Heterodimerization of Calcium Sensing Receptors with Metabotropic Glutamate Receptors in Neurons

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Calcium sensing (CaR) and Group I metabotropic glutamate receptors exhibit overlapping expression patterns in brain, and share common signal transduction pathways. To determine whether CaR and Group I metabotropic glutamate receptors (mGluRs) (mGluR1 and mGluR5) can form heterodimers, we immunoprecipitated CaR from bovine brain and observed co-precipitation of mGluR1α. CaR and mGluR1α co-localize in hippocampal and cerebellar neurons, but are expressed separately in other brain regions. In vitro transfection studies in HEK-293 cells established the specificity and disulfide-linked nature of the CaR:mGluR1α (CaR: mGluR5) interactions. CaR:mGluR1α (CaR:mGluR5) heterodimers exhibit altered trafficking via Homer 1c when compared with CaR:CaR homodimers. CaR becomes sensitive to glutamate-mediated internalization when present in CaR:mGluR1α heterodimers. These results demonstrate cross-family covalent heterodimerization of CaR with Group I mGluRs, and increase the potential role(s) for CaR in modulating neuronal function.

Calcium sensing receptors (CaR)3 are critically involved in regulating organ ischemic mineral ion homeostasis (1). Recent evidence suggests, however, that CaR regulates a variety of processes independent of Ca2+ homeostasis, including secretion, cell proliferation and/or differentiation, apoptosis, and membrane excitability (2–4). In brain, calcium sensing receptors are expressed, in a developmentally regulated manner, at substantial levels in the subfornical organ, olfactory bulbs, hippocampus, striatum, and cerebellum (5–8). CaR RNA and/or immunoreactivity has been identified in neurons, oligodendroglia, and microglia (8–10). In isolated hippocampal pyramidal neurons, CaR agonists activate non-selective cation and Ca2+-activated K+ channels (11); these responses are not observed when cells are isolated from mice having a targeted disruption of the CaR gene (12). These studies suggest potential role(s) for CaR in modulating neuronal excitability, as well as local ion homeostasis in the brain. Interestingly, CaR expression is present in many brain regions overlaps with that established for group I metabotropic glutamate receptors (mGluRs) (13–17) and Gαq/Go11 (18), the G protein implicated in mediating both CaR and group I mGluR activation of downstream signaling pathways.

CaR is a member of Family C of the G protein-coupled receptor superfamly, as are mGluRs, γ-aminobutyric acid receptors (GABAB1Rs), and putative pheromone/taste receptors. CaR and mGluR1α are both activated by extracellular Ca2+ over similar concentration ranges (19, 20), although the mechanism(s) involved in Ca2+-mediated activation may differ (21). Although CaR shares only a modest level of amino acid identity with mGluRs (<24%), critical structural similarities as well as overlapping agonist sensitivities hint at a common ancestry. In particular, CaR and Group I mGluRs have a large extracellular domain which incorporates both the agonist-binding site, sharing structural homology with bacterial periplasmic binding proteins and all other members of Family C (22), and a cysteine-rich domain involved in signal transmission from the binding domain to the transmembrane helices (23).

Both CaR and mGluRs function as disulfide-linked dimers (24–28). Recent studies on the structurally related GABAB1Rs have shown that these receptors function only as heterodimers, incorporating GABAB1 and GABAB2 subunits (29–31). Chimeras of CaR and group I mGluRs are functional (32, 33), suggesting that despite minimal sequence identity, the domains derived from distinct receptors can form productive interactions within the chimera. Given the overlapping expression of CaR and Group I mGluRs in brain, and their ability to form functional chimeras, we determined whether CaR and Group I mGluRs formed heterodimers in vivo. Here we demonstrate that heterodimers of these two distinct classes of G protein-coupled receptors form in vivo and in vitro, and demonstrate that trafficking of CaR within heterologously expressed CaR:mGluR heterodimers is influenced by glutamate and Homer 1c.

