Interaction of Calmodulin with the Serotonin 5-Hydroxytryptamine2A Receptor

A PUTATIVE REGULATOR OF G PROTEIN COUPLING AND RECEPTOR PHOSPHORYLATION BY PROTEIN KINASE C*

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The 5-hydroxytryptamine2A (5-HT2A) receptor is a Gα13-coupled serotonin receptor that activates phospholipase C and increases diacylglycerol formation. In this report, we demonstrated that calmodulin (CaM) co-immunoprecipitates with the 5-HT2A receptor in NIH-3T3 fibroblasts in an agonist-dependent manner and that the receptor contains two putative CaM binding regions. The putative CaM binding regions of the 5-HT2A receptor are localized to the second intracellular loop and carboxyl terminus. In an in vitro binding assay peptides encompassing the putative second intracellular loop (i2) and carboxyl-terminal (ct) CaM binding regions bound CaM in a Ca2+-dependent manner. The i2 peptide bound with apparent higher affinity and shifted the mobility of CaM in a nondenaturing gel shift assay. Fluorescence emission spectral analyses of dansyl-CaM showed apparent K_D values of 65 ± 30 nM for the i2 peptide and 168 ± 38 nM for the ct peptide. The ct CaM-binding domain overlaps with a putative protein kinase C (PKC) site, which was readily phosphorylated by PKC in vitro. CaM binding and phosphorylation of the ct peptide were found to be antagonistic, suggesting a putative role for CaM in the regulation of 5-HT2A receptor phosphorylation and desensitization. Finally, we showed that CaM decreases 5-HT2A receptor-mediated [35S]GTPγS binding to NIH-3T3 cell membranes, supporting a possible role for CaM in regulating receptor-G protein coupling. These data indicate that the serotonin 5-HT2A receptor contains two high affinity CaM-binding domains that may play important roles in signaling and function.

The serotonin 5-hydroxytryptamine2A (5-HT2A) receptor is a prototypical G protein-coupled receptor (GPCR) that plays diverse roles in both the central nervous system and peripheral vasculature. In the central nervous system these receptors are widely distributed, being expressed in the neocortex, claustrum, mammillary nuclei, basal ganglia, and anterior cingulate cortex (1). 5-HT2A receptors are also highly expressed in vascular smooth muscle and renal mesangial cells, where they mediate contraction and proliferation (2–4), and platelets, where they contribute to aggregation and adherence (5), as well as in kidney (6) and skeletal muscle (7, 8). The heterogeneous expression of the 5-HT2A receptor is accompanied by a diverse array of pathophysiological implications for 5-HT2A receptor signaling, including roles in sleep, hallucinogenesis, schizophrenia, appetite control, neuroendocrine secretions, hypertension, and depression (9–12). The 5-HT2A receptor is involved in the mechanism of action of hallucinogens, atypical neuroleptics, antidepressants, and other psychoactive drugs.

5-HT2A receptors signal primarily through heterotrimERIC proteins of the Gα13 subfamily to the activation of phospholipase C, and the subsequent formation of diacylglycerol and activation of protein kinase C (13–15). Other second messengers and effectors regulated by the 5-HT2A receptor include phospholipase A2 (16–18), phospholipase D (19), Ca2+ channels (20–22), reactive oxygen and nitrogen species (23, 24), and Na+/H+ exchange (25, 26). In essentially all cases, activation of downstream signaling molecules has been shown to be mediated by heterotrimeric G proteins.

Calmodulin (CaM) is a small (148 amino acids, ~17 kDa), soluble protein, which functions as the major calcium sensor in most cells (27). As a prototypical member of the EF-hand family of Ca2+-binding proteins, CaM can bind up to four Ca2+ ions, which subsequently extend the protein to expose hydrophobic patches capable of binding cellular targets (28, 29). These targets number well over one hundred and include enzymes, ion channels, transcription factors, and cytoskeletal proteins. Recently, CaM has been shown to bind to several plasma membrane receptors, including the epidermal growth factor receptor, platelet glycoprotein VI, and some GPCRs (30, 31). The first GPCR that was shown to interact with CaM was the metabotropic glutamate subtype 5 receptor, which contains a nase; GPCR, G protein-coupled receptor; i2, 20-amino acid peptide fragment from the second intracellular loop of the human serotonin 5-HT2A receptor; ct, 20-amino acid peptide fragment from the juxtamembrane region of the carboxyl terminus of the human serotonin 5-HT2A receptor; MEK, mitogen-activated protein kinases kinase; MOPS, 3-(N-morpholino)propanesulfonic acid; PKA, protein kinase A; PKC, protein kinase C; PVPF, polyvinylidene fluoride; GTPγS, guanosine 5’-3-O-(thio)triphosphate; ct-P, a synthetic peptide identical to the ct peptide but containing a phosphorylated threonine at residue 386.
CaM-binding site in a region of the extended carboxyl terminus of the receptor also known to bind G protein βγ subunits (32). Subsequently, the interaction of CaM with the third intracellular loop of D_{1}-dopamine and μ-opicoid receptors was shown to regulate receptor coupling to Pertussis-toxin-sensitive heterotrimeric G proteins (33, 34), whereas Nichols et al. (35) showed that the interaction of CaM with the V_{y}-vasopressin receptor modulates ligand-induced elevations in intracellular calcium. Our group recently showed that CaM interacts with the G_{i3}-coupled 5-HT_{2A} receptor at two distinct sites in the receptor third intracellular loop in regions that overlap with protein kinase C phosphorylation sites (36). Interestingly, binding of CaM to synthetic peptides corresponding to these regions prevented phosphorylation of the peptides by protein kinase C. Furthermore, binding of CaM to the 5-HT_{2A} receptor decreases G protein coupling as assayed by GTPγS binding to crude membrane preparations. These examples indicate that CaM interactions may play important and diverse roles in GPCR signaling.

