Elucidation of the Role of Peptide Linker in Calcium-sensing Receptor Activation Process

Received for publication, October 11, 2006, and in revised form, December 11, 2006 Published, JBC Papers in Press, December 21, 2006, DOI 10.1074/jbc.M609610200

Kausik Ray, Kaylin A. Adipietro, Claudia Chen, and John K. Northup
From the Laboratory of Cellular Biology, NIDCD, National Institutes of Health, Bethesda, Maryland 20892

Family 3 G-protein-coupled receptors (GPCRs), which includes metabotropic glutamate receptors (mGluRs), sweet and “umami” taste receptors (T1Rs), and the extracellular calcium-sensing receptor (CaR), represent a distinct group among the superfamily of GPCRs characterized by large amino-terminal extracellular ligand-binding domains (ECD) with homology to bacterial periplasmic amino acid-binding proteins that are responsible for signal detection and receptor activation through as yet unresolved mechanism(s) via the seven-transmembrane helical domain (7TMD) common to all GPCRs. To address the mechanism(s) by which ligand-induced conformational changes are conveyed from the ECD to the 7TMD for G-protein activation, we altered the length and composition of a 14-amino acid linker segment common to all family 3 GPCRs except GABAB receptor, in the CaR by insertion, deletion, and site-directed mutagenesis of specific highly conserved residues. Small alterations in the length and composition of the linker impaired cell surface expression and abrogated signaling of the chimeric receptors. The exchange of nine amino acids within the linker of the CaR with the homologous sequence of mGluR1, however, preserved receptor function. Ala substitution for the four highly conserved residues within this amino acid sequence identified a Leu at position 606 of the CaR critical for cell surface expression and signaling. Substitution of Leu606 for Ala resulted in impaired cell surface expression. However, Ile and Val substitutions displayed strong activating phenotypes. Disruption of the linker by insertion of nine amino acids of a random-coiled structure uncoupled the ECD from regulating the 7TMD. These data are consistent with a model of receptor activation in which the peptide linker, and particularly Leu606, provides a critical interaction for the CaR signal transmission, a finding likely to be relevant for all family 3 GPCRs containing this conserved motif.

The human extracellular calcium-sensing receptor (CaR) is a novel cation-sensing G-protein-coupled receptor (GPCR) in parathyroid cells and plays a central role in the regulation of extracellular [Ca2+]o homeostasis by controlling the rate of parathyroid hormone secretion (1). The CaR may also be involved in other physiological regulation in organs such as bone, brain, kidney, and intestine. Activation of CaR by elevated levels of [Ca2+]o stimulates phospholipase C via the Gs subfamily of G-proteins resulting in the increase of phosphoinositide (PI) hydrolysis and subsequently in the release of intracellular Ca2+ from stores in the endoplasmic reticulum.

CaR is a member of the family 3 GPCR gene family that includes eight metabotropic glutamate receptors (mGluR1–8), two γ-aminobutyric acid receptor subunits (GABA_{B1} and GABA_{B2}), three sweet and umami taste receptors (T1R1, T1R2, and T1R3), several putative rodent pheromone receptors (V2Rs), and orphan receptors (GPRC6A, GPRC5B–5D) (2). All family 3 GPCRs possess a large amino-terminal extracellular ligand-binding domain (ECD) that share structural similarity to the bi-lobed Venus flytrap domain motif (VFTM) of bacterial periplasmic binding proteins connected to a seven-transmembrane helical domain (7TMD) prototypical for all GPCRs responsible for G-protein activation (1, 2). Many of the GPCRs in this family are covalently joined homodimers with two monomers being linked by one or more disulfide bridges in the VFTMs. This has been rigorously demonstrated for the CaR and mGluRs (3–5). The GABA_{B} receptor, in contrast, is an obligate heterodimer composed of GABA_{B1} and GABA_{B2} subunits stabilized by a carboxyl-terminal coiled-coil interaction (6).

