COMPLEX FORMATION WITH THE TYPE B \(\gamma\)-AMINOBUTYRIC ACID RECEPTOR AFFECTS THE EXPRESSION AND SIGNAL TRANSDUCTION OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR: STUDIES WITH HEK-293 CELLS AND NEURONS

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Running title: Complex formation between GABA-B-Rs and CaRs

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SUMMARY

We co-immunoprecipitated the Ca\(^{2+}\)-sensing receptor (CaR) and type B \(\gamma\)-aminobutyric acid receptor (GABA-B-R) from human embryonic kidney (HEK)-293 cells expressing these receptors and from brain lysates where both receptors are present. CaRs extensively co-localized with the two subunits of the GABA-B-R (R1 and R2) in HEK-293 cell membranes and intracellular organelles. Coexpressing CaRs and GABA-B-R1’s in HEK-293 cells suppressed the total cellular and cell-surface expression of CaRs and inhibited phospholipase C (PLC) activation in response to high extracellular [Ca\(^{2+}\)]\(_e\) ([Ca\(^{2+}\)]\(_e\)). In contrast, coexpressing CaRs and GABA-B-R2’s enhanced CaR expression and signaling responses to raising [Ca\(^{2+}\)]\(_e\). The latter effects of the GABA-B-R2 on the CaR were blunted by coexpressing the GABA-B-R1. Coexpressing the CaR with GABA-B-R1 or R2 enhanced the total cellular and cell-surface expression of the GABA-B-R1 or R2, respectively. Studies with truncated CaRs indicated that the N-terminal extracellular domain of the CaR participated in the interaction of the CaR with the GABA-B-R1 and R2. In cultured mouse hippocampal neurons, CaRs co-localized with the GABA-B-R1 and R2. CaRs and GABA-B-R1’s also co-immunoprecipitated from brain lysates. The expression of the CaR was increased in lysates from GABA-B-R1-knockout mouse brains and in cultured hippocampal neurons with their GABA-B-R1 genes deleted in vitro. Thus, CaRs and GABA-B-R subunits can form heteromeric complexes in cells and their interactions affect cell-surface expression and signaling of CaR, which may contribute to extracellular Ca\(^{2+}\)-dependent receptor activation in target tissues.

INTRODUCTION

Receptors in family C of the G protein-coupled receptor (GPCR) superfamily mediate critical functions in a wide range of tissues. Family C consists of the type B (or metabotropic) \(\gamma\)-aminobutyric acid (GABA) receptor (GABA-B-R) subunits (R1 and R2), the extracellular Ca\(^{2+}\)-sensing receptor (CaR), several metabotropic glutamate receptors (mGluRs), a large group of taste receptors, and many at present orphan receptors (1).

GABA-B-Rs are critical molecules in the central nervous system (CNS) where their function tends to be suppression of neuronal activity (2,3). GABA-B-Rs are also expressed in peripheral tissues including the heart, spleen, lung, liver, stomach, kidney, adrenal gland, bone, and cartilage where their functions are unknown (4-14).

CaRs control systemic Ca\(^{2+}\) balance via the regulation of parathyroid hormone (PTH) secretion and renal Ca\(^{2+}\) excretion (15). CaRs are also expressed in many other tissues like the brain where their functions are uncertain. In neurons, astrocytes, and neuroglia, CaRs modulate the activity of K\(^+\) and non-selective cation channels and cell growth (16). In osteoblasts and chondrocytes, CaRs activate signaling responses...
and mediate high extracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)\(_e\)])-induced changes in cell differentiation (17-19).

It has been difficult to determine specific functions for CaRs in vivo in tissues like the brain, bone, or cartilage. Both human and murine models of CaR inactivation lack phenotypic features that can be clearly assigned to tissues beyond the parathyroid gland and kidney. One reason is that there may be redundancy in Ca\(^{2+}\)-sensing molecules in vivo. Other receptors or signaling mechanisms may assume Ca\(^{2+}\)-sensing in vivo when the wild-type (wt) CaR is partially inactivated (20).

Molecules that are reasonable candidates for Ca\(^{2+}\) sensors are other members of family C. Increasing evidence supports the idea that family C receptors like the GABA-B-R can form multimeric complexes (21,22). The GABA-B-R1 and R2 are two distinct gene products that heterodimerize to form fully functional GABA-B-Rs capable of ligand-binding and signal transduction (23). Structure-function studies with the R1 and R2 suggest that their dimerization is essential for trafficking to the cell membrane and stable cell-surface expression of functional receptors (24,25).

CaRs are also thought to function as homodimers in target tissues such as the parathyroid gland and kidney. This idea is largely based on studies with mutant CaRs whose ability to alter the signaling of wt CaRs is best explained by the presence of heteromeric wt-mutant CaR signaling complexes at the membrane (26,27). CaRs also heterodimerize with mGluRs (28). Gama et al showed that the CaR co-immunoprecipitated with the mGluR1 and R5 in HEK-293 cells, co-localized with mGluRs in specific populations of neurons in brain sections, and could be co-immunoprecipitated with mGluR1 from whole brain lysates (28). Although CaRs and GABA-B-Rs are present in many of the same tissues (e.g., brain, kidney, parathyroid, bone, and cartilage), it is an open question whether these receptors can physically associate endogenously and, if so, whether such associations produce changes in cell function.

These studies tested whether GABA-B-Rs associated with CaRs in brain lysates and in HEK-293 cells transfected with receptor cDNAs and whether their putative interactions impacted on the function of the CaR. We found that GABA-B-Rs physically interacted with CaRs in HEK-293 cells but more importantly also in the brain. Both receptors are strongly co-expressed throughout the brain, and their ability to interact to influence levels of CaR expression and thereby modulate cellular sensitivity to extracellular Ca\(^{2+}\) and other CaR ligands could begin to explain the significant effects of Ca\(^{2+}\) on key CNS functions.

### EXPERIMENTAL PROCEDURES

#### Materials

Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA). The human (h) GABA-B-R1a, GABA-B-R1b, and GABA-B-R2 cDNAs cloned into pcDNA3.1/hygro or pcDNA3.1/neo plasmids were gifts from Drs. Edward Nemeth and Karen Krapcho (NPS Pharmaceuticals, Inc, Salt Lake City, UT). The hGABA-B-R1a and R1b are two alternatively spliced forms of the hGABA-B-R1, which bind the same ligands and activate the same signaling responses (2). cDNAs encoding the rat GABA-B-R1a and R2 fused with a HA-epitope at their amino (N)-termini (HA-GABA-B-R1a and HA-GABA-B-R2) were cloned into pcDNA 3.1 as previously described (24). The pcDNA3.1/V5-His expression vector, rat anti-HA, and mouse anti-V5 antisera were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-CaR (#21825A) antiserum were raised against an extracellular epitope of the bovine parathyroid CaR (29).