CaM has previously been shown to be a major target for 5-HT_{2A} receptor signaling. Agonist-mediated up-regulation of the 5-HT_{2A} receptor is dependent upon both CaM and CaM-dependent kinase 2 (37). Berg et al. (38) showed that CaM is required for 5-HT_{2A} receptor-mediated activation of AMPA receptors in A1A1 cells. Likewise, the CaM-dependent enzymes CaM-dependent kinase 2 and calcineurin (a CaM-dependent phosphatase) play roles in 5-HT-induced cyclooxygenase 2 mRNA expression in renal mesangial cells (39, 40). Finally, the 5-HT_{2A} receptor activates signal-regulated protein-activated protein kinases through the intermediate action of CaM^2/ CaM (1).

Our group previously reported that CaM interacts with the G_{i3}-coupled 5-HT_{2A} receptor third intracellular loop at two distinct sites and that the interaction of CaM with those sites may play a role in regulating receptor phosphorylation and desensitization induced by protein kinase C (36). We were interested in establishing whether the G_{i3}-coupled 5-HT_{2A} receptor could also interact with CaM, and if so, what consequences this binding might have on receptor function. In the current work, we report that, in NIH-3T3 fibroblasts, CaM co-immunoprecipitates in an agonist-dependent manner with the 5-HT_{2A} receptor. A search of the primary sequence revealed the presence of CaM-binding motifs, located in the second intracellular loop and the juxtamembrane region of the carboxyl terminus of the receptor. Both motifs contain consensus phosphorylation sites and are important for G protein coupling, indicating that interaction of CaM with those sites could play roles in regulating receptor function. In this report, we sought to characterize the putative CaM-binding sites in the 5-HT_{2A} receptor and to determine their functional significance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified bovine brain calmodulin, biotinylated calmodulin, and purified rat brain PKC were obtained from Calbiochem (La Jolla, CA). Rabbit polyclonal antibody directed against the amino terminus (amino acids 22–41) of the rat 5-HT_{2A} receptor was purchased from Calbiochem. Rabbit polyclonal antibody directed against the carboxyl terminus of the rat 5-HT_{2A} receptor (amino acids 428–443) was kindly provided by Ryan Strachan (Cleveland, OH) and Dr. Bryan Roth (Cleveland, OH). Mouse anti-CaM antibodies were from Upstate Biotechnology (Charlottesville, VA).Dansyl chloride was purchased from Molecular Probes (Eugene, OR) and [35S]GTPγS was purchased from PerkinElmer Life Sciences.

**Synthesis of 5-HT_{2A} Receptor Peptides**—Peptides derived from the amino acid sequence of the second intracellular loop (amino acids 183–202, HSRFPNSRTKAFKHLHAVWITI) and carboxyl terminus (amino acids 377–396, PLVYTFLNKYRRSAFIRYIQ) of the human 5-HT_{2A} receptor were synthesized using standard solid-phase methods on a Rainin PS3 automated peptide synthesizer by the Medical University of South Carolina Peptide Synthesis Facility. Peptide sizes and purity were verified using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. When necessary, peptides were purified on a Waters Delta Prep 3000 chromatography system using a C-18 silica column and elution across a linear gradient of acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid (Emory University Microchemical Facility, Atlanta, GA).

**Cell Culture—**NIH-3T3 cells were maintained in minimum essential medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). Cells were incubated 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).

**Immunoprecipitation—**Quiescent NIH-3T3 cell monolayers grown on 100-mm dishes were treated with agonist (1 μM 5-HT) or vehicle for the appropriate time then lysed in 500 μl of modified radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 1 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Lysates were homogenized, clarified by centrifugation at 14,000 g for 15 min, and subjected to immunoprecipitation with protein A/G-agarose for 30 min at 4 °C. Pre-cleared lysates were then incubated with commercial anti-5-HT_{2A} receptor antibody overnight at 4 °C. Immunoprecipitates were then washed three times with radioimmunoprecipitation assay buffer and twice with phosphate-buffered saline. Immunoprecipitates were then resuspended in Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Gels were analyzed by immunoblot with either anti-CaM antibody or a second anti-5-HT_{2A} receptor antibody.