Another major difference in the structure of the GABA_{B} receptor and many other family 3 GPCRs including mGluRs, CaR, sweet/umami taste receptors, and putative pheromone receptors is the presence of a distinct highly conserved nine-cysteine domain after the VFTM (called NCD) with a carboxyl-terminal extension of a 14-amino acid linker after the ninth cysteine connecting to the first transmembrane helix of the 7TMD in the later receptors. Structural predictions suggest that NCD may possess four β-strands and three disulfide bridges and for the CaR this domain seems to be essential for transmission of signals from the ECD to the 7TMD (7, 8). Although the linker connecting the NCD with the first transmembrane helical domain contains no cysteines for disulfide linkages, the length of this 14-residue linker is highly conserved in NCD-containing receptors indicating a stringent structural constraint. Thus, we and others hypothesized that this peptide linker might contribute to the signal transmission of family 3 GPCRs.
GPCRs (9, 10). To address how the ligand-induced conformational changes of the VFTM might be transmitted for G-protein coupling, the peptide linkers of the GABA<sub>B</sub> receptor heterodimer were examined (11). Modification of two GABA<sub>B</sub> receptor subunit linkers by changes in sequence and/or length were mostly tolerated and thus the linker regions in GABA<sub>B</sub> receptors were predicted to act only as tethers for the VFTMs to the 7TMD, supporting a direct contact model of receptor activation. In this model illustrated in Fig. 1A, a, receptor activation occurs predominantly through contacts between the ligand-bound VFTM and exo-loops of the 7TMD and the linker acts solely to keep the VFTM in proximity to the 7TMD. Alternatively, x-ray crystallographic analyses revealed that the distance between the VFTM carboxyl termini in the dimers of the mGluR1 decrease upon glutamate binding in the closed state of the two VFTMs suggesting that the two 7TMDs may be drawn closer to VFTMs through the linkers upon ligand activation (12). As depicted in Fig. 1A, b, this alternative model referred to as the peptide-linker model of receptor activation predicts that the structure and conformational change of the linker is important for receptor activation. In a combination model, a direct contact of the VFTM and 7TMD is mediated by a linker conformational change (Fig. 1A, c).

To identify the role of the peptide linker in the activation process of the CaR and to test among the different proposed receptor linker activation models, we created deletions and insertions to modify the length and also changed the composition by chimeric substitution of homologous sequences between CaR and mGluR1 and introducing changes in residues by point mutation within this linker of the CaR. Our analysis of the expression and signaling properties of these receptor constructs suggests an essential role for highly conserved amino acids within this structure.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis of the Chimeric and Point Mutant Receptors—The coding sequences for the human CaR was inserted into the pCR3.1 expression plasmid. Mutations were introduced in the sequences encoding the linker region of the CaR using the QuikChange site-directed mutagenesis kit (Stratagene) as described previously (3). Briefly, a pair of complementary primers with 65–70 bases was designed for each chimeric receptor construct with targeted residues placed in the middle of the primers. In case of point mutation, a pair of complementary primers with 30–35 bases was similarly constructed with the residue change placed in the middle of the primers. The overlapping oligonucleotide sequences used for site-directed mutagenesis and construction details of the chimeric and point mutant constructs are available upon request. The mutations were confirmed by automated DNA sequencing using a Taq Dye Deoxy terminator cycle sequencing kit and ABI Prism 377 DNA sequencer (Applied Biosystems). All five chimeric constructs were completely sequenced to confirm the absence of mutations in other regions of these mutant constructs. For each point mutant construct sequenced, we analyzed at least two independent clones and confirmed that they had identical expression and functional characteristics.

Transient and Stable Expression of Receptors in Mammalian Cells—HEK293 and HeLa cells were transfected with the pCR3.1 expression plasmid encoding the CaR receptor constructs using Lipofectamine (Invitrogen). To achieve optimal expression, 90% confluent cells in 80-cm<sup>2</sup> flasks were transfected with an optimized amount of plasmid DNA (8 μg/flask) diluted in Dulbecco’s modified Eagle’s medium, mixed with diluted Lipofectamine (Invitrogen), and incubated at room temperature for 30 min and added to cells. After 5 h of incubation, the transfection medium was replaced with complete Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. This amount of DNA was determined to generate optimal transfection efficiency and the highest expression level of the CaR. Clonal HEK293 cell lines expressing the Gly-link9ins chimeric CaR was selected by isolating Geneticin or G418 (800 μg/ml)-resistant independent clonal isolates 3 weeks after transfection. The clonal cell line (Gly-link9insC10) was selected and maintained in media containing 400 μg/ml G418 for functional studies on the basis of the highest cell surface expression determined by immunoblot analysis with monoclonal antibody, ADD, clone 5C10 (Affinity BioReagents), raised against a synthetic peptide from the CaR ECD.