Guinea pig anti-GABA-B-R1 antiserum was raised against a peptide (CPSEPDPRLSSDGSRV-HLLYY) in the C-terminal tail of both GABA-B-R1a and R1b. Guinea pig anti-GABA-B-R2 antiserum was obtained from US Biological, Inc (Swampscott, MA). Fluorescein-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-rat or mouse IgG for immunocytochemistry were obtained from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxidase (HRP) and Texas Red-conjugated goat anti-guinea pig IgG antisera were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Protein A/G-conjugated beads for immunoprecipitation and SuperSignal chemiluminescence substrate were from Pierce (Rockford, IL).

#### Subcloning of cDNA constructs

The cDNA for a human CaR fused to the V5 epitope at its C-terminus (hCaR-V5) was...
constructed by in-frame ligation of a Xba I-Hind III cDNA fragment encoding residues # 1-1071 of the receptor with the pcDNA3.1/V5-His expression vector. cDNA encoding a truncated bovine parathyroid CaR (T866-CaR), lacking the entire C-terminal tail of the receptor, was subcloned into pcDNA1/Amp as described (30). cDNA encoding the N-terminal extracellular domain (ECD, residues 1 to 579) of the hCaR was ligated in-frame with the 5′-end of the cDNA encoding the firefly green fluorescent protein (GFP) in a pcDNA3.1 vector to generate the ECD-GFP construct (ECD-GFP/pcDNA3.1).

**Cell culture and transfection**

HEK-293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Inc., Herndon, VA) + FBS (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml) and transfected with cDNAs using the Ca2+ phosphate method (29). Briefly, cultures at ~50-70% confluency were incubated with a mixture of cDNAs encoding CaRs and GABA-B-Rs (10 µg per construct) as specified for 24 hrs. For controls, vector (pcDNA3.1/hygro, pcDNA3.1/neo, or pcDNA1/Amp) DNA was added to standardize the total amount of DNA in each transfection. Transfected cells were replated and cultured for an additional 48 hrs.

Mouse hippocampal neurons were cultured from wt Black Swiss mice and mice carrying GABA-B-R1 genes flanked with loxP sequences (floxed-GABA-B-R1) according to published protocols (31,32). Briefly, hippocampus was dissected and digested with protease XXIII (3 mg/ml; Sigma, St, Louis, MO) in a neuron maintenance media [NMM; Neural Basal media (Invitrogen) plus serum-free B27 supplement (Invitrogen), L-glutamine (0.5 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml)] at 37°C for 15 min. After 2 washes with NMM, cells were dissociated by passing the digested tissues through Pasteur pipettes with gradually reduced, fire-polished tip openings (500 to 200 µM in diameter). Cells were cultured in poly D-lysine-coated (0.1 mg/ml) plastic dishes and or glass coverslips in NMM for 3 days and supplemented with cytosine β-D-arabinofuranoside (3 µM; Sigma, St. Louis, MO) for an additional 3-4 days before immunocytochemical staining or viral infections.

To assess the effects of blocking the expression of the GABA-B-R1 on the expression of the CaR in neurons, hippocampal neurons from floxed-GABA-B-R1 mice (33) were infected with adenoviruses carrying a cDNA encoding bacterial Cre recombinase (Ad-Cre, Microbix Inc, Toronto, Ontario, Canada) or empty viral vector (Ad-Cont). The titer for infection (8 pfu/cell) had been validated in pilot experiments and found not to alter neuron survival or gross morphology (data not shown). Controls included infection of hippocampal neurons from wt mice with Ad-Cre or Ad-Cont as shown.

**Immunoprecipitation and immunoblotting**

The following steps were performed in the presence of a Complete™ protease inhibitor cocktail (Roche, Indianapolis, IN) without reducing agents, unless specified.

HEK-293 cells: Total protein lysates were extracted from transfected cells with nonionic detergent Nonidet P-40 (1%) in phosphate-buffered saline (PBS) (NP40-PBS). Protein lysates (500 µg) were incubated with anti-HA or anti-V5 antisera (3 µg) in NP40-PBS (500 µl) for 1 hr and then with Protein A/G-conjugated beads (20 µl) overnight. Beads with immunoprecipitates were washed with ice-cold NP40-PBS (1 ml) 5 times, collected by centrifugation, and eluted by incubating with a sample buffer [Tris-HCl (300 mM, pH 6.8), SDS (10%), bromophenol blue (0.01%), glyc erol (50%), and dithiothreitol (100 µM)] at 37°C for 30 min.

Mouse brain and hippocampal neurons: Crude membranes were prepared from the brains of wt Black Swiss mice and from hippocampal neurons infected with adenoviruses from floxed-GABA-B-R1 mice as described (18,29). Proteins were extracted from these membrane preparations with NP40-PBS and immunoprecipitated (1 mg protein/500 µl NP40-PBS) with anti-GABA-B-R1 antibodies (5 µg) as described above.

Immunoprecipitates, crude membranes, and protein lysates which were previously incubated with sample buffer containing DTT (100 µM) at 37°C for 30 min were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes (18,29). Nitrocellulose membranes were blotted with anti-HA (2 nM), anti-V5 (2 nM), anti-GABA-B-R1 (10 nM), anti-GABA-B-R2 (10 nM), or anti-CaR (50 nM) plus corresponding...
HRP-conjugated secondary antibodies (18,29,34). Signals were detected by a SuperSignal chemiluminescent substrate and Kodak X-ray films.

**Biotinylation of cell-surface proteins**

HEK-293 cells transfected with different combinations of hCaR-V5 and HA-GABA-B-R1 and R2 were washed with ice-cold PBS 3 times and incubated with an EZ-Link biotin reagent [Sulfo-Succinimidyl 6-(biotinamido) Hexanoate, (Pierce, Foster City, CA)] in PBS (1mg/ml) on ice for 90 min. Labeled cells were washed with a glycine reagent (50 mM in PBS) for 10 min 3 times to remove unbound biotin reagent and with PBS twice, and protein lysates were extracted as described above. Biotinylated proteins were precipitated with streptavidin-conjugated beads (50 µl/500 µg protein in 500 µl NP40-PBS) and immunoblotted with anti-HA and anti-V5 as described above.

**InsP assay**

Total InsP accumulation was assessed in triplicate as described (29). Briefly, transfected HEK-293 cells were labeled with [³H]-myo-inositol (2 µCi/ml) for 18 to 24 hrs and then exposed to different [Ca²⁺]e in the presence of LiCl (10 mM) for 60 min at 37°C. Total [³H]-InsPs were analyzed by anion-exchange chromatography and presented as the fold-increase over the basal levels at 0.5 mM Ca²⁺ and 0.5 mM Mg²⁺.

**Immunocytochemistry**

For dual-fluorescence immunocytochemistry, transfected HEK-293 cells grown on coverslips were fixed with paraformaldehyde (4%) for 20 min and permeabilized with methanol (80%) (34). After overnight incubation at 4°C with rat anti-HA (10 nM) and mouse anti-V5 antisera (10 nM), cells were washed and incubated with fluorescein and Texas Red-conjugated anti-IgG antibodies for 60 min at room temperature (18,29). After washing, coverslips with cells were mounted on glass slides using Gel Mount (Biomed, Foster City, CA) and examined with a Leica TCS confocal microscope (Laboratory for Cell Imaging, San Francisco Department of Veterans Affairs Medical Center). Fluorescent images were obtained sequentially, and their overlays are presented.