**Gel Shift Assays—**Gel shift analyses of CaM-peptide complexes were performed using urea-polyacrylamide gel electrophoresis, as described by Erickson-Vitanen and Delgrado (41). Reactions (30 μl of total volume) containing 300 pmol of CaM (~5 μg) and increasing amounts of 5-HT_{2A} peptide (0–200 pmol) were incubated in 100 mM NaCl, 4 mM 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 mM urea and either 0.1 mM CaCl₂ or 1 mM EGTA in the running buffer. Protein was visualized by staining with Gel-code blue (Pierce) 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 100 mM Tris-HCl, pH 7.5, containing 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. Biotinylated membranes were washed 3× in PBS, then washed with PBS containing 0.1% Tween 20, followed by incubation with alkaline phosphatase-conjugated avidin for 1 h at room temperature. Detection was with a chemiluminescent reagent.

**Fluorometric Measurements with Dansyl-CaM—**Dansyl-CaM was synthesized according to the method of Bertrand et al. (42). Briefly, 10 mg of CaM was incubated with ~1 mg of dansyl chloride for 1 h at 4 °C. Dansyl-CaM was purified from unincorporated dye using a Centricron™ 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 400–600 nm using a SLM 8000™ C spectrophuorometer (AMINCO-Bowman) with an excitation wavelength of 340 nm. Test peptides (0–2 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 K₈₅ values for each concentration of dansyl-CaM were fit to the Hill equation by linear regression to calculate the affinities.

**In Vitro Kinase Assays—**Thirty-five ng (~0.56 unit) of purified rat brain PKC was incubated with increasing concentrations of ct peptide (0–6 μM) in kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM CaCl₂, 0.1 mg/ml phosphatidylyserine, 0.01 mg/ml diacylglycerol, 100 μM [γ-32P]ATP) in a total volume of 25 μl. In some cases, purified bovine brain calmodulin (0–20 μM) was added to reactions. Assays were started by the addition of 5 μM of [γ-32P]ATP,
buffer and resuspended in 50 mM Tris HCl, pH 7.4, 2.5 mM MgCl2 at a

and then incubated at 30 °C for 1 h. Reactions were then transferred to

**Preparation of NIH-3T3/5-HT2A Receptor Cell Membranes—** NIH-3T3 cells overexpressing the human 5-HT2A receptor (8 pmol/mg) were kindly provided by Dr. Elaine Sanders-Bush (Nashville, TN). Cells were grown to confluence in 100-mm dishes, incubated in serum-free medium for 16–24 h, scraped into lysis buffer (100 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM EGTA, 1 µg/ml each of aprotinin, leupeptin, and pepstatin), then subjected to homogenization by twenty strokes in a Dounce homogenizer. Lysed cells were centrifuged at 1,000 × g for 10 min to remove whole cells and nuclear debris, and the supernatant was centrifuged at 37,000 × g for 20 min. The resulting pellet was washed twice in lysis buffer and resuspended in 50 mM Tris HCl, pH 7.4, 2.5 mM MgCl2 at a final protein concentration of ~4 mg/ml. Membranes were frozen in liquid nitrogen, and stored at −80 °C for future use.

**[^35S]GTPγS Binding to NIH-3T3/5-HT2A Receptor Cell Membranes—** The protocol for measurement of [^35S]GTPγS binding to crude cell membranes was adapted from the methods of Cussac et al. (44) and Adlersberg et al. (44). Briefly, 50-µg aliquots of NIH-3T3/5-HT2A receptors cell membranes were added to 500 µl of assay buffer (20 mM HEPES, pH 7.4, 6 mM MgCl2, 50 µM GDP, 100 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM CaCl2), in the presence or absence of increasing concentrations of i2 or ct peptides, CaM, or S-100 protein, and then incubated for 15 min to allow for GDP loading. Reactions were initiated by the addition of [^35S]GTPγS (1250 Ci/mmol) to a final concentration of 200 pM, in the presence or absence of 10 µM 5-HT, and incubated for an additional 1 h at 25 °C. Bound and free nucleotides were then separated by filtration over glass fiber filters, and bound radioactivity was determined by 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 5-HT2A Receptor in NIH-3T3 Fibroblasts—Several groups, including ours, have previously shown that CaM plays a role in numerous 5-HT2A receptor signaling pathways, including the activation of mitogen-activated protein kinases (1) and Na+/H+ exchange (26, 45). As a receptor that couples to Gq/11 type proteins, the 5-HT2A receptor is capable of stimulating phosphoinositide turnover and to subsequently increase intracellular Ca2+ levels. We wondered whether the 5-HT2A receptor might exert some of its Ca2+-sensitive and/or -insensitive intracellular effects by directly interacting with CaM. Other GPCRα, including the D2 dopamine (32), µ-opioid receptors (34), and 5-HT1A receptor (36), have been shown to directly interact with CaM through their third intracellular loops. We treated wild-type NIH-3T3 cells and NIH-3T3 cells overexpressing the 5-HT2A receptor, with 1 µM serotonin for 5 min, and immunoprecipitated the receptor with a commercial 5-HT2A receptor antibody. As shown in Fig. 1, a CaM-specific mouse monoclonal antibody detected a band at ~19 kDa in NIH-3T3–5-HT2AR cell immunoprecipitates, which corresponds to the Ca2+-bound form of CaM. This immunoreactive band was only moderately detectable in immunoprecipitates from untreated NIH-3T3/5-HT2AR cells, but was significantly increased in immunoprecipitates from cells treated with 5-HT. A second anti-5-HT2AR antibody detected an immunoreactive band at ~60 kDa in immunoprecipitates from receptor-expressing NIH-3T3 cells that was unchanged after treatment with 5-HT. No immunoreactive bands were detected with either antibody in immunoprecipitates from wild-type NIH-3T3 cells. These data suggest that the 5-HT2A Receptor can complex with CaM in an agonist-dependent manner in NIH-3T3 fibroblasts.