Immunoblot Analyses with Detergent-solubilized Whole Cell Extracts—Whole cell extracts were prepared as described previously (3). Confluent cells in 80-cm<sup>2</sup> flasks were rinsed with ice-cold phosphate-buffered saline, scraped, and solubilized in solution B (20 mM Tris-HCl, pH 6.8, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, and 1% Triton X-100) with freshly added protease inhibitor mixture (Roche Applied Sciences). For cleavage with endoglycosidase-H (Endo-H), Triton X-100-solubilized proteins were treated for 30 min with test agents in PI buffer. The reactions were then depleted of extracellular [Ca<sup>2+</sup>]<sub>e</sub>-free phosphate-buffered saline, followed by a 30-min incubation and wash with PI buffer (120 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl<sub>2</sub>, 20 mM LiCl, and 25 mM PIPES, pH 7.2) containing no Ca<sup>2+</sup>. This solution was removed and cells were then treated for 30 min with test agents in PI buffer. The reactions...
Role of Linker in CaR Activation

A

CaR

mGluR1

mGluR5

V2R2

r V2R2

h T1R1

h T1R2

h T1R3

B

h CaR
r mGluR1
r mGluR1
r mGluR5
m V2R2
r V2R2
h T1R1
h T1R2
h T1R3

C

A

F

L

E

R1-link9ins

Gly-link9ins

R1-link9

Gly-link9

Δ-link9

FIGURE 1. A, models of family 3 receptor activation. The direct contact model (a) is shown here for CaR as initially proposed for the GABA<sub>A<sub> heterodimer (11). In contrast to GABA<sub>A<sub> receptor subunits, the CaR, like mGluRs are disulfide-linked homodimers shown schematically here as two subunits in black. The Ca<sup>2+</sup> ligand is shown as light gray spheres bound to the VFTM stabilizing the closed conformation of the two VFTM protomers as seen in the glutamate-bound crystal structures of mGluR1 VFTM. The binding of ligand associated with the closed conformational change in the VFTM is transmitted to the 7TMD of the receptor through direct contacts of the ECD and 7TMD on processing/cell surface expression and signaling of the CaR. Amino acid sequence alignment among divergent members of family 3 GPCRs showed that the 14-amino acid chain length among all members between the last cysteine (Cys<sup>598</sup> in CaR) of the NCD and the first amino acid of the transmembrane domain (Gly<sup>613</sup> in CaR) is strictly maintained among the CaR, mGluR1, mGluR5, and sweet, umami taste (T1R1–3), and a putative pheromone receptor (V2R2), whereas there are significant divergence in the amino acid compositions of the linkers (Fig. 1B). We modified the linker length and composition of the CaR as shown in Fig. 1C. As described in this figure, two chimeric CaR receptor constructs were generated in which the linker lengths were increased with the insertion of either the corresponding nine-residue sequence of rat mGluR1 sequence (IPVRYLEWFS) or with GGGASASGG, a Gly sequence predicted to form a random coil structure in the middle of the 14-amino acid CaR linker and these two chimeric receptor constructs were named R1-link9ins and Gly-link9ins, respectively. Two other chimeras (R1-link9 and Gly-link9, respectively) were generated that changed the composition of the linker sequences without changing the CaR linker peptide length. In these constructs, nine residues of the 14-amino acid CaR linker were changed between the proteom carboxyl termini and the linkers draw the VFTM/NCD and 7TMDs closer to trigger G-protein signaling. For completeness, we provide a combination model (c) in which the ligand-bound conformational changes in the VFTM/NCD draw the 7TMDs closer as in the peptide-linker model with movements of the linkers required for altered contacts of the VFTM/NCD and 7TMDs underlying receptor activation. B, sequence alignment of the linker regions for family 3 GPCRs. The sequences of 14-amino acid long linkers of 10 members of family 3 GPCRs are shown. The alignment is based on the CaR linker sequence from Cys<sup>598</sup>, the last cysteine in the NCD and Gly<sup>613</sup>, the first residue in the 7TMD. The underlined amino acids of the CaR and mGluR1 are the nine residues exchanged between these receptors within the linker peptide. Identical and highly conserved residues are boxed. C, five CaR mutants constructed to change the length and composition of the linker peptide. In R1-link9ins and Gly-link9ins chimeric constructs, the homologous nine amino acids from mGluR1 (underlined in B) or a nine-amino acid long random-coil peptide, GGGASASGG, was inserted between Phe<sup>605</sup> and Leu<sup>606</sup>, respectively, which resulted in linker extensions. In R1-link9 chimera, the homologous nine amino acids from mGluR1 (underlined in B) replaced the corresponding nine residues in CaR keeping the linker length constant. Similarly, in the Gly-link9 chimera, the corresponding nine amino acid residues of the CaR were deleted and replaced simultaneously with the GGGASASGG peptide so that the length of the linker was kept intact. A deletion mutant (Δ-link9) of the CaR nine residues (underlined in B), resulted in shortened length of the peptide linker.