MATERIALS AND METHODS

Receptor Constructs—Human CaR (hCaR) was obtained from Dr. Klaus Seuwen (Novartis Pharma, AG, Basel, Switzerland), rat mGluR1α from Dr. Thomas P. Segerson (Oregon Health Sciences University), rat mGluR5 and Homer 1c from Dr. Paul Worley (Johns Hopkins University), β2-adrenergic receptor from Dr. David Yue (Johns Hopkins University), and pDsRed1N1 was purchased from CLONTECH. hCaR-EGFP was prepared as described (26). Rat mGluR1a was subcloned from pBluescript into pCDNA3.1 (Invitrogen) with NotI-BamHI site in place of the stop codon, using the original mGluR1α in pBluescript as the template. After amplification, the product was cut with NotI-BamHI and subcloned in-frame into pEGFPN1 (CLONTECH). Double-tagged proteins (Flag-hCaR-EGFP and Flag-mGluR1α-EGFP) were generated by inserting

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3 The abbreviations used are: CaR, calcium sensing receptor; mGluRs, metabotropic glutamate receptors; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; GPCR, G-protein coupled receptor.
the Flag protein epitope sequence in-frame after the respective signal peptide (MAFYSFCCWLLALTHTWSAGY for hCaR and MVRLLLFFPMIFLEMSILP for mGluR1α) by inverse polymerase chain reaction (34), using hCaR-EGFP and mGluR1α-EGFP as templates. The same technique was used to insert the Flag epitope sequence at the carboxyl terminus of wild-type β-adrenergic receptor, mGluR1α, mGluR5, and hCaR, to produce single epitope proteins.

Cell Transfection—Plasmids were introduced into HEK-293 cells by the liposomal formulation NovaFECT® (Venn-Nova, Pompano Beach, FL) according to the manufacturer’s protocol. A total of 2 μg of plasmid mixture was diluted in Opti-MEM® (Life Technologies, Inc., Rockville, MD) and mixed with 5 μl of transfection agent. When necessary, dPSRed1N1, which generates a soluble control protein, was used to maintain a constant amount of DNA (and protein expression) for each transfection.

Immunoprecipitation—Bovine brain was freshly obtained from a slaughterhouse and kept at 4 °C in Hanks’ balanced salt solution until dissection. Tissue was homogenized in lysis buffer (1 mM EDTA, 100 mM iodoacetamide, 0.5% Triton X-100 in PBS) containing protease inhibitors (Complete, Roche Molecular Biochemicals, Indianapolis, IN) using a Polytron homogenizer. After centrifugation at 20,000 rpm for 40 min, the supernatant was stored in 500-μl aliquots at −80 °C. Transfected HEK-293 cells were rinsed with PBS containing 1 mM EDTA, and after brief centrifugation at 500 rpm, the pellet was suspended in lysis buffer with protease inhibitors as described above and incubated at 4 °C for 30 min. The tubes were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was used for immunoprecipitation. To minimize nonspecific binding, 25 μl of Protein G-Sepharose (Sigma), for ADD (monoclonal, obtained from Dr. A. Spiegel) and anti-Flag antibodies (Sigma) or Protein A-agarose (Invitrogen, Carlsbad, CA), for PAI–934 antibody (ABR, Golden, CO) was added to each tube (brain homogenate or transfected cells) and incubated for 1 h at 4 °C. For immunoprecipitation from brain, 5 μl of ADD (1 μg/ml) or PAI–934 were added to each supernatant. For immunoprecipitation from transfected HEK-293 cells, 5 μl of anti-Flag antibody (Sigma) was added to each supernatant and incubated for 2 h at 4 °C. 25 μl of resin (either Protein G-Sepharose or Protein A-agarose, depending upon the antibody) was added to each tube and incubated for 1 h at 4 °C. The resin was washed 3 times with ice-cold wash buffer A (150 mM NaCl, 1% Triton X-100 in 50 mM Tris–HCl, pH 7.4) and incubated in 30 μl of Western blot loading buffer (12 M urea, 4% SDS, 0.01% bromophenol blue, 100 mM 8-mercaptoethanol in 200 mM Tris) for 30 min at room temperature. Samples were run on 4–15% SDS-polyacrylamide gels as previously described (26) and blots probed with anti-GFP antibody at 1:2000 dilution (Molecular Probes, Eugene, OR).