Identification of Putative CaM Binding Regions in the 5-HT2A Receptor—NMR studies have shown that CaM binds target peptides via hydrophobic interactions and via salt bridges typically involving glutamate residues in the EF-hand regions (47, 48). Predictably, identified CaM binding regions conform to short peptides that form amphipathic α-helices composed of hydrophobic and positively charged amino acid residues (49). Using a web-based computer search program, which identifies such regions based on evaluation criteria such as hydrophathy, α-helical propensity, residue charge, helical class, residue weight, and hydrophilic residue content (50), we identified two putative CaM binding regions in the protein sequence of the 5-HT2A receptor. The illustration in Fig. 2A indicates that the putative CaM-binding domains are localized to the second intracellular loop and the juxta membrane region of the carboxyl terminus of the receptor. Both regions contain a propensity of hydrophobic and basic amino acids, typical of standard CaM binding regions. Although CaM binding regions do not conform to a linear arrangement of amino acids, several different CaM-binding motifs have been identified based on distances between key hydrophobic residues. The CaM binding region in the second intracellular loop of the 5-HT2A receptor was classified as a 1-8-14 motif, which consists of hydrophobic residues at positions 1, 8, and 14; whereas the putative CaM-binding site in the carboxyl terminus of the 5-HT2A receptor was classified as a 1-10 motif (Fig. 2B), with key hydrophobic residues separated by eight amino acids. As shown in Fig. 2B, the 5-HT2A receptor CaM binding regions could be aligned with other well defined CaM binding motifs from other proteins.

To illustrate the amphipathic nature of the putative CaM-binding sequences of the 5-HT2A receptor, we created helical wheel diagrams using a web-based modeling program (50). As expected, like most CaM-binding sites, both helical wheels showed clusters of positively charged amino acids on one side of the α-helix, with mostly hydrophobic amino acids concentrated on the opposite side (data not shown).

CaM Binds to Peptides Derived from the Second Intracellular Loop and Carboxyl Terminus of the 5-HT2A Receptor—Using synthetic peptides encompassing amino acids 183–202 (i2) and 377–396 (ct) of the 5-HT2A receptor, we tested the ability of either or both regions to interact with CaM with a modified blot overlay technique. Increasing amounts of each peptide (1–100 nmol) were slot-blotted to PVDF membranes and incubated with biotinylated CaM. To separate specific from nonspecific binding, we used a negative control peptide corresponding to the 17-amino acid CaM binding region of myosin light chain kinase, which contains a point mutation that renders it unable to bind CaM. Both the i2 and ct peptide bound biotinylated CaM in buffer containing 0.1 mM Ca2+, whereas the myosin light chain kinase control peptide showed no binding (Fig. 3). Binding was significantly reduced when Ca2+ was removed from the buffer and replaced with 1 mM EGTA. Interestingly, the i2 peptide appeared to bind more readily to CaM than did
the ct peptide, indicating that, although both peptides bind CaM in a Ca\(^{2+}\)-sensitive manner, they may do so with differing affinities.

We next analyzed the peptide-CaM complexes using polyacrylamide gel electrophoresis in the presence of 4M urea. This technique eliminates lower affinity and nonspecific protein-protein interactions (KD ≈ 100 nM). Increasing amounts of peptide (75–3000 pmol) were incubated with a constant amount of CaM (300 pmol) and subsequently analyzed by non-denaturing gel electrophoresis. In the presence of Ca\(^{2+}\), the addition of the i2 peptide produced an upward shift in the migration of CaM (Fig. 4A). In contrast, no peptide-CaM complexes were formed when Ca\(^{2+}\) was chelated with EGTA. The ct peptide produced no readily apparent shift in the mobility of CaM (Fig. 4B). This suggests that the ct peptide probably binds CaM with an affinity weaker than 100 nM, however the density of the CaM band decreased consistently with increasing peptide concentration, indicating that some shift may have occurred without detection. The incomplete gel shift induced by the i2 peptide, even at a peptide-CaM molar ratio of 10:1, is in line with published reports of other CaM binding regions that utilized this method under the same conditions (33, 36, 52). Likewise, incubation with a peptide derived from the CaM binding region of myosin light chain kinase, which binds CaM with very high affinity (≈ 6 pM), was also unable to induce a complete shift in CaM mobility in our hands (Fig. 4C).