For cultured hippocampal neurons, immunocytochemical detection was performed with rabbit anti-CaR (500 nM), guinea pig anti-GABA-B-R1 (100 nM) antisera, and guinea pig anti-GABA-B-R2 (100 nM) antisera respectively, and corresponding fluorescein- and Texas Red-conjugated anti-IgG antibodies as described above.

Parathyroid glands from newborn calves and kidneys, growth plate cartilage, and bones from 2-4 week old mice were dissected, fixed in 4% paraformaldehyde in PBS, decalcified in 10% EDTA in PBS (for cartilage and bones only), and cut into 4 µm sections. Immunoreactivity in sections was detected with anti-GABA-B-R1 (100 nM) and HRP-conjugated goat anti-guinea pig IgG antisera after the sections were subjected to antigen retrieval with trypsin (0.01%) at 37°C for 10 minutes.

**Statistics**

Statistical differences were tested by ANOVA with the t-test using Excel 98 (MicroSoft, Seattle, WA).

**RESULTS**

**Immunoprecipitation, co-localization, and membrane expression of the CaR and GABA-B-R1 in HEK-293 cells**

To test whether CaRs physically interact with GABA-B-Rs, we transfected HEK-293 cells with cDNAs encoding hCaR-V5, HA-GABA-B-R1a, or both. The CaR and GABA-B-R1 and their associated proteins were immunoprecipitated from cell lysates by anti-V5 (Figures 1a, 1d) or anti-HA antisera (Figures 1b, 1e), respectively. Immunoblots of the proteins pulled down by anti-V5 antisera demonstrated co-precipitation of hCaR-V5 and HA-GABA-B-R1a from lysates of cells expressing both receptor cDNAs (Figures 1a, 1d; lane 1). Conversely, anti-HA antisera pulled down the HA-GABA-B-R1a together with hCaR-V5 from the same cell lysates (Figures 1b, 1e; lane 2). These immunoprecipitations were judged to be specific according to the following criteria. (1) Lysates from cells transfected with hCaR-V5 cDNA alone, immunoprecipitated with anti-V5 antibodies, did not contain any proteins immunoreactive with anti-HA antibodies (Figure 1d, lane 1). (2) Immunoprecipitations from lysates of cells transfected only with HA-GABA-B-R1a and performed with anti-HA antibodies likewise
did not contain proteins immunoreactive with anti-V5 or anti-CaR antisera (data not shown). (3) Lysates from mock-transfected HEK-293 cells, subjected to immunoprecipitation with either anti-V5 or anti-HA antibodies, did not bring down proteins capable of cross-reacting with either anti-V5, anti-HA, anti-GABA-B-R1, or anti-CaR antibodies (data not shown). (4) When lysates from HEK-293 cells separately transfected with HA-GABA-B-R1a or hCaR-V5 cDNAs were mixed and then immunoprecipitated with anti-HA antiserum, we pulled down only the HA-GABA-B-R1a but not the hCaR-V5 (data not shown). This indicates that complex formation occurs only when both receptors are synthesized and processed in the same cells and does not occur non-specifically in the lysate. On the basis of these controls, we judged the physical interaction between GABA-B-R1a and CaR to be specific and cell-dependent.

On denaturing gels, hCaR-V5’s appeared as the expected 140, 160, and >205 kD bands in the immunoprecipitates (Figure 1a) or in the input lysates prior to immunoprecipitation (Figure 1c) (29). These bands represent different glycosylated variants of a core protein with an estimated molecular weight of 120 kD and their aggregates (35). The GABA-B-R1 migrated as an 110 kD monomer and as larger aggregates of 250 kD (Figure 1d-f; lane 2) comparable to previous reports (24). The identities of these bands were confirmed by immunoblotting with antisera against the epitope tags as shown and also by antisera raised against endogenous epitopes in the CaR and GABA-B-R1 (data not shown). We consistently noted that the 140 kD form, but not the 160 kD form of the CaR, was preferentially co-immunoprecipitated with HA-GABA-B-R1a using anti-HA antiserum (Figure 1b, lane 2). This further supports a specific interaction between the 140 kD CaR and GABA-B-R1.

Dual-fluorescence immunocytochemistry with anti-V5 and anti-HA antisera revealed extensive co-localization of the hCaR-V5 and the HA-GABA-B-R1a in the cell membranes (Figure 1g, arrowheads), peri-nuclear regions (double arrowheads), and intracellular organelles (arrows) in transfected HEK-293 cells, further supporting the biochemical association of these receptors that we noted in immunoprecipitations.

Co-expressing the GABA-B-R1 altered the cell-surface expression of the hCaR-V5. We demonstrated this by immunoblotting surface proteins from HEK-293 cells, previously labeled with biotin reagent, and precipitated with avidin-conjugated beads (Figures 1h, 1i). In lysates from cells expressing both hCaR-V5 and HA-GABA-B-R1a cDNAs, the levels of biotin-labeled CaRs were decreased by 70% (Figure 1i, lane 2) compared to levels in cells expressing hCaR-V5 alone (Figure 1i, lane 1). This change was accompanied by a reduction in total hCaR-V5 protein expression averaging 70% (for the 140 and 160 kD forms) in cells co-expressing HA-GABA-B-R1a (Figures 1h, 1c lane 2 vs 1), demonstrating a regulation of CaR expression by the GABA-B-R1.

Effects of co-expression of CaRs and GABA-B-Rs on signal transduction

We next addressed whether co-expressing GABA-B-R1’s and CaRs affected signal transduction by CaRs. We tested the effects of raising [Ca\(^{2+}\)]\(_e\) on the accumulation of total InsPs as an index of PLC activation in HEK-293 cells. Cells expressing cDNAs encoding either the hGABA-B-R1a or the hGABA-B-R1b showed no InsP response to raising [Ca\(^{2+}\)]\(_e\) from 0.5 to 10 mM in the presence or absence of the GABA-B-R agonists baclofen (10^{-4} M) or GABA (3x10^{-4} M) (data not shown). Similar negative results were also obtained in cells co-expressing cDNAs for both the hGABA-B-R1 (1a or 1b) and R2 (data not shown). In contrast, raising [Ca\(^{2+}\)]\(_e\) increased total InsP accumulation in a dose-dependent manner in cells expressing the hCaR-V5 alone (Figure 1j). Interestingly, high [Ca\(^{2+}\)]\(_e\)-induced InsP accumulation was significantly blunted in cells co-expressing both the CaR and the HA-GABA-B-R1a cDNA (Figure 1j). Maximal responses to > 10 mM Ca\(^{2+}\) were decreased by 40% (p<0.001), and there was a modest but statistically significant shift to the right in the ED\(_{50}\) for Ca\(^{2+}\) from 3.6 to 5.1 mM (p<0.01; N=10 transfections) (Figure 1j). Co-expression of cDNAs for the two human GABA-B-R1 splice forms -- hGABA-B-R1a and hGABA-B-R1b -- had the same effects on the signaling responses of the hCaR-V5 (data not shown).
Immunoprecipitation, co-localization, and membrane expression of the CaR and GABA-B-R2 in HEK-293 cells