ELISA—After 24 h of incubation at 37 °C, transfected cells were replated into a 96-well plate pre-coated with rat tail collagen. Experiments were performed on the third day after transfection. Before experiments, cells were incubated with Dulbecco’s modified Eagle’s medium/zero glutamate for 4 h. Wells were treated with various agonists, and reactions were halted by fixing for 10 min at 22–24 °C with 4% paraformaldehyde in PBS, rinsed twice with PBS, and blocked for 30 min with PBS, 5% fetal bovine serum. The blocking solution was replaced by 50 μl of 1:4000 dilution of anti-Flag antibody (Sigma) conjugated with horseradish peroxidase. After 3 rinses with wash buffer B (0.5% Triton X-100 in PBS), the plate was developed with 50 μl of 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma). After 20–30 min, the substrate solutions from each well were transferred to a new plate containing 50 μl of H2SO4 (1 x). The plate was analyzed at 450 nm on a SpectraMax 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA). The original plate (containing the fixed cells) was washed 3 times with PBS, and then assayed for protein (Micro BCA, Pierce, Rockford, IL) to permit normalization.

Immunofluorescence Localization—Fisher F344 rats (21, 28, and 35 days of age, 5 rats per group) were deeply anesthetized with methoxyflurane, and blood removed from tissues by intracardiac perfusion with 25 ml of cacodylate buffer, pH 7.4. Tissues were then fixed in situ by perfusion with 40 ml of 4% paraformaldehyde in cacodylate buffer. After 18 h post-fixation within the skull, the brains were dissected out and embedded in gelatin (15 brains per block). Serial 40-μm thick cryosections were cut (Neomax Technologies, Inc., Rockville, MD). Floating sections (5 sections per rat brain) were incubated with mild agitation in: 1) 0.05% Triton X-100 in PBS pH 7.4 (PBS-T) for 20 min; 2) 5% normal donkey serum (Jackson ImmunoResearch Labs, Inc., West Grove, PA) in PBS (blocking solution) for 30 min; and 3) primary antibodies (10 μg/ml, diluted in blocking solution) for 18–48 h at room temperature. Primary antibodies were mouse monoclonal anti-CaR (ADD) and rabbit anti-mGluR1α (Chemicon Intl., Temecula, CA). Sections were then washed 3 times for 10 min with PBS-T before incubation with secondary conjugates (10 μg/ml, diluted in blocking solution). Conjugates used were donkey anti-mouse IgG labeled with fluorescein and donkey anti-rabbit IgG labeled with rhodamine (Jackson ImmunoResearch Labs, Inc.). Conjugate specificity was confirmed by abolition of detectable staining when the primary antibody was omitted. Sections were washed 3 times for 10 min with PBS-T, dipped in DAB, and overslipped with Vectashield (Vector Labs, Burlingame, CA), sealed with nail polish. Images were collected using a Zeiss LSM 510 confocal microscope using a 4-μm optical slice and final magnification of × 25–50.

RESULTS

Isolation of CaR:mGluR1α Heterodimers from HEK-293 Cells and Bovine Brain—Two different antibodies against CaR were used in immunoprecipitation studies from bovine brain. A monoclonal anti-CaR antibody, ADD, was obtained from Dr. Alan Spiegel (35), and a polyclonal anti-CaR antibody, PAI–934, purchased from Affinity BioReagents. To confirm the specificity of both antibodies in immunoprecipitation studies, CaR-EGFP was heterologically expressed in HEK-293 cells, membranes solubilized, and immunoprecipitation performed with either ADD or PAI–934. Western blots were probed with an anti-GFP antibody (Molecular Probes), and confirmed that both ADD and PAI–934 immunoprecipitated a band having a molecular mass appropriate to the GFP-tagged CaR (~190 kDa), Fig. 1A.