Interaction of 5-HT\(_{2A}\) i2 and ct Peptides with Dansyl-CaM—Precise affinities are often difficult to calculate using gel-shift assays or in vitro binding techniques. Thus, we evaluated the binding affinities of the i2 and ct peptides for CaM by measuring changes in the fluorescence emission spectrum of dansyl-CaM. Ligand binding to dansyl-CaM enhances the fluorescence emission and blue-shifts the emission peak to a lower wavelength. As shown in Fig. 5A, dansyl-CaM displays weak fluorescence in the absence of Ca\(^{2+}\), with an emission peak of ∼520 nm. In the presence of Ca\(^{2+}\), the fluorescence emission is en-
presence of 4M urea and either 0.1 mM CaCl$_2$ or 1 mM EGTA. Complexes amounts (0, 75, 150, 300, 600, 1500, and 3000 pmol) of peptide in the synthetic peptides.

biotinylated CaM. with 0.5 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 CaCl$_2$ 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 i2 and ct peptides, but not to a myosin light chain kinase-negative control peptide, in the presence of Ca$^{2+}$. CaM binding was significantly reduced in the presence of EGTA. Data are representative of three separate experiments.

FIG. 3. Interactions of 5-HT$_{2A}$ 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 CaCl$_2$ 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 i2 and ct peptides, but not to a myosin light chain kinase-negative control peptide, in the presence of Ca$^{2+}$. CaM binding was significantly reduced in the presence of EGTA. Data are representative of three separate experiments.

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A

FIG. 4. Complex formation between CaM and 5-HT$_{2A}$ receptor 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 mM urea and either 0.1 mM CaCl$_2$ or 1 mM EGTA. Complexes were then resolved by nondenaturing polyacrylamide gel electrophoresis, in the presence of 4 mM urea and either CaCl$_2$ or EGTA. Gels were stained with GelCodeBlue staining reagent. The i2 peptide (A) and the authentic myosin light chain kinase peptide (C), but not the ct peptide (B), caused a shift in the migration of CaM in the presence of Ca$^{2+}$, but not in the presence of EGTA. Data are representative of three separate experiments.

B

Complex formation between CaM and 5-HT$_{2A}$ receptor 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 mM urea and either 0.1 mM CaCl$_2$ or 1 mM EGTA. Complexes were then resolved by nondenaturing polyacrylamide gel electrophoresis, in the presence of 4 mM urea and either CaCl$_2$ or EGTA. Gels were stained with GelCodeBlue staining reagent. The i2 peptide (A) and the authentic myosin light chain kinase peptide (C), but not the ct peptide (B), caused a shift in the migration of CaM in the presence of Ca$^{2+}$, but not in the presence of EGTA. Data are representative of three separate experiments.

C

Enhanced and shifted to a lower wavelength. As expected, both i2 and ct peptides further enhanced dansyl-CaM fluorescence and shifted the emission peak to ~495 nm, whereas the negative control myosin light chain kinase mutant peptide was completely ineffective (data not shown). The amounts of i2 and ct peptides were then varied to generate typical concentration-dependent saturation curves. The fluorescence emission spectra of dansyl-CaM (0.1, 0.2, 0.3, 0.4, and 0.5 µM) were measured in the presence of increasing concentrations of i2 or ct peptides (0–2 µM), and resulting data points were fit by nonlinear least squares analysis to the Scatchard one-site binding equation (Fig. 5, B and C). As expected, higher concentrations of dansyl-CaM required increased concentrations of peptide to enhance fluorescence and reach saturation, resulting in variations in EC$_{50}$ measurements. This results from a depletion of free peptide, a phenomenon which is most apparent at low concentrations of dansyl-CaM. By calculating EC$_{50}$ values over a range of dansyl-CaM concentrations it was possible to extrapolate to an infinitely low dansyl-CaM concentration, at which point depletion is essentially nonexistent. As shown in Fig. 5D and 5E, EC$_{50}$ measurements derived from peptide binding curves were plotted against the concentration of dansyl-CaM. These values fell onto straight lines with slopes of close to one, indicative of a 1:1 binding stoichiometry. Extrapolating the lines to the y-axis gave “true affinities” of 65 ± 30 nM and 168 ± 38 nM for the i2 and ct peptides, respectively.

