to occur in living cells. The interactions between the 5-HT1A receptor and CaM depend on Ca2+ binding and activation of CaM in that the Ca2+-chelator, BAPTA-AM, and three CaM inhibitors can abrogate the BRET signal. Moreover, the interaction between CaM and the 5-HT1A receptor appears to be constitutive, in that agonist treatment does not alter significantly either the amount of CaM in 5-HT1A receptor immunoprecipitates, or the BRET signal between 5-HT1A receptor-eYFP and CaM-luciferase.

A wide variety of CaM-binding domains have been described in various proteins. The two putative CaM-binding sites parallel typical CaM-binding domains, forming predicted amphipathic α-helices composed of basic and hydrophobic amino acid residues. In addition, both sites could be aligned with well established CaM binding motifs based on the location of key hydrophobic residues. The CaM binding region in the C-terminal portion of the the 5-HT1A receptor i3 loop was classified as a 1-8-14 motif, in that it aligns well with other well defined 1-8-14 CaM binding motifs, such as those for neuronal and endothelial nitric-oxide synthase. The putative CaM-binding domain in the N terminus of the 5-HT1A receptor i3 loop is similar in location to a CaM-binding domain identified in the

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**Fig. 9. Effect of CaM on phosphorylation of i3N and i3C peptides by PKC.** A and C, increasing concentrations of i3N (0–5 μM) and i3C (0–10 μM) peptides were incubated with [γ-32P]ATP and purified rat brain PKC for 1 h at 30 °C and incorporated radioactivity was measured as described in "Experimental Procedures." Data was expressed as the percentage of maximum phosphorylation for each peptide and fit to the one-site binding equation. Data and K_{d}/V_{max} values represent the mean ± S.E. for at least three separate experiments for each peptide. B and D, *in vitro* phosphorylation assays were repeated in the presence of varying concentrations of CaM (0, 4, 10, and 20 μM for i3N; 0, 40, 80, and 120 μM for i3C). Results shown represent the mean ± S.E. for at least three separate experiments with each peptide.
D$_2$ dopamine receptor, but the two sites show little sequence homology. The N-terminal 5-HT$_{1A}$ receptor i3 loop binding region was classified into a relatively newly defined class of CaM binding motifs characterized by core hydrophobic residues at positions 1 and 12, and this peptide was well aligned with other 1-12 motif-containing proteins such as caldesmon, the N-methyl-D-aspartate receptor, and the phosphodiesterases. Although originally classified as a type 1B 1-8-14 motif (sequence read in the C- to N-terminal direction), the D$_2$ dopamine CaM binding region has been re-classified as a basic motif, characterized by a preponderance of positively charged lysine and arginine residues within the CaM-binding sequence.

The role of the 5-HT$_{1A}$ receptor CaM-binding sites is unknown; however, the juxtamembrane N- and C-terminal regions of the 5-HT$_{1A}$ receptor third intracellular loop have been implicated in numerous receptor functions. Both sites reside in regions of the i3 loop important for contacting heterotrimERIC G proteins, with work by Albert et al. suggesting a model in which amphiphilic a-helical regions of the i2 and i3 loop align to form hydrophobic G protein interaction sites (45). Structural predictions based on this model would likely suggest an interaction with CaM as well, based on similar hydrophobic-binding pockets. This is supported in part by studies showing that certain CaM-binding motifs can serve as substrates for rat brain PKC-induced phosphorylation (46). Moreover, CaM decreases the ability of PKC to phosphorylate either peptide in a concentration-dependent manner. Furthermore, interaction of CaM with the a-opioid (OP$_3$) and D$_2$ dopamine receptors has previously been shown to regulate G protein activation (29). In the case of the OP$_3$ receptor, CaM was shown to compete with G protein binding, whereas in the case of the D$_2$ receptor, CaM was shown to block the receptor-operated G protein activation switch without uncoupling the receptor from G protein. It is likely that CaM plays similar roles in 5-HT$_{1A}$ receptor signaling to G protein subunits.

The 5-HT$_{1A}$ receptor i3N and i3C regions also contain phosphorylation sites previously implicated in receptor desensitization (36, 41, 49). Lembo et al. showed that phorbol ester-induced receptor desensitization could be reversed by mutation of three putative PKC sites in the i3 loop of the 5-HT$_{1A}$ receptor (43). Two of these sites, R277KTVK$_{281}$ and R341KTVK$_{345}$, reside in the i3N and i3C regions, respectively. New data in this report shows that both 5-HT$_{1A}$ receptor i3N and i3C peptides can serve as substrates for rat brain PKC-induced phosphorylation. Moreover, CaM decreases the ability of PKC to phosphorylate either peptide in a concentration-dependent manner. This finding suggests that constitutive binding of CaM to either juxtamembrane region of the 5-HT$_{1A}$ receptor i3 loop could prevent or greatly attenuate phosphorylation of the receptor by PKC, and if so, then CaM should also serve to attenuate PKC-induced desensitization of the receptor.