CaR, mGluR1α, or CaR plus mGluR1α (1:1 cDNA ratio) were transiently expressed in HEK-293 cells, and immunoprecipitation was carried out with either ADD or PAI–934. Western blots were probed with anti-mGluR1α antibody (Chemicon), and as illustrated in Fig. 1B, only cells which had been transfected with both CaR and mGluR1α (lanes labeled C:O) yielded a band at the molecular mass consistent with mGluR1α (approximately 185 kDa). The anti-Flag antibody was used as a negative control for immunoprecipitation, and did not bring down mGluR1α. These results clearly demonstrate that stable CaR:mGluR1α heterodimers form when heterologously expressed in HEK-293 cells. To determine whether CaR:mGluR heterodimers were formed in vivo, CaR from bovine brain was immunoprecipitated with either ADD or PAI–934. Western blots were probed with anti-mGluR1α antibodies (Fig. 1C). Significant immunoreactivity was observed at a molecular weight consistent with fully glycosylated mGluR1 when immunoprecipitation was carried out with either ADD or PAI–934, but not when the anti-Flag antibody was used. The results illustrated in Fig. 1 indicate CaR:mGluR1 heterodimers form in vivo and in vitro.

Immunolocalization of CaR and mGluR1α in Rat Brain—To define the brain regions in which CaR:mGluR1α heterodimerization might be important, we performed immunofluorescence localization studies with the monoclonal anti-CaR antibody ADD and a rabbit polyclonal anti-mGluR1α antibody (Chemicon) in juvenile rat brain. We examined 3–5 serial brain sections from F344 rats at 21, 28, or 35 days of age (n = 5 rats/age group). The immunoreactivity patterns observed for either receptor did not differ among these age groups. The expression patterns of both CaR and mGluR1α observed were consistent with previous studies (5, 36, 37). Representative images from 28-day-old rats are shown in Fig. 2.

The regions in which CaR and mGluR1α immunoreactivity were most highly expressed together were the cerebellum and the hippocampus. In the cerebellum, overlapping expression of CaR and mGluR1α was observed by confocal microscopy in cerebellar purkinje cells (Fig. 2A, arrows) and molecular layer (Fig. 2A, M). Immunoreactivity for mGluR1α only was observed in the granule cell layer (Fig. 2A). In the hippocampus,
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FIG. 1. CaR immunoprecipitates with mGluR1α. A, the specificity of two antibodies against CaR, ADD and PAI–934, was established by immunoprecipitation of CaR-EGFP transiently expressed in HEK-293 cells. Samples were run on 4–15% SDS-polyacrylamide electrophoresis gels, and Western blots were probed with anti-GFP antibody. B, ADD co-immunoprecipitates CaR and mGluR1α from transfected cells. HEK-293 cells in 25-mm dishes were transfected with 2 μg of CaR, mGluR1α, or both (C:G). Cells were harvested, and solubilized membranes were immunoprecipitated with ADD, anti-Flag, or PAI–934 antibodies. Western blots were probed with anti-mGluR1α antibody. C, ADD co-immunoprecipitates CaR and mGluR1α from bovine thalamus. 1 ml of bovine thalamus homogenate (500 μg/ml) was immunoprecipitated with ADD, anti-Flag, or PAI–934 antibodies. Samples were run on 4–15% SDS-polyacrylamide gel electrophoresis, and blot was probed with anti-mGluR1α antibody. In all blots involving PAI–934, the intense band at 130 kDa is due to antibody.

In Vitro Characterization of CaR:mGluR1α Heterodimerization—The data in Figs. 1 and 2 strongly suggest that CaR and mGluR1α are co-localized, particularly in the hippocampus and cerebellum, and that they can be co-immunoprecipitated from brain homogenates. When heterologously expressed in the same cells, CaR and mGluR1α can also be co-immunoprecipitated. Further experiments were therefore designed to test the specificity of the association and nature of the interaction.

To determine the specificity of CaR interactions with Group I mGluRs, CaR-Flag was expressed in HEK-293 cells with various receptors tagged at their carboxy termini with EGFP (CLONTECH). Membranes were isolated and CaR-Flag was immunoprecipitated with an anti-Flag antibody. Western blots of the immunoprecipitates were probed with an anti-GFP antibody. Western blots were probed with anti-mGluR1α antibody.