Functional Overlap of CaM-binding Domain and PKC Phosphorylation Site within the 5-HT$_{2A}$ Receptor ct Peptide—Most GPCRs can be modulated by kinase-directed phosphorylation of threonine and serine residues, with the second messenger-dependent kinases (PKA, PKC, and others) predominating at low agonist concentrations, and the G protein-coupled receptor kinases predominating at high agonist concentrations and over extended periods of time (53). Although direct phosphorylation of the 5-HT$_{2A}$ receptor has yet to be reported, both 5-HT$_{2A}$ receptor internalization and desensitization appear to be highly dependent on PKC activation (54, 55). The 5-HT$_{2A}$ receptor contains six putative PKC phosphorylation sites, five of which are localized to the receptor third intracellular loop. Interestingly, the only putative PKC phosphorylation site in the carboxyl terminus ($^{384}$NKTYR$^{385}$) is centrally located within the ct CaM binding region (Fig. 6A). We consequently wondered whether binding of CaM to this region might regulate phosphorylation of this residue by PKC and, conversely, whether phosphorylation of this residue might inhibit CaM binding. Because typical CaM binding regions contain few or no acidic (negatively charged) amino acids, phosphorylation of the target threonine residue in the ct CaM binding region would add a negative charge and would consequently be expected to perturb CaM binding. We tested this hypothesis by evaluating the fluorescence emission spectrum of dansyl-CaM in the presence of a synthetic peptide identical to the ct peptide, but which contains a phosphorylated threonine at residue 386 (ct-P). As shown in Fig. 6B, the increase in fluorescence induced by the phosphorylated peptide was highly diminished as compared with the shift induced by the unphosphorylated peptide, suggesting that phosphorylation of the ct peptide inhibits CaM binding.

We next assessed whether the peptide could actually be phosphorylated by PKC in vitro, and if so, whether this phosphorylation could be inhibited by CaM. Purified rat brain PKC (composed primarily of PKC-α and PKC-β isozymes) readily phosphorylated the ct peptide ($K_{cat} = 8.83 ± 2.31$ µM, $V_{max} = 48.85 ± 7.94$ nmol/min/mg). In contrast, phosphorylation of the 5-HT$_{2A}$ receptor i2 peptide was not observed under identical conditions (data not shown). Interestingly, phosphorylation of the ct peptide was dose-dependently decreased in the presence of CaM, with a 20 µM concentration of CaM able to inhibit ct phosphorylation by ~80%. These data suggest that phosphorylation of the ct peptide by PKC and peptide binding to CaM are likely to be mutually inhibitory.
CaM Inhibits 5-HT_{2A} Receptor Coupling to G Proteins—The 5-HT_{2A} receptor modulates the activity of numerous second messengers and effectors, primarily through the \( \alpha \) subunits of \( G_{\alpha q/11} \) heterotrimers. Although the G protein contact sites of the 5-HT_{2A} receptor have yet to be detailed experimentally, the G protein-coupling sites of other GPCRs have been mapped to diverse regions of the second intracellular loop, third intracellular loop, and carboxyl terminus (56–60). Binding of CaM to intracellular regions of the 5-HT_{2A} receptor might be expected to sterically hinder receptor access to G protein subunits. This appears to be the case with some \( G_{\alpha q/11} \)-coupled receptors, including the \( D_2 \) dopamine (33) and \( \mu \)-opioid (34) receptors, both of which interact with CaM via their extended third intracellular loops. However, a similar observation has yet to be reported for \( G_{\alpha q/11} \)-coupled receptors. We assessed 5-HT_{2A} receptor coupling to heterotrimeric G proteins by measuring the binding of non-hydrolyzable \[^{35}S\]GTP\_S to cell membranes. For these experiments we used NIH-3T3 cells, which significantly overexpress...
the 5-HT2A receptor. The use of an artificial receptor expression system was essential to achieve an adequate signal-to-noise ratio, due largely to the fact that Goq11 proteins are relatively low in abundance, and have much lower rates of nucleotide exchange than Goq9 proteins. Stimulation of NIH-3T3 membranes with 10 μM 5-HT produced an ~25% increase in [35S]GTPγS binding (Fig. 7A). Interestingly, CaM dose-dependently decreased [35S]GTPγS binding, with concentrations of 1–10 μM reducing [35S]GTPγS binding to basal levels or lower. This effect was not due to nonspecific effects of the CaM protein, in that membranes incubated with the Ca2+-binding protein S-100 (which has a similar molecular weight to CaM but different target binding proteins) did not attenuate the 5-HT-induced increase in [35S]GTPγS binding (data not shown). These data suggest that interaction of CaM with the 5-HT2A receptor may regulate receptor coupling to heterotrimeric G proteins. However, it remained unclear whether this effect was via CaM binding to the second intracellular loop, carboxyl terminus, or both regions of the receptor.