We next sought to test whether the GABA-B-R2, a partner of the GABA-B-R1 in vivo, also associated with the CaR in HEK-293 cells. Antiserum to the hCaR-V5 co-immunoprecipitated the HA-tagged rat GABA-B-R2 (HA-GABA-B-R2) from lysates of HEK-293 cells co-expressing these receptor cDNAs, strongly suggesting a physical interaction between these receptors. Anti-V5 antiserum pulled down the hCaR-V5 (Figure 2b, lane 2) together with the HA-GABA-B-R2 (Figure 2f, lane 2) from cells expressing both receptors but not from the cells expressing hCaR-V5 only (Figure 2f, lane 1). Similarly, anti-HA antiserum pulled down the HA-GABA-B-R2 (Figure 2g, lane 2) together with the hCaR-V5 (Figure 2c, lane 2). Like the HA-GABA-B-R1, as noted above, the HA-GABA-B-R2 associated preferentially with the 140 kD form of the hCaR-V5 in the protein complexes pulled down by anti-HA antiserum (Figure 2c; lane 2).

Co-expressing cDNAs encoding HA-GABA-B-R2 and hCaR-V5 also impacted the expression and signal transduction of the CaR. In contrast to inhibition by the presence of GABA-B-R1 on CaR signaling, co-expressing the HA-GABA-B-R2 with the CaR not only increased the expression of CaR protein by \( \approx 100\% \) by immunoblotting (Figures 2a, 2b, lane 2 vs lane 1) but also enhanced cell-surface CaR expression. In the presence of the HA-GABA-B-R2, CaR surface expression was increased 2-fold, as quantified by surface biotin labeling (Figure 2d, lane 2 vs lane 1). Consistent with increased CaR expression, co-expressing the HA-GABA-B-R2 with the CaR enhanced InsP accumulation in cells in response to high [Ca\(^{2+}\)]\(_e\) (Figure 2i). In cells expressing both the hCaR-V5 and the HA-GABA-B-R2, maximal responses to \( \geq 10 \) mM Ca\(^{2+}\) were increased by \( >100\% \) (N=7; p<0.001) (Figure 2i), compared to cells expressing the hCaR-V5 alone (Figure 2i). The ED\(_{50}\) for Ca\(^{2+}\), however, was unchanged (Figure 2i). These data support a role for the GABA-B-R2 in promoting and/or stabilizing membrane CaR expression and thereby its signaling capacity.

Reciprocal effects of CaRs on GABA-B-R1 or R2 expression

We next determined whether expressing CaRs along with either GABA-B-R1’s or R2’s in HEK-293 cells could alter GABA-B-R1 or R2 expression. Immunoblots indicated that co-expressing CaRs strongly increased total immunoreactivity and cell-surface expression by biotin labeling of either the HA-GABA-B-R1 (Figures 3a, 3b) or R2 (Figures 3c, 3d) in cells expressing these cDNAs.

Because the GABA-B-R1 normally complexes with the R2 to form fully functional receptors, we tested whether co-expressing the HA-GABA-B-R1 can interfere with the interaction between the hCaR-V5 and the hGABA-B-R2. In these experiments, we used untagged hGABA-B-R2 to be able to distinguish it unequivocally from the HA-GABA-B-R1 during immunoprecipitation or immunoblotting using anti-HA antisera. Equal quantities by mass of receptor cDNA were included in each transfection.

As shown in Figure 4a, co-expressing cDNAs for the hGABA-B-R2 and hCaR-V5 in HEK-293 cells enhanced high [Ca\(^{2+}\)]\(_e\)-induced InsP accumulation, compared to the responses in cells expressing hCaR-V5 alone. This effect was blocked when HA-GABA-B-R1 cDNA was also included in the transfections. Co-expressing the HA-GABA-B-R1 also blocked the ability of the hGABA-B-R2 to enhance total immunoreactivity and cell-surface expression of the hCaR-V5 (Figures 4b, 4c). These data suggested that there was a competitive interaction between the GABA-B-R1 and R2 to influence the effects of the opposite receptor subunit on CaR-induced InsP accumulation and on cell-surface and total cellular CaR expression.

Interactions among the GABA-B-R1 and R2 and the CaR

To test the possibility that the HA-GABA-B-R1 might interfere with the physical association between the hCaR-V5 and hGABA-B-R2, we performed immunoprecipitations using anti-V5 (Figures 5a, 5b) or anti-HA (Figures 5c, 5d) antisera on lysates from cells expressing the hGABA-B-R2 alone (lane 1 in each panel), hGABA-B-R2 plus hCaR-V5 (lane 2 in each panel), or hGABA-B-R2 plus hCaR-V5 and HA-GABA-B-R1 (lane 3 in each panel). Expression levels for the hGABA-B-R2, HA-GABA-B-R1, and hCaR-V5 in the immunoprecipitates were assessed by immunoblotting with anti-GABA-B-
R2 (Figures 5a, 5c), anti-HA (Figure 5b), and anti-V5 (Figure 5d), respectively. A reduction in hGABA-B-R2 immunoreactivity was observed in the hCaR-associated complexes pulled-down by anti-V5 antisera from cells expressing all 3 receptor cDNAs (Figure 5a, lane 3), compared to that of cells expressing just the hGABA-B-R2 plus hCaR-V5 (Figure 5a, lane 2). This suggested a block in the interaction between the hGABA-B-R2 and hCaR-V5 when the HA-GABA-B-R1 was overexpressed. In addition, in precipitates brought down by anti-V5, we observed significant immunoreactivity detected by anti-HA antisera (Figure 5b, lane 3), indicating the presence of HA-GABA-B-R1 in these complexes and, thereby, an interaction between hCaR-V5 and HA-GABA-B-R1. This was confirmed by the presence of hCaR-V5 in the HA-GABA-B-R1-associated complexes pulled-down with anti-HA in these triply transfected cells (Figure 5d, lane 3). In these R1-associated complexes, we also observed substantial hGABA-B-R2 immunoreactivity (Figure 5c, lane 3), indicating at least an interaction between the GABA-B-R1 and R2. This suggested the potential for the formation of a complex containing GABA-B-R1 and R2 and did not rule out the possibility that the CaR, GABA-B-R1 and R2 associated within a single complex.