FIG. 2. CaR and mGluR1α are co-localized in juvenile rat brain. CaR and mGluR1α immunoreactivity was detected in serial brain sections from 28-day-old F344 rats (n = 5). Confocal microscopy revealed co-localization (yellow) of CaR (green) and mGluR1α (red) in cerebellar Purkinje cells (A, arrows) and molecular layer, and in hippocampal dentate gyrus (B). CaR immunoreactivity alone is observed in neuronal cell bodies in the brain stem (C), and mGluR1α immunoreactivity alone detected in the cerebellar granular cell layer (A). The brain sections (40-μm thick cryostat) were incubated with 10 μg/ml mouse monoclonal anti-CaR and rabbit anti-mGluR1α for 36 h. Omission of the specific receptor antibodies abolished staining (not shown). Abbreviations: Ml, molecular layer; Gel, granular cell layer; Pc, Purkinje cell layer; DG, dentate gyrus; PoDG, polymorph layer dentate gyrus. Images were collected using a Zeiss LSM 510 confocal microscope using 4-μm optical slices. Original magnification was × 50 for A and C, and × 25 for B.
polyacrylamide gel electrophoresis and blots were probed with anti-
H11001 antibodies, individual dishes of HEK-293 cells were transfected with either mGluR1 (G1:C) coexpressed in the same cells, and CaR-Flag and Flag antibody, and then solubilized and electrophoresed in the mGluR1 with CaR-Flag and CaR-EGFP (C:C) or CaR-Flag and mGluR1-EGFP (G1:C). For membrane mixing experiments, individual dishes of HEK-293 cells were transfected with either mGluR1-Flag or CaR-EGFP (G1/C) and then membranes were mixed prior to immunoprecipitation. B, CaR and mGluR1a form heterodimers by disulfide bonds. HEK-293 cells were transiently transfected with CaR-Flag + CaR-EGFP (C:C), mGluR1a-Flag + CaR-EGFP (G1/C), or mGluR5-Flag + CaR-EGFP (G5/C). For membrane mixing experiments, individual dishes of HEK-293 cells were transfected with either mGluR1a-Flag or CaR-EGFP (G1/C) and then membranes were mixed prior to immunoprecipitation. B, CaR and mGluR1a form heterodimers by disulfide bonds. HEK-293 cells were transiently transfected with CaR-Flag + CaR-EGFP (C:C) or mGluR1a-Flag + CaR-EGFP (G5/C). The pelleted resin was resuspended in loading buffer in the absence or presence of 100 mM β-mercaptoethanol (β-ME), and incubated 30 min at room temperature. Locations of monomers and dimers are indicated.

To determine whether the interaction between CaR and Group I mGluRs was stabilized via a covalent disulfide linkage, as is observed for CaR:CaR or mGluR:mGluR homodimers (26, 28), membranes isolated from HEK-293 cells co-transfected with CaR-Flag and CaR-EGFP (C:C) or CaR-Flag and mGluR1a-EGFP (G1/C) were immunoprecipitated with anti-Flag antibody, and then solubilized and electrophoresed in the absence or presence of β-mercaptoethanol. The Western blot in Fig. 3B illustrates that in the absence of β-mercaptoethanol, GFP immunoreactivity appears predominantly as molecular weight consistent with CaR-Flag:CaR-EGFP or CaR-Flag: mGluR1-EGFP dimers (and higher oligomers), while in the presence of β-mercaptoethanol, the preponderance of GFP immunoreactivity is present at a molecular weight consistent with the respective monomers. These results strongly suggest that CaR:mGluR heterodimers are stabilized by covalent disulfide bonds, as has been demonstrated for the respective homodimers.

Altered Surface Expression of CaR:mGluR1a Heterodimers in the Presence of Homer 1c—CaR mutants which are misfolded or incorrectly dimerized are not fully glycosylated and do not traffic to the plasma membrane (33, 38, 39). Western blots derived from immunoprecipitation of CaR-Flag plus Group I mGluRs exhibit predominantly the fully glycosylated forms of both CaR and mGluR1a or mGluR5 (Fig. 3A), suggesting complete processing of the heterodimers and localization to the plasma membrane. As a second test of plasma membrane localization of the CaR/mGluR heterodimers, we took advantage of the family of Homer/vesl proteins which interact with and specify subcellular localization of Group I mGluRs (40). In particular, Homer 1c has been shown both in vivo (41) and in vitro (42) to stabilize Group I mGluRs at the plasma membrane.