Previous studies have shown that synthetic peptides derived from specific receptor-G protein interface sites can mimic the activated receptors themselves. This methodology has been used extensively to characterize G protein interaction domains for multiple receptors, including those for angiotensin II (60), glucagon-like peptide (57), and dopamine (61). We investigated the ability of the 5-HT2A receptor CaM-binding domains to stimulate G protein coupling by measuring [35S]GTPγS binding to NIH-3T3 cell membranes in the presence of increasing concentrations of i2 or ct peptides (0.1–10 μM). Data are expressed as percentage of basal binding in unstimulated cells (n = 6).

**FIG. 7. Effect of CaM on 5-HT2A receptor coupling to heterotrimeric G proteins.** A, [35S]GTPγS binding to cell membranes derived from NIH-3T3 cells overexpressing the 5-HT2A receptor (generous gift from Dr. Elaine Sanders-Bush, Vanderbilt University) was evaluated in the absence and presence of increasing concentrations of CaM (0.1–10 μM). GTPγS binding was determined in the presence of 50 μM GDP, with or without 10 μM 5-HT, and expressed as a percentage of basal binding in unstimulated cells. Data are expressed as percentage of basal binding in unstimulated cells (n = 6).

**DISCUSSION**

What is new about this work is that we have characterized two putative CaM-binding domains in the 5-HT2A receptor, and have shown that the interaction of CaM with these domains likely attenuates G protein coupling to the second intracellular loop and phosphorylation of the carboxyl terminus of the receptor by PKC. To our knowledge, this is the first demonstration that CaM attenuates G protein coupling of a Goq11-prefering receptor. We showed that CaM co-immunoprecipitates with the 5-HT2A receptor from NIH-3T3 fibroblasts and that the amount of CaM co-immunoprecipitating with the receptor
was increased after treatment with 5-HT, suggesting that CaM association is dependent on receptor activation. We subsequently identified two putative CaM-binding sites located within the second intracellular loop and carboxyl terminus of the receptor. These sites have properties typical of CaM-binding domains, including putative amphipathic α-helical structures composed of basic and hydrophobic amino acid residues. In addition, both sites could be aligned with well established CaM-binding motifs based on the locations of key hydrophobic residues. Synthetic peptides derived from the i2 and ct regions bound to CaM in a Ca\(^{2+}\)-sensitive manner with average KD values of 65 and 168 nM, respectively. Interestingly, many GPCRs appear to contain one or more putative CaM-binding sites, suggesting that interaction of CaM with different receptor regions may represent a common mechanism through which GPCR-dependent signaling pathways may be regulated (Table I).

The functions of the 5-HT\(_{2A}\) receptor CaM-binding domains have not been completely elucidated. However, these regions have previously been implicated in numerous signaling pathways and processes. The importance of the second intracellular loop in G protein coupling has been experimentally confirmed for other GPCRs, including the bradykinin B\(_2\) (62), α\(_{2A}\) adrenergic (63), and type B endothelin receptors (64). Using molecular modeling, Prado et al. (62) produced evidence that the distal i2 loop and proximal carboxyl terminus of the bradykinin B\(_2\) receptor may interact with each other to mediate receptor internalization and resensitization, in addition to G protein coupling. Berg et al. (65) showed that the i2 loop of the 5-HT\(_{2A}\) receptor is important for G\(\alpha_q\) coupling, and that different isoforms of the receptor produced via RNA editing in regions of the second intracellular loop varied in their ability to induce second messengers.

Previous work has demonstrated that CaM binding to the receptor third intracellular loops can attenuate the interactions between G\(\alpha\) and several receptors, including the 5-HT\(_{1A}\) receptor (36), μ-opioid receptor (34), D\(_2\) dopamine receptor (33), and the group III metabotropic glutamate receptor, mGlur7a (66, 67). New evidence in the current report suggests that CaM binding to the second intracellular loop of the 5-HT\(_{2A}\) receptor is likely to play a role in attenuating signal initiation from the 5-HT\(_{2A}\) receptor second intracellular loop by dampening interactions with heterotrimeric G proteins.

One general mechanism of receptor desensitization involves phosphorylation of intracellular residues by second messengers such as PKC, PKA, and the G protein-coupled receptor kinases. Our group previously showed that CaM binding to synthetic

### Table I

Location of putative CaM binding regions in GPCR families

| Gene Family | CaM Binding Sites |
|------------|--------------------|
| Serotonin  | 5-HT\(_{1D}\) (148-183), 5-HT\(_{1E}\) (123-142), 5-HT\(_{2A}\) (184-201), 5-HT\(_{2C}\) (162-180) |
| Adrenergic | α\(_{2A}\) (131-145), α\(_{2B}\) (106-123), α\(_{3}\) (189-208), α\(_{7}\) (234-250), α\(_{1}\) (290-308), β\(_{1}\) (241-259), β\(_{2}\) (211-239), β\(_{3}\) (227-243) |
| Muscarinic | M\(_{1}\) (199-218), M\(_{2}\) (352-371), M\(_{3}\) (378-395), M\(_{4}\) (480-496), M\(_{5}\) (388-405), M\(_{6}\) (426-495), M\(_{8}\) (505-522) |
CaM and 5-HT<sub>2A</sub> Receptor