Interaction of CaR mutants with GABA-B-Rs

We next tested whether the C-terminal tail of the CaR is required for complex formation with the GABA-B-R1. We co-transfected cDNAs encoding the HA-GABA-B-R1 and a truncated bovine parathyroid CaR mutant (T866) missing the entire C-terminal tail of the receptor (residues #867-1085) (30). Immunoreactive bands of sizes compatible with the truncated CaRs were detected in complexes pulled-down from lysates of cells co-expressing these receptors using an anti-HA antiserum (Figure 6b, lane 2). Furthermore, immunoblots of the input lysates from the transfected cells prior to immunoprecipitation demonstrated that co-expressing HA-GABA-B-R1 also suppressed the total amount of the truncated CaR that was expressed (Figure 6a, lane 2 vs lane 1). This finding was similar to what we noted with the full-length CaR. Thus, the extracellular and transmembrane domains of the CaR are sufficient to allow the CaR and GABA-B-R1 to interact.

A cardinal feature of the CaR and other family C receptors is the large extracellular domain (ECD). To determine whether this domain in the CaR is involved in the interaction with the GABA-B-Rs, we transfected cells with a cDNA encoding the ECD (residues 1 to 579) of the hCaR fused to GFP (ECD-GFP) and cDNAs encoding either HA-GABA-B-R1 or R2. Immunoprecipitations of cell lysates were performed with anti-HA antisera and the expression of ECD-GFP was determined by immunoblotting with either anti-CaR (Figures 6c, 6d) or anti-GFP antisera (data not shown). The ECD-GFP co-immunoprecipitated with the HA-GABA-B-R1 (Figure 6d, lane 4) as well as with HA-GABA-B-R2 (Figure 6d, lane 5).

Interactions of CaRs and GABA-B-Rs in native tissues

To determine whether physical interactions between CaRs and GABA-B-Rs might occur in native tissues, we performed immunocytochemistry to detect GABA-B-Rs in the parathyroid gland, kidney, bone, cartilage, and hippocampal neurons where CaRs are known to be expressed (15,18,36,37). GABA-B-R1 immunoreactivity was diffusely distributed in the parathyroid gland, likely in the chief cells that make PTH (Figure 7a, arrowheads). In kidney cortex, extensive GABA-B-R1 immunoreactivity was found in tubular cells (36) (Figure 7b, arrowheads). In bone sections (Figure 7c), GABA-B-R1 immunoreactivity was present in osteoblasts (black arrowheads), osteocytes (black arrows), osteoclasts (double arrows), and other bone marrow cells (red arrowheads), where we and others have detected CaR immunoreactivity and transcripts (18,37,38). In growth plate cartilage, GABA-B-R1 immunoreactivity was scattered in proliferating cells (Figure 7d; red arrowheads), present at higher levels in maturing cells (red arrows), and at the highest levels in hypertrophic chondrocytes (black arrowheads). This pattern mirrors CaR expression in mouse, rat, and bovine growth plate cartilage (18). Immunoreactivity in these tissues was judged to be specific, since it was absent in bone and cartilage sections treated with anti-GABA-B-R1 antiserum preabsorbed with the peptide against which it was raised (data not shown).

Co-expression of the CaR and the GABA-B-R1 or R2 in cultured hippocampal neurons was...
confirmed by dual-fluorescence immunocytochemistry. Co-localization of the CaR (Figure 8a, green) and GABA-B-R1 (red) was demonstrated in cell bodies (arrows) and processes (arrowheads) of the neurons. The larger flattened cells underneath the neurons, presumably astrocytes and other types of neuroglia, were negative (double arrows). These latter cells showed substantial expression of CaRs but not of GABA-B-R1’s. A similar pattern of co-localization was observed for the CaR and GABA-B-R2 (Figure 8b).

To examine whether the GABA-B-R1 physically interacts with the CaR in the brain, we immunoprecipitated the GABA-B-R1 and associated proteins. In these immunoprecipitates, we identified GABA-B-R1’s with predominant sizes of $\approx 110$ and $130$ kD and larger aggregates ($>200$ kD) (Figure 8c, anti-R1). This immunoreactivity was absent when anti-GABA-B-R1 antiserum was omitted from the immunoprecipitation [Figure 8c, (-)Ab]. In the complexes pulled-down by anti-GABA-B-R1, we also detected a predominant band with a size of $\approx 110$ kD and a fainter band with a size of $\approx 130$ kD which were both immunoreactive with the anti-CaR antibody, supporting a physical interaction between the GABA-B-R1 and CaR in the brain (Figure 8d, lane 2).

Effects of knockout of the GABA-B-R1 in the brain and cultured hippocampal neurons

We next tested whether the knockout of the GABA-B-R1 altered the expression of the CaR in two animal models. We first compared the expression of the CaR protein in lysates from the brains of wt and GABA-B-R1 knockout mice [GABA-B-R1(-/-)] (33). Immunoblots of protein lysates from wt mouse brains showed 3 protein bands of $\approx 110$, $120$, and $130$ kD reactive with anti-CaR antiserum (Figure 9a). These bands were absent in blots probed with anti-CaR antiserum preabsorbed with immunizing peptide (data not shown). Bands of the same sizes were also observed in lysates from GABA-B-R1(-/-) mice, but the intensities of the $\approx 110$ and $120$ kD bands were consistently increased (by $\approx 75\%$, $p<0.05$). Interestingly, there were two larger aggregates with sizes $>200$ kD that were not easily detected in lysates from wt mouse brain (Figure 9a, arrows). The expression of the $130$ kD band did not differ in lysates from wt and GABA-B-R1(-/-) mice.

To confirm these observations, we knocked-out the GABA-B-R1 genes in cultured hippocampal neurons by Cre-lox recombination and looked at its effect on the expression of the CaR. Cultured hippocampal neurons from mice carrying homozygous floxed GABA-B-R1 genes (33) were infected with adenoviruses expressing bacterial Cre recombinase cDNA. Real-time PCR showed a decrease of $\approx 75\%$ in the RNA levels for the GABA-B-R1 in floxed-GABA-B-R1 neurons infected with Ad-Cre, when compared to cells infected with control viruses (data not shown). In cells infected with Ad-Cre viruses, the expression of GABA-B-R1 protein was also reduced (by $\approx 45$-65\%) when compared to cells infected with Ad-Cont viruses (Figure 9b). As predicted, this was accompanied by an increase in the expression of total cellular CaR averaging $\approx 40\%$ in Ad-Cre-infected cells compared to control virus-infected neurons ($p<0.05$; $N=3$ independent infections) (Figure 9c). These findings support the idea that there is a reciprocal relationship between CaR and GABA-B-R1 expression in mouse brain along the lines of what we observed in HEK-293 cells overexpressing these receptors.

DISCUSSION

Oligomerization of either the identical or different receptor subunits is an established feature of GPCRs. Homodimerization of $\beta$-adrenergic receptors and heterodimerization between the angiotensin II receptor type 1 (AT1) and the bradykinin B2 receptors and between the purinergic $P_{2Y1}$ and the adenosine A1 receptors are clear examples of this phenomenon (39). The formation of receptor oligomers plays a critical role in modulating the function of family C receptors. This is best illustrated by the GABA-B-Rs in which there is an absolute need for heterodimerization between the R1 and R2 subunits to produce a fully functional GABA-B-R (40,41).