An ELISA was used to quantify plasma membrane localization of CaR:CaR and mGluR1a:mGluR1a homodimers, as well as CaR:mGluR1a heterodimers; in each transfection, the first partner in the pair contained an amino-terminal Flag epitope. Coexpression of Flag-mGluR1a plus Homer 1c in HEK-293 cells resulted in a significant increase in plasma membrane localization of Flag-mGluR1a, when compared with Flag-mGluR1a alone (Fig. 4A), as has been previously shown (42). CaR does not contain the -PPSFPR- epitope, present near the carboxyl terminus of Group I mGluRs, which is required for interaction with the Homer EVH1 domain (69). In confirmation, the degree of plasma membrane localization of Flag-CaR in HEK-293 cells was not affected by coexpression with Homer 1c (Fig. 4B). However, when Flag-CaR plus mGluR1a were coexpressed, Homer 1c significantly increased the amount of plasma membrane-localized Flag-CaR (Fig. 4B). These results suggest that localization of Flag-CaR:mGluR1a heterodimers at the plasma membrane is stabilized in the presence of Homer 1c. In addition, the results imply that only a single -PPSFPR- epitope is required within the receptor dimer for Homer 1c-mediated enhancement of plasma membrane localization, since the increase in plasma membrane localization of the Flag-mGluR1a:mGluR1a dimers is comparable to that observed for the Flag-CaR:mGluR1a heterodimers (compare Fig. 4, A and B). Coexpression of Flag-CaR with mGluR5 significantly in-
increased Flag-CaR at the plasma membrane in both the absence and presence of Homer 1c (Fig. 4C).

Internalization of CaR:mGluR1α Heterodimers in Response to Both Ca2+ and Glutamate—Coexpression of CaR and mGluR1α with Homer 1c increases CaR localization to the plasma membrane, presumably within the CaR:mGluR1α heterodimer (Fig. 4B). Conversely, decreases in plasma membrane localization occur as a result of agonist exposure for many GPCRs; therefore, the effects of Ca2+ or glutamate were examined in cells expressing Flag-CaR:CaR, Flag-mGluR1α: mGluR1α, Flag-CaR:mGluR1α, or Flag-mGluR1α:CaR dimers. For each transfection pair, plasma membrane localization of the Flag-tagged receptor was determined by ELISA. Transfected cells were exposed to either control (0.5 mM Ca2+, no glutamate), 10 mM Ca2+, 1 mM glutamate, or 10 mM Ca2+ plus 1 mM glutamate, for 15 min. The results are illustrated in Fig. 5.

Cells expressing Flag-CaR:CaR (Fig. 5A) responded to 10 mM Ca2+ with a 57% decrease in plasma membrane localization of receptor dimers, but 1 mM glutamate had no statistically significant effect. The response to both 10 mM Ca2+ plus 1 mM glutamate was indistinguishable from the response to Ca2+ alone. Internalization of Flag-CaR:mGluR1α heterodimers was sensitive to either 10 mM Ca2+ or 1 mM glutamate to a similar extent, while there was no further potentiation of internalization when both agonists were added together (Fig. 5B). These results indicate that heterodimerization of CaR with mGluR1α confers glutamate sensitivity to CaR internalization.

Cells expressing Flag-mGluR1α:mGluR1α (Fig. 5C) responded to 1 mM glutamate with a 57% decrease in plasma membrane-localized receptor dimers, while 10 mM Ca2+ induced a 39% decrease. mGluR1α activation can be induced by either Ca2+ or glutamate (19, 20), and these results demonstrate that Ca2+ can also induce mGluR1α internalization. Both 10 mM Ca2+ and 1 mM glutamate added together had an effect comparable to that of 1 mM glutamate (59% decrease). Cells expressing Flag-mGluR1α:CaR exhibited a statistically significant increase in response to 10 mM Ca2+ (39.4% in the homodimer versus 62% reduction in the heterodimer, p < 0.01). The Flag-mGluR1α:CaR heterodimer was equally sensitive to each agonist alone or both agonists added simultaneously. Results from both Flag-CaR:mGluR1α or Flag-mGluR1α:CaR heterodimers indicate an increased sensitivity to desensitization, i.e. internalization, in response to exposure to the agonist which activates the non-tagged heterodimer partner.