peptides derived from the 5-HT<sub>1A</sub> receptor third intracellular loop could inhibit phosphorylation by protein kinase C (36). Interestingly, the 5-HT<sub>2A</sub> receptor carboxyl terminus CaM binding region contains a consensus PKC phosphorylation site, K<sup>384</sup>NKTYR<sup>388</sup>. It is well established that the 5-HT<sub>2A</sub> receptor can activate PKC via the hydrolysis of phosphatidylinositol bisphosphate and the subsequent formation of diacylglycerol. Berg et al. (55) showed that agonist-induced desensitization of the 5-HT<sub>2A</sub> receptor was dependent on the activity of PKC and could be modulated by pharmacological inhibitors of CaM. Likewise, activation of PKC in HEK293 cells induced the internalization and recycling of a 5-HT<sub>2A</sub> receptor green fluorescent protein fusion protein (54). The current work also supports a potential role for CaM in modulating phosphorylation of the 5-HT<sub>2A</sub> receptor. We showed for the first time that the putative PKC phosphorylation site in the 5-HT<sub>2A</sub> receptor carboxyl terminus is an excellent PKC substrate in vitro. Furthermore, CaM was capable of concentration-dependently decreasing the ability of PKC to phosphorylate a peptide derived from the ct region. The apparent agonist-dependent binding of CaM to the 5-HT<sub>2A</sub> receptor carboxyl terminus suggests a possible mechanism by which this receptor may be partially protected from PKC-dependent phosphorylation and perhaps subsequent internalization and desensitization. Furthermore, we speculate that the failure of several groups to demonstrate PKC-mediated phosphorylation of the 5-HT<sub>2A</sub> receptor in vivo may be due, in part, to steric blockade of PKC sites by CaM in live cells. This suggests that a complex contribution of both CaM and PKC may regulate 5-HT<sub>2A</sub> receptor internalization and desensitization.

Mutation of Ser<sup>188</sup> in the second intracellular loop has been shown to attenuate 5-HT<sub>1A</sub> receptor desensitization (68). Interestingly, this residue is predicted to be a phosphorylation site for the dual-specificity kinase Clk-2. The S<sup>188A</sup> mutation also interestingly, this residue is predicted to be a phosphorylation site that the failure of several groups to demonstrate PKC-dependent desensitization. The second intracellular loop of the 5-HT<sub>2A</sub> receptor contains a DRY motif similar (I/V)<sup>XX</sup> which has been proposed as an important regulator of receptor trafficking in yeast (72) and human epithelial cells (73). Our group reported a role for CaM in 5-HT<sub>1A</sub> receptor internalization, a required step for MEK and subsequent ERK activation (74). Conversely, pharmacological inhibitors of CaM were found to inhibit recycling and degradation of the EGF receptor without affecting internalization, resulting in the accumulation of receptors in enlarged endosomal structures (75). Thus, it is conceivable that CaM could regulate 5-HT<sub>2A</sub> receptor internalization and/or trafficking through direct binding to the receptor.

The current work has significance that extends beyond the 5-HT receptor family in that many GPCRs possess putative CaM binding domains (Table I) in intracellular juxtamembrane regions of the second and third intracellular loops and carboxyl termini. Thus far, these motifs have been implicated in G protein coupling and PKC-induced phosphorylation of both Go<sub>a</sub> and Go<sub>q/11</sub>-coupled receptors, for which CaM appears to dampen both processes. Because CaM has been implicated in various other GPCR processes such as ERK activation, stimulation of Na<sup>+</sup>/H<sup>+</sup> exchange, and receptor trafficking (1, 26, 72–77), direct binding of CaM to GPCRs could modulate any number of receptor processes. Additionally, the presence of putative CaM binding motifs in G<sub>s</sub>-coupled receptors such as the β<sub>1</sub>-, β<sub>2</sub>-, and β<sub>3</sub>-adrenergic and 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> receptors (Table I) raises the possibility that CaM might influence cAMP accumulation by directly binding to those receptors, although that possibility remains to be verified experimentally.

In conclusion, we have identified for the first time the presence of CaM-binding sites in the 5-HT<sub>2A</sub> receptor. These sites reside in juxtamembrane regions of the second intracellular loop and carboxyl terminus of the receptor. To our knowledge, this represents the first example of a GPCR with a CaM-binding site in the second intracellular loop and the first demonstration that CaM binding can attenuate G protein coupling to a Go<sub>q/11</sub>-preferring receptor. In addition, the work extends the concept that CaM binding to GPCR can inhibit receptor phosphorylation by PKC. These results suggest that CaM may serve as a valuable regulator and/or second messenger of 5-HT<sub>2A</sub> receptor function.

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Interaction of Calmodulin with the Serotonin 5-Hydroxytryptamine2A Receptor: A PUTATIVE REGULATOR OF G PROTEIN COUPLING AND RECEPTOR PHOSPHORYLATION BY PROTEIN KINASE C

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