RESULTS

Expression and Function of CaR Peptide Linker Chimeric Receptors—This study was designed to determine the impact of the length and composition of the peptide linker connection between the VFTM/NCD and 7TMD on processing/cell surface expression and signaling of the CaR. Amino acid sequence alignment among divergent members of family 3 GPCRs showed that the 14-amino acid chain length among all members between the last cysteine (Cys<sup>598</sup> in CaR) of the NCD and the first amino acid of the transmembrane domain (Gly<sup>613</sup> in CaR) is strictly maintained among the CaR, mGluR1, mGluR5, and sweet, umami taste (T1R1–3), and a putative pheromone receptor (V2R2), whereas there are significant divergence in the amino acid compositions of the linkers (Fig. 1B). We modified the linker length and composition of the CaR as shown in Fig. 1C. As described in this figure, two chimeric CaR receptor constructs were generated in which the linker lengths were increased with the insertion of either the corresponding nine-residue sequence of rat mGluR1 sequence (IPVRYLEWFS) or with GGGASASGG, a Gly sequence predicted to form a random coil structure in the middle of the 14-amino acid CaR linker and these two chimeric receptor constructs were named R1-link9ins and Gly-link9ins, respectively. Two other chimeras (R1-link9 and Gly-link9, respectively) were generated that changed the composition of the linker sequences without changing the CaR linker peptide length. In these constructs, nine residues of the 14-amino acid CaR linker were changed between the proteom carboxyl termini and the linkers draw the VFTM/NCD and 7TMDs closer to trigger G-protein signaling. For completeness, we provide a combination model (c) in which the ligand-bound conformational changes in the VFTM/NCD draw the 7TMDs closer as in the peptide-linker model with movements of the linkers required for altered contacts of the VFTM/NCD and 7TMDs underlying receptor activation. B, sequence alignment of the linker regions for family 3 GPCRs. The sequences of 14-amino acid long linkers of 10 members of family 3 GPCRs are shown. The alignment is based on the CaR linker sequence from Cys<sup>598</sup>, the last cysteine in the NCD and Gly<sup>613</sup>, the first residue in the 7TMD. The underlined amino acids of the CaR and mGluR1 are the nine residues exchanged between these receptors within the linker peptide. Identical and highly conserved residues are boxed. C, five CaR mutants constructed to change the length and composition of the linker peptide. In R1-link9ins and Gly-link9ins chimeric constructs, the homologous nine amino acids from mGluR1 (underlined in B) or a nine-amino acid long random-coil peptide, GGGASASGG, was inserted between Phe<sup>605</sup> and Leu<sup>606</sup>, respectively, which resulted in linker extensions. In R1-link9 chimera, the homologous nine amino acids from mGluR1 (underlined in B) replaced the corresponding nine residues in CaR keeping the linker length constant. Similarly, in the Gly-link9 chimera, the corresponding nine amino acid residues of the CaR were deleted and replaced simultaneously with the GGGASASGG peptide so that the length of the linker was kept intact. A deletion mutant (Δ-link9) of the CaR nine residues (underlined in B), resulted in shortened length of the peptide linker.

were terminated by addition of 1 ml of HCl/methanol (1:1000, v/v). Total inositol phosphates (IP) were isolated by chromatography on Dowex 1-2X8 columns.