Significant evidence suggests that phosphorylation of CaM-binding domains and the binding of CaM to a target protein can be mutually exclusive and in some cases antagonistic. Phosphorylation of target proteins by PKC has been shown to reduce binding of CaM to myosin 1 (50), the plasma membrane Ca$^{2+}$ pump (51, 52), and calcium transport-like (CaT1) protein (53). Likewise, binding of CaM to neural tissue-enriched acidic protein (NAP-22) inhibits phosphorylation by PKC (54). Minakami et al. showed that phosphorylation and CaM binding to peptides derived from the mGluR5 are antagonistic, indicating a possible regulatory role for CaM in GPCR signaling and responses (40). We have shown that phosphorylated peptides derived from the N- and C-terminal CaM binding regions of the 5-HT$_{1A}$ receptor i3 loop have weakened affinities for CaM. In addition, association of CaM with the i3N and i3C peptides inhibited phosphorylation by PKC. These data suggest that PKC-induced phosphorylation and CaM binding to the juxtamembrane regions of the 5-HT$_{1A}$ receptor are antagonistic. Interestingly, the affinities of the i3N and i3C peptides for CaM (~100 nM and 1 pM) are both significantly stronger than their corresponding affinities for PKC (~5 pM and 1 pM). This indicates that CaM could reasonably be expected to blunt phosphorylation of both i3N and i3C peptides by PKC within the 5-HT$_{1A}$ hororeceptor in vivo.

Taken in aggregate, our findings implicate constitutive direct binding of CaM to juxtamembrane regions of the i3 loop of the 5-HT$_{1A}$ receptor in blunting the phosphorylation of the receptor by PKC. The findings further suggest that CaM plays a role in preventing or attenuating PKC-induced (heterologous) desensitization of the 5-HT$_{1A}$ receptor in CHO cells. Indeed, the 5-HT$_{1A}$ receptor in CHO cells is relatively resistant to desensitization in that pretreatment with phorbol esters shifts the concentration-response curve for the inhibition of adenyl cyclase less than one log to the right, without altering the maximum response (36).

Our findings do not rule out other roles for CaM in regulating the propagation of signals from the 5-HT$_{1A}$ receptor or in regulating other processes such as receptor internalization. In that regard, we have previously shown that CaM is critical for the 5-HT$_{1A}$ receptor to activate ERK, in a process involving agonist-induced receptor internalization (30). Similarly, Melien et al. (55) showed that ERK activation induced by norepinephrine and prostaglandin P2o was sensitive to pharmacological inhibitors of CaM in hepatocytes. Likewise, CaM has been shown to mediate activation of ERK by the a-opioid receptor through a pathway involving the transactivation of the epidermal growth factor (EGF) receptor (56). A role for CaM has also been proposed in the general process of receptor endocytosis and trafficking in numerous models, including yeast (57) and human epithelial cells (32). Our group reported a role for CaM in 5-HT$_{1A}$ receptor internalization, a required step for MEK and subsequent ERK activation (30). Conversely, pharmacological inhibitors of CaM have been shown to inhibit recycling and degradation of the EGF receptor without affecting its internalization, resulting in the accumulation of receptors in enlarged endosomal structures (48). Although roles for direct binding of CaM to a GPCR in signal initiation or in internalization and/or trafficking of the receptor have yet to be reported, we speculate that these effects are possible, if not likely.

In conclusion, we have identified the presence of two distinct CaM-binding sites in the serotonin 5-HT$_{1A}$ receptor. These sites reside in juxtamembrane regions at the N and C termini of the large third intracellular loop of the receptor. In addition, we have shown that the 5-HT$_{1A}$ receptor interacts with CaM in intact, living cells. Finally, we have shown that binding of CaM and phosphorylation by PKC of the 5-HT$_{1A}$ receptor third intracellular loop juxtamembrane peptides are antagonistic in vitro. These results suggest that CaM may serve as a critical regulator of 5-HT$_{1A}$ receptor function.

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Calmodulin Interacts with the Third Intracellular Loop of the Serotonin 5-Hydroxytryptamine 1A Receptor at Two Distinct Sites: PUTATIVE ROLE IN RECEPTOR PHOSPHORYLATION BY PROTEIN KINASE C

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