Work by Bai and colleagues (35,42,43) supports the idea that homodimerization of CaRs is also critical to their function. In their studies, co-expression of wt and signaling-deficient mutant CaRs in HEK-293 cells produced a population of receptors with markedly reduced responses to $[Ca^{2+}]_{o}$ (42). This supports a critical interaction
between the wt and mutant CaRs at the cell-surface that modifies CaR function. This group was further able to co-immunoprecipitate wt and mutant CaRs, identified by different epitopes tags, which further supported either a direct physical association between the two receptors or an association within a larger signaling complex (42,44).

Studies by Gama et al (28) showed that CaRs have the capacity to form heteromeric complexes with mGluRs in HEK-293 cells and in the brain by co-immunoprecipitation studies. Our observations in transfected HEK-293 cells and brain lysates support the formation of heteromeric complexes between CaRs and GABA-B-Rs. These interactions clearly add another dimension to models of how signaling by family C receptors in the CNS might be regulated. The biological importance of the interactions among family C GPCRs could be substantial. In particular, any interaction between the CaR and GABA-B-R1 and R2 could have broad impact because of their wide distribution in many cell types and their ability to respond to ubiquitous ligands.

Our data indicate that the GABA-B-R1 and R2 critically regulate the expression of CaR protein but do so in opposing directions. Because the cDNAs we used contain a strong viral (CMV) promoter, transcription of the cDNAs is considered to be constitutive. The impact of GABA-B-Rs on CaR expression that we observed is likely occurring at early post-translational steps. Extensive co-localization of these receptors beginning in the perinuclear compartments, likely endoplasmic reticulum, in HEK-293 cells suggests that these receptors begin to interact immediately after their synthesis. The latter notion is further supported by the observation that GABA-B-R1 and R2 preferentially interact with the ≈140 kD form of the CaR which is thought to be a less mature, high-mannose form of the receptor. Our data further indicate that cell-surface expression of the 140 kD CaR was enhanced by co-expression of the GABA-B-R2. Thus, complex formation between GABA-B-Rs and CaRs might be influencing post-translational modification (e.g., glycosylation), receptor stability, and turnover (trafficking and degradation) of these receptors.

What special functions could be served by the formation of heteromeric complexes between CaRs and GABA-B-Rs? Studies with the GABA-B-R1 and R2 suggest that their heterodimerization is absolutely critical for efficient receptor trafficking to the cell membrane, formation of ligand-binding sites, and coupling to downstream effectors. When the GABA-B-R1 is expressed alone in HEK-293 cells or Xenopus oocytes, the R1 protein is restricted to the endoplasmic reticulum (ER) and does not traffic to the cell membrane or mediate signal transduction. This is due to the presence of an ER retention sequence (R-S-R-R) in the C-terminal tail of the GABA-B-R1 (24,41,45). When this sequence is mutated, the GABA-B-R1 trafficks to the membrane, but it lacks signaling ability. This ER retention signal is absent in the GABA-B-R2, which trafficks normally to the cell membrane when expressed alone, but the GABA-B-R2 by itself does not activate signaling pathways due to the lack of a ligand-binding site. It has been proposed that the formation of heteromeric complexes between R1 and R2 allows the C-terminal tail of the GABA-B-R2 to “mask” the ER retention domain in the R1. This permits the trafficking of heterodimers to the cell-surface, binding of ligands to the R1 subunit, and coupling of the R2 to signaling molecules (24). This “masking” effect is thought to occur via a coiled-coiled interaction between two predicted α-helical domains in the C-terminal tails of the GABA-B-R1 and R2 (2,24). A putative ER retention sequence (RXR) comparable to the one in the GABA-B-R1 is also present in the CaR along with a similarly located α-helical domain (1, 30). Our previous studies showed that mutations of residues in this α-helical region completely blocked targeting of the receptor to the cell-surface (30).

Based on the above studies, we speculate that CaR may also be stabilized and signal more efficiently by forming a complex with other CaRs. The co-association in complexes of CaRs with other members of family C, such as GABA-B-R1 or R2, however, may change the ligand sensitivity, G-protein coupling, and specificity of signaling responses of the CaR in as yet unknown ways. These possibilities will require formal testing.

Findings in the present study indicate that expression of GABA-B-Rs influences the overall levels of the CaR and its membrane targeting. Complex formation between the CaR and the GABA-B-R2 promotes the membrane CaR expression and thus enhances signal transduction.
by this mechanism. Possibly CaR/GABA-B-R2 complexes may be more stable than CaR homodimers. It is likely that the GABA-B-R2 may produce a better “masking” effect on a putative ER-retention signal in the CaR than another CaR. In remarkable contrast to this paradigm, the GABA-B-R1 appears to interfere with CaR homodimerization either by competing for CaR subunits or by producing less stable complexes (i.e., CaR/R1) that eventually target CaRs for degradation. The R2/CaR complexes also appear to be more stable and traffic more efficiently than R2 by themselves according to our data. Likewise, forming complexes with the CaR stabilizes the GABA-B-R1 and increases its expression on the cell-surface. Whether this is due to the ability of CaR to mask the ER retention signal in the R1 will require further investigation.

We further demonstrated that the ability of the GABA-B-R2 to form complexes with and to stabilize the CaR was significantly blunted by co-expression of the R1. This supports competition between the R1 and R2 subunits for the CaR and suggests that the R1/CaR complex may be a stronger association than the R2/CaR complex. Competition among different receptors for partners is one explanation for our data. We, however, have no direct proof for it, as we did not examine the effects of varying amounts of cDNA transfected. Equal amounts (10 µg) of each cDNA were transfected, and the amount of cDNA used was well above the levels needed for maximal signaling responses in these cells. Furthermore, we did not directly assess whether these receptors are expressed with equal efficiency or at the same molar concentrations under our conditions. Nor were absolute levels of CaRs vs GABA-B-R1 and R2 subunits in the brain and in hippocampal neurons assessed in the present study. In the latter tissue and cells, we observed co-immunoprecipitation of the GABA-B-R1 and the CaR and a response to knocking-down GABA-B-R1 expression on total CaR expression. This suggested to us that our observations in HEK-293 cells, where receptors were overexpressed, might be relevant to native tissues.