Data Analysis—The [Ca<sup>2+</sup>], saturation profiles of PI hydrolysis were analyzed using Graphpad Prism software (version 3). Data curve fits for single-site and other ligand-binding models were compared for minimum residual errors. The coefficients for the best-fit models for an individual experiment were used to compute EC<sub>50</sub> values reported in Table 1. For the L606A mutant, the data were best-fit to a single site non-cooperative model.
Role of Linker in CaR Activation

Table 1

| Receptor          | IP formation | EC50 (nM) | Emax (%) |
|-------------------|--------------|-----------|----------|
| CaR               | 4.76 ± 0.08  | 75.0 ± 6.7 (5) |
| R1-link9          | 5.61 ± 0.19  | 100       |
| I603A             | 5.72 ± 0.12  | 78.8 ± 4.1 (5) |
| F605A             | 5.24 ± 0.14  | 93.3 ± 8.7 (4) |
| L606A             | 3.35 ± 0.17  | 31.4 ± 2.8 (5) |
| W608A             | 4.25 ± 0.10  | 110.3 ± 12.7 (4) |
| L606F             | 4.48 ± 0.15  | 122.6 ± 6.5 (5) |
| L606I             | 3.07 ± 0.14  | 116.8 ± 16.3 (3) |
| L606V             | 2.76 ± 0.12  | 105.2 ± 4.0 (5) |

Maximal response and EC50 values of [Ca2+]o on CaR and its mutant receptors.

The maximal IP production measured with 10 mM Ca2+ in cells transiently expressing the indicated receptor is expressed as percentage of maximal wild type (wt) CaR response and (n) designates the number of independent experiments performed in triplicate or duplicate.

Figure 2. Analysis of CaR linker chimeric receptors. A, presents the data from immunoblot analysis of cell surface expression of CaR and linker chimeric receptors. The positions of the 150- and 130-kDa bands representing differentially N-glycosylated CaR forms are indicated and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**). The positions of the 150- and 130-kDa bands representing differentially N-glycosylated CaR forms are indicated and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**). The positions of the 150- and 130-kDa bands representing differentially N-glycosylated CaR forms are indicated and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**). The positions of the 150- and 130-kDa bands representing differentially N-glycosylated CaR forms are indicated and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**).

either to the corresponding nine mGluR1 sequence or to Gly sequence as shown above. A fifth construct (Δ-link9) with the deletion of nine residues was also created with a shortened length of the CaR linker peptide. The analysis of the expression patterns of the wild type CaR and these five chimeric mutants is shown in Fig. 2A. Immunoblotting experiments for chimeric receptors transiently expressed in HEK293 cells showed that both the wild type CaR and R1-link9 receptor expressed efficiently at the cell surface by immunoblotting with the monoclonal antibody ADD (Fig. 2A). As seen in this figure, under reducing conditions, ADD antibody detected two major monomeric bands of ~150 and 130 kDa molecular mass in wild type receptor expressing cells. We and others (13, 14) have previously reported that the 150-kDa band identifies CaR forms expressed at the cell surface, modified with complex carbohydrates by N-glycosylation of several Asp residues in the ECD and is resistant to Endo-H digestion. The 130-kDa band contains high mannose-modified forms, trapped intracellularly, and Endo-H digestion reduces these high mannose-modified receptor forms to non-glycosylated forms shifted to a 120-kDa band on immunoblots. Similarly, the ADD antibody detected both bands in R1-link9 chimeric receptor-expressing cells and the upper 150-kDa band showed resistance to Endo-H digestion, whereas the lower 130-kDa band was sensitive to Endo-H. HEK293 cells consistently expressed a lower intensity of signal for the 150-kDa forms of R1-link9 compared with the wild type CaR, probably indicating a lower number of this chimeric receptor at the cell surface. Surprisingly, other chimeric mutant receptors showed no detectable 150-kDa band with mostly a single Endo-H-sensitive 130-kDa band. However, the Gly-link9ins mutant in several immunoblotting experiments showed the presence of a faint upper 150-kDa band, indicating some forms of this mutant receptor may reach the cell surface. Next, each mutant receptor was expressed transiently in HEK293 cells and the capacity of each mutant receptor to generate intracellular signals for IP accumulation in response to [Ca2+]o was compared with that of a wild type CaR. With the exception of R1-link9, all of the chimeric receptors failed to show any response even at a saturating concentration (10 mM) of Ca2+.

The maximal responses (Emax) for these mutants were indistinguishable from the basal level and none of the mutants displayed constitutive activity (data not shown). Among these chimeric receptors, the Gly-link9ins mutant that showed some cell surface-expressed receptor forms also failed to respond to [Ca2+]o in the PI assay (Fig. 2B). The saturation curves for wild type CaR and R1-link9 receptor are shown in Fig. 2B. The R1-link9 receptor exhibited similar sigmoidal saturation as the wild type CaR but with somewhat reduced sensitivity to [Ca2+]o (EC50 of 5.6 mM versus wild type CaR EC50 of 4.7 mM, Table 1).