**DISCUSSION**

As early as the 1980s, oligomers of β2-adrenergic receptor (43) and muscarinic receptors (44) were observed, but their functional significance was uncertain. More recently, oligomerization has been suggested to account for the multiple agonist affinity states observed for GPCRs (45, 46). Convincing functional evidence for β2-adrenergic receptor dimerization is derived from complementation studies, where wild type receptors exert a dominant positive effect on mutant receptors (47) or coexpression of two deficient mutants or chimeras restores receptor function (48, 49). Dimersization of β2-adrenergic receptor is required for efficient signaling via Gα (50). Internalization of δ-opioid receptors depends upon agonist-induced conversion of receptor dimers to monomers, a process which is sensitive to agonist structure (51).

Heterodimerization has been observed between subtypes of somatostatin (52) and opioid receptors (53, 54), and is required for formation of functional GABAβ receptors (29–31). Receptors belonging to distinct families, i.e. the angiotensin II receptor AT1 and bradykinin receptor B2 (55), the dopamine D1 and adenosine A1 receptors (56), and mGluR1α and adenosine A1 receptors (57) have recently been shown to heterodimerize. Heterodimerization among subtypes of a single class or among multiple classes of GPCRs can lead to signal enhancement, altered agonist sensitivities, and/or differences in trafficking or desensitization pathways.

In the present study, covalent heterodimerization of CaR and Group I mGluRs has been demonstrated both in vivo and in HEK-293 cells. In particular, immunoprecipitation of CaR from bovine brain resulted in co-precipitation of mGluR1α. In rat brain, CaR and mGluR1α were co-localized throughout the hippocampus and cerebellum, suggesting the potential for heterodimerization. In transfected HEK-293 cells, CaR and either mGluR1α or mGluR5 were co-precipitated when expressed in the same cells, but not when membranes expressing the two types of receptors were mixed prior to immunoprecipitation. In both types of heterologous expression experiments, CaR did not co-precipitate with β2-adrenergic receptor, a receptor from a distinct GPCR family.

CaR:mGluR1α heterodimers are sensitive to reducing agents such as β-mercaptoethanol. While sensitivity to reducing agents does not necessarily imply covalent heterodimerization, both mGluR and CaR homodimers are stabilized by disulfide bonds. The cysteine residue responsible for homodimerization of mGluRs, C140 (58), is localized to an unstructured loop in the mGluR structure (59). Cysteine residues within the mGluR Cys129, corresponding to Cys140 of mGluR1, is responsible for formation of functional GABAB receptors (29–31). Receptors belonging to distinct families, i.e. the angiotensin II receptor AT1 and bradykinin receptor B2 (55), the dopamine D1 and adenosine A1 receptors (56), and mGluR1α and adenosine A1 receptors (57) have recently been shown to heterodimerize. Heterodimerization among subtypes of a single class or among multiple classes of GPCRs can lead to signal enhancement, altered agonist sensitivities, and/or differences in trafficking or desensitization pathways.

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![Image](http://www.jbc.org/)

**Fig. 5. Glutamate decreases surface localization of CaR in the presence of mGluR1α.** Transiently transfected HEK-293 cells were exposed to 0.5 mM Ca2+ (black), 10 mM Ca2+ (red), 1 mM glutamate (green), or 10 mM Ca2+ + 1 mM glutamate (yellow) for 15 min at 37 °C. Reactions were halted by fixing cells with paraformaldehyde, and ELISA were developed with anti-Flag/horseradish peroxidase conjugate (1:4000). Panels indicate cells transfected with: A, Flag-CaR-EGFP alone (+pDsRed1N1); B, Flag-CaR-EGFP + mGluR1α; C, Flag-mGluR1α-EGFP alone (+pDsRed1N1); or D, Flag-mGluR1α-EGFP + CaR. Surface localization was quantified by normalizing the ELISA data (optical density reading at 450 nm) by the protein data (optical density reading at 550 nm) for each well. Significance was determined by unpaired t test, at p < 0.01. * indicates significance in comparison with control (0.5 mM Ca2+) for each panel, while # indicates significance in comparison with 1 mM glutamate or 10 mM Ca2+ + 1 mM glutamate.