What role might the formation of complexes between the CaR and GABA-B-R serve in target tissues? Complexes between the P2Y1 and A1 receptors display different sensitivities to antagonists and activate different G proteins, compared to pure populations of P2Y1 or A1 receptors (46). Along these lines, we observed a rightward-shift in the dose-response curve for high [Ca^{2+}]_e to activate PLC in HEK-293 cells co-expressing the GABA-B-R1 and CaR, supporting a possible change in the pharmacological properties of CaR/R1 heterodimers. Although the shift in EC_{50} was modest, it may reflect a more significant alteration in pharmacologic properties of CaR/GABA-B-R1 heterodimers. The normal signaling of homodimers, the more prevalent CaR form, would tend to mask the change. Furthermore, we cannot rule out the possibility that reduced receptor numbers contributed to the changes in signaling, because total and cell-surface expression of CaRs was reduced in cells co-expressing the GABA-B-R1. We think, however, that this is less likely, as GABA-B-R2 overexpression increased both CaR expression and maximal signaling capacity without affecting the EC_{50} of high [Ca^{2+}]_e-induced signaling responses. In addition, our previous studies, in which we compared CaR signaling when different levels of CaR expression were achieved in HEK-293 and COS-1 cells, showed that the EC_{50}’s for Ca^{2+} were relatively constant despite different levels of CaR expression (unpublished data).

It is well documented that CaRs are expressed in various parts of the brain, where GABA-B-Rs are present (2,16). By confocal microscopy we co-localized these receptors in cultured neurons and immunoprecipitated them, confirming complex formation between the GABA-B-R1 and CaR in brain lysates. Our data indicate that the GABA-B-R1 appears to interact preferentially with CaRs of lower molecular weight (i.e., ≈110, 120, and 130 kD). The exact sequences of CaR species in the brain remain unclear. These receptor forms tend to be smaller than the recombinant CaRs expressed in HEK-293 cells. This may be due to differential glycosylation, alternative splicing, and/or proteolytic cleavage in these tissues. Further studies are required to distinguish among these possibilities.

In addition to the brain, we have found that GABA-B-R1 and CaRs also co-immunoprecipitated in chondrocytes (manuscript in preparation). We further found that knocking-out GABA-B-R1 genes altered the potency of high [Ca^{2+}]_e to activate ERK1/2 in cultured chondrocytes but not the ability of high [Ca^{2+}]_e to
activate PLC -- a distinct effect from what we observed in HEK-293 cells (manuscript in preparation). These data taken together suggested that the GABA-B-R1 may affect the coupling of CaRs to downstream signaling molecules and the effect may depend on the composition of signaling molecules in each cell type, in this case chondrocytes vs HEK-293 cells. It is also plausible that complex formation between GABA-B-Rs and CaRs may alter the ability of CaRs to sense changes in $[\text{Ca}^{2+}]_c$ in their milieu as well as the signaling of other endogenous receptors. These studies raise the possibility that interactions among family C GPCRs could modulate signaling networks in tissues like the brain. Furthermore, heterodimeric complexes between family C GPCRs (and other associated molecules) may represent new therapeutic targets for the development of specific and effective treatments for neurologic disorders and modulating CNS responses that are mediated by GABA and Ca$^{2+}$. 
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**FIGURE LEGENDS**

**Figure 1.** Immunoprecipitation, membrane localization, and signal transduction of the hCaR-V5 and HA-GABA-B-R1. (a-f) Immunoblotting (IB) of immunoprecipitates (Imppts) (a,b,d,e) and input lysates prior to immunoprecipitation (c,f) prepared from HEK-293 cells transiently expressing cDNAs for hCaR-V5 (lane 1) or hCaR-V5 plus HA-GABA-B-R1a (lane 2). Immunoprecipitation was performed using anti-V5 (a,d) and anti-HA (b,e) antisera. Anti-V5 (a,b,c) and anti-HA (d,e,f) antisera were used to detect the hCaR-V5 and HA-GABA-B-R1a, respectively. Blots are representative of 12 independent experiments. (g) Detection of the CaR (green) and GABA-B-R1 (red) by dual-fluorescence immunocytochemistry with confocal microscopy in HEK-293 cells transfected with cDNAs encoding both receptors. Co-localization of the receptors was indicated by yellow color in overlay images. Bottom micrographs in panel g are digitally enlarged images of two regions of interest indicated by square boxes in the overlay panel. The receptors were co-localized in the cell membrane (arrowheads), perinuclear ER (double arrowheads), and other intracellular organelles (arrows) with “N” indicating the nucleus. (h,i) Blotting of precipitates (ppts) pulled-down by avidin-conjugated beads and input lysates prior to precipitation from HEK-293 cells transiently expressing cDNAs encoding hCaR-V5 alone (lane 1) or hCaR-V5 plus HA-GABA-B-R1a (lane 2). Cells were exposed to biotin reagents as described in Materials and Methods before lysates were harvested. (j) Effects of raising \([\text{Ca}^{2+}]_e\) from 0.5 mM to the concentrations shown on total \(^3\text{H}\)-InsP accumulation in HEK-293 cells transiently expressing cDNAs encoding hCaR-V5 (●) or hCaR-V5 with HA-GABA-B-R1a (○). Changes in \(^3\text{H}\)-InsPs at different \([\text{Ca}^{2+}]_e\) are presented as the fold-increase over basal levels at 0.5 mM Ca\(^{2+}\) (N=10 experiments in triplicate).

**Figure 2.** Immunoprecipitation, cell-surface expression, and signal transduction of the CaR and the GABA-B-R2. (a-h) Immunoblotting (IB) of input lysates prior to immunoprecipitation (a,e), immunoprecipitates (Imppts) pulled-down by anti-V5 (b,f) and anti-HA (c,g) antisera, and precipitates (ppts) pulled-down from lysates from biotinylated HEK-293 cells transiently expressing cDNAs encoding the hCaR-V5 (lane 1) or hCaR-V5 plus HA-GABA-B-R2 (lane 2) using avidin-conjugated beads (d,h). Blotting was performed using anti-V5 (a-d) and anti-HA (e-h) to detect hCaR-V5 and HA-GABA-B-R2, respectively, and represents 1 of 6 independent experiments. (i) Effects of raising \([\text{Ca}^{2+}]_e\) from 0.5 mM to the concentrations shown on total \(^3\text{H}\)-InsP accumulation in HEK-293 cells expressing cDNAs encoding hCaR-V5 (●) or hCaR-V5 and HA-GABA-B-R2 (○). \(^3\text{H}\)-InsP accumulation at different \([\text{Ca}^{2+}]_e\) is presented as the fold-increase over basal levels at 0.5 mM Ca\(^{2+}\) (N= 7 experiments in triplicate).

**Figure 3.** Effects of co-expressing the CaR and either the HA-GABA-B-R1 or HA-GABA-B-R2 cDNAs on receptor association and cell-surface expression. Immunoblots of the input lysates before (a,c) and after (b,d) precipitation with avidin beads of lysates from biotinylated HEK-293 cells transiently expressing cDNAs encoding HA-GABA-B-R1a (HA-R1a) (lane 1), HA-R1a plus hCaR-V5 (lane 2), HA-GABA-B-R2 (HA-R2) (lane 3), or HA-R2 plus hCaR-V5 (lane 4). Detection was with anti-HA for HA-R1a or HA-R2 depending on the transfection protocol (#1-4) (N=4 independent experiments).