Signaling Properties of Chimeric Receptor Gly-link9ins—Because of the low level of cell surface expression in HEK293 cells transiently expressing Gly-link9ins receptor, we tested varying amounts of the Gly-link9ins plasmid DNA during transient transfection (1, 2, and 3 µg of DNA/well of a six-well plate) and decreasing the wild type CaR plasmid DNA amount (0.25 and 1 µg of DNA/well) to achieve comparable cell surface receptor levels of the two receptors. Based on the intensity of the 150-kDa band in immunoblots, comparable cell surface expression was achieved by transfecting cells with 0.25 µg of DNA/well for wild type CaR and 3 µg of DNA/well for Gly-link9ins receptor. Under these conditions, the wild type CaR showed 3–4-fold increases in IP accumulation in response to 10 mM Ca2+, whereas Gly-link9ins receptor showed no increase in inositol phosphate formation (data not shown). To confirm unambiguously that Gly-link9ins chimeric receptor expressed at the cell surface consistently expressed a lower intensity of signal for the 150-kDa forms of R1-link9 compared with the wild type CaR, probably indicating a lower number of this chimeric receptor at the cell surface. Surprisingly, other chimeric mutant receptors showed no detectable 150-kDa band with mostly a single Endo-H-sensitive 130-kDa band. However, the Gly-link9ins mutant in several immunoblotting experiments showed the presence of a faint upper 150-kDa band, indicating some forms of this mutant receptor may reach the cell surface. Next, each mutant receptor was expressed transiently in HEK293 cells and the capacity of each mutant receptor to generate intracellular signals for IP accumulation in response to [Ca2+]o was compared with that of a wild type CaR. With the exception of R1-link9, all of the chimeric receptors failed to show any response even at a saturating concentration (10 mM) of Ca2+. The maximal responses (Emax) for these mutants were indistinguishable from the basal level and none of the mutants displayed constitutive activity (data not shown). Among these chimeric receptors, the Gly-link9ins mutant that showed some cell surface-expressed receptor forms also failed to respond to [Ca2+]o in the PI assay (Fig. 2B). The saturation curves for wild type CaR and R1-link9 receptor are shown in Fig. 2B. The R1-link9 receptor exhibited similar sigmoidal saturation as the wild type CaR but with somewhat reduced sensitivity to [Ca2+]o (EC50 of 5.6 mM versus wild type CaR EC50 of 4.7 mM, Table 1).
Role of Linker in CaR Activation

A

FIGURE 3. NPS-R568 and [Ca\(^{2+}\)]\(_o\) synergism of the 7TMD of Gly-link9ins chimeric receptor. A, stimulation of IP formation of untransfected HEK293 (light gray) and a stable HEK293 clonal line Gly-link9insC10 expressing Gly-link9ins receptor (dark gray) was assessed in the presence of 0.5 and 10 mM Ca\(^{2+}\) or 1 \(\mu\)M NPS-R568 alone, or in combination. The values shown are the means of triplicate determinations from one representative experiment. B, stimulation of PI hydrolysis in the T903-Rhoc2.1 cells stably expressing the T903-Rhoc receptor (light gray) or 1 \(\mu\)M NPS-R568 (dark gray) was assessed in the presence of 0.5 and 10 mM Ca\(^{2+}\) or in combination. The values shown are the means of triplicate determinations from two representative experiments. C, the [Ca\(^{2+}\)]\(_o\)-elicited response of the Gly-link9insC10 cells in the presence of 1 \(\mu\)M NPS-R568 was determined for the indicated concentrations of Ca\(^{2+}\) (Fig. 4, A–C). The shape of [Ca\(^{2+}\)]\(_o\)-elicited response was calculated EC50 values for [Ca\(^{2+}\)]\(_o\) ranging from 4.2 to 5.7 mM (Fig. 4, A–C, Table 1). In contrast, the L606A mutant displayed markedly attenuated [Ca\(^{2+}\)]\(_o\)-elicited response with a maximal response diminished to 31% of the wild type response at 10 mM Ca\(^{2+}\) (Fig. 4B, Table 1). The shape of [Ca\(^{2+}\)]\(_o\)-saturation curve of L606A changed to hyperbolic from the steeply sigmoidal saturation profile of the wild type CaR. Immunoblot experiments confirmed that the highly reduced functional response of L606A was due to deficiencies in cell surface expression of this mutant as seen by the presence of little or none of the cell surface expressed 150-kDa forms compared with the wild type and other mutant receptors tested (Fig. 4D).