**Figure 4.** PLC activation in response to raising \([\text{Ca}^{2+}]_e\) and changes in CaR cell-surface expression due to the presence of the hGABA-B-R2 with or without the HA-GABA-B-R1. (a) Effects of different \([\text{Ca}^{2+}]_e\) on total \(^3\text{H}\)-InsP accumulation in HEK-293 cells expressing cDNAs encoding the hCaR-V5 (●), hCaR-V5 with hGABA-B-R2 (hR2) (○), or hCaR-V5 with both hR2 and HA-R1a (●). Changes in \(^3\text{H}\)-InsPs at different \([\text{Ca}^{2+}]_e\) are presented as the fold-increase over basal levels at 0.5 mM Ca\(^{2+}\) (N= 9 experiments in triplicate). (b,c) Immunoblotting of lysates before (a) and after (b) precipitation with avidin from biotinylated HEK-293 cells transiently expressing cDNAs encoding hCaR-V5 (lane 1), hCaR-V5 plus hR2 (lane 2), hCaR-V5 plus hR2 and HA-R1a (lane 3). Blots were done with anti-V5 to detect the hCaR-V5 (N= 4 experiments).

**Figure 5.** Effects of expressing the HA-GABA-B-R1 on the association between the hGABA-B-R2 (hR2) and the hCaR-V5. Blotting of immunoprecipitates (Imppt) brought down by anti-V5 (a,b) and anti-
HA (c,d) antisera from HEK-293 cells transiently expressing cDNAs encoding hR2 (lane 1), hR2 plus hCaR-V5 (lane 2), or hR2 plus hCaR-V5 and HA-R1a (lane 3). Immunoblotting was performed using anti-GABA-B-R2 (a,c), anti-HA (b), and anti-V5 (d) antisera to detect hR2, HA-R1a, and hCaR-V5, respectively (N=6 independent experiments).

**Figure 6.** A C-terminally truncated CaR and the extracellular domain of the CaR co-immunoprecipitate the GABA-B-R1 and R2. Blots of input lysates (a,c) and immunoprecipitates (b,d) performed with anti-HA (b,d) antisera from HEK-293 cells transiently expressing cDNAs encoding the tail-truncated bovine CaR (T866-CaR) (lane 1, panels a and b), T866-CaR plus HA-R1a (lane 2, panels a and b), ECD-GFP (lane 3, panels c and d), ECD-GFP plus HA-R1a (lane 4, panels c and d), or ECD-GFP plus hR2 (lane 5, panels c and d). These blots are representative of 3 independent experiments.

**Figure 7.** Endogenous GABA-B-R1 expression in bovine and murine tissues. GABA-B-R1s were detected in sections of bovine parathyroid glands (a), mouse kidney cortex (b), mouse tibia (c), and mouse epiphyseal growth plate (d) using anti-GABA-B-R1 antibodies as indicated by brown DAB staining. For details of staining patterns in each tissue see the Results section. Bars, 50 µm.

**Figure 8.** Expression of the CaR and the GABA-B-R1 and R2 in hippocampal neurons and brain lysates by immunocytochemistry and immunoblotting and their co-association in brain lysates by immunoprecipitation. (a) Dual-fluorescence confocal microscopy indicates the presence of CaR (green) and GABA-B-R1 (red) along with their localization by image overlay (yellow). (b) Confocal imaging demonstrates the CaR (green) and GABA-B-R2 (red) along with their localization by image overlay (yellow). CaRs and GABA-B-Rs [both R1 (panel a) and R2 (panel b)] are co-localized in cell bodies (arrows) and processes (arrowheads) of the neurons as indicated in the overlays (yellow) at either 40 or 200x magnification. Double arrows depict a large flattened astrocyte and other types of neuroglia, which stain positively for CaR but not for GABA-B-R1 or R2 (a,b; overlay images). Bars: 40x, 50 µm; 200x, 10 µm. (c, d) Immunoblotting of mouse brain lysates before (Input) and after immunoprecipitation (Imppt) with (anti-R1) or without [(-)Ab] anti-GABA-B-R1 antisera. Blotting was performed with the GABA-B-R1 (c) or CaR (d). Blots represent 3 lysates.

**Figure 9.** Effects of knocking-out the GABA-B-R1 on CaR expression in the brain and in cultured hippocampal neurons. (a) Immunoblots of brain lysates from wt and GABA-B-R1 knockout [R1(-/-)] mice demonstrated expression of multiple CaR-specific bands. There was a modest increase in the intensity of the ≈110 and 120 kD bands of ≈75% in the R1(-/-) lysate with β-actin serving as a protein loading control (N=2 lysates). (b) Immunoblots of lysates from floxed-GABA-B-R1 hippocampal neurons 4-5 days after infection with Ad-Cont (lane 1) or Ad-Cre (lane 2) adenoviruses. Control levels of GABA-B-R1 expression (lane 1) were reduced by ≈50% after knocking down the GABA-B-R1 genes by the expression of Cre recombinase in hippocampal neurons (lane 2). (c) Blots depict changes in CaR expression in hippocampal neurons with their GABA-B-R1 genes knocked down, compared to levels in control cells, in 3 independent experiments (Exp#1-3). There was an average ≈40% decrease in CaR expression in Ad-Cre vs Ad-Cont virus-infected cells. CaR expression in panels (a) and (c) was quantified by densitometry and normalized to the expression of β-actin as described in Results.
Figure 1

Transfection protocol:
1. CaR-V5
2. CaR-V5 + HA-GABA-B1a

j

h

hCaR-V5
hCaR-V5 + HA-GABA-B1a

[Ca^{2+}]_o, mM
Figure 2

Transfection protocol: 1. CaR-V5  2. CaR-V5+HA-GABA-B-R2
Figure 3

Transfection protocol:
1. HA-R1a
2. HA-R1a+CaR-V5
3. HA-R2
4. HA-R2+CaR-V5

IB: anti-HA
Figure 4

(a) Graph showing the fold increase of \(^{3}\text{H}\)-InsP in relation to the calcium concentration ([Ca\(^{2+}\)])

(b) Western blot of input samples with anti-V5 antibody

(c) Western blot of samples precipitated with avidin

Transfection protocol:
1. CaR-V5
2. CaR-V5+hR2
3. CaR-V5+hR2+HA-R1a
Figure 5

Transfection protocol:
1. hR2
2. hR2+CaR-V5
3. hR2+CaR-V5 +HA-R1a
Figure 6

Transfection protocol:
1. T866-CaR
2. T866-CaR +HA-R1a
3. ECD-GFP
4. ECD-GFP +HA-R1a
5. ECD-GFP +HA-R2
Figure 8

(a) CaR, GABA-B-R1, Overlay

40x

200x

(b) CaR, GABA-B-R2, Overlay

40x

200x

(c) Input, Imppt, Anti-R1, (-)Ab

(d) Input, Imppt, Anti-R1, (-)Ab

IB: anti-GABA-B-R1

IB: anti-CaR
Complex formation with the type B γ-aminobutyric acid receptor affects the expression and signal transduction of the extracellular calcium-sensing receptor: Studies with HEK-293 cells and neurons

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