These observations indicate that a severe constraint on the amino acid side chain at this 606 position may be critical for the presence of 1 \(\mu\)M NPS-R568 was determined for the indicated concentrations of Ca\(^{2+}\). Data are represented as a percentage of maximal response at 10 mM Ca\(^{2+}\) and are representative of the results obtained from two similar experiments. The curve shown is the best-fit for this data using a single-site binding model.
Role of Linker in CaR Activation

FIGURE 4. Analysis of CaR linker point mutants. A, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in transiently transfected HEK293 cells expressing wild type CaR (○) or L603A (●) was determined for the indicated concentrations of Ca\(^{2+}\). B, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in transiently transfected HEK293 cells expressing wild type CaR (○), F605A (▲), and L606A (●) mutants was determined for the indicated concentrations of Ca\(^{2+}\). C, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in HEK293 cells transiently transfected with wild type CaR (○) and W608A (●) mutant was determined for the indicated concentrations of Ca\(^{2+}\). All data are expressed as a percentage of the maximal response of the wild type CaR observed with 10 mM Ca\(^{2+}\). Values are the mean ± S.E. for triplicate determinations of a representative experiment with three or more independent experiments showing similar results. D, immunoblot analysis of wild type CaR, I603A, F605A, L606A, and W608A expression. The cell surface-expressed 150-kDa receptor forms and intracellularly trapped 130-kDa receptor forms are marked on the right.

FIGURE 5. Analysis of Leu\(^{606}\) point mutants. A, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in transiently transfected HEK293 cells expressing wild type CaR (○) and L606F (▲) was determined for the indicated concentrations of Ca\(^{2+}\). B, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in transiently transfected HEK293 cells expressing wild type CaR (○) and L606I (●) was determined for the indicated concentrations of Ca\(^{2+}\). C, the \([\text{Ca}^{2+}]_o\) saturation profile for IP formation in transiently transfected HEK293 cells expressing wild type CaR (○) and L606V (▲) and L606A (●) was determined for the indicated concentrations of Ca\(^{2+}\). All data are expressed as a percentage of maximal response of the wild type CaR observed with 10 mM Ca\(^{2+}\). Values are the mean ± S.E. for triplicate determinations of a representative experiment with three or more independent experiments showing similar results. D, immunoblot analysis of wild type CaR, L606I, L606V, and L606A mutant receptor expression was determined with (+) and without (−) digestion with Endo-H. The migration of 150- and 130-kDa forms of the CaR and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**). The migration of 150- and 130-kDa forms of the CaR and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**). The migration of 150- and 130-kDa forms of the CaR and the Endo-H-digested nonglycosylated band migration is shown as asterisks (**).

cell surface expression of the CaR. Alanine at this position possibly provides a less bulky side chain and perhaps failed to produce a critical Van der Waals contact provided by a leucine residue at position 606. To evaluate the importance of Leu\(^{606}\), we next engineered three point mutants, L606F, L606I, and L606V, in which the replaced amino acids (Phe, Ile, and Val) preserved the hydrophobicity but changed the length of the side chains of these amino acids. Because of similarities between the side chains of the alphatic amino acids Leu, Ile, and Val, we believed the Ile and Val substitution mutants would fold and function normally at the cell surface. Phe has a rigid aromatic group on the side chain but like Leu is hydrophobic with similar side chain length, so we predicted this mutant may also be functional. As opposed to the L606A receptor, mutants replacing Leu at position 606 with Phe, Ile, and Val exhibited increased sensitivity to \([\text{Ca}^{2+}]_o\) with maximal responses similar to the wild type CaR (Fig. 5, A–C, Table 1). Both the L606I and L606V mutant receptors exhibited substantial increases in sensitivity to \([\text{Ca}^{2+}]_o\) (EC\(_{50}\) values of 3.0 and 2.7 mM, respectively, versus wild type CaR EC\(_{50}\) value 4.7 mM) with left-shifted dose-response curves. The response of the L606F receptor was also left-shifted but less activating with an EC\(_{50}\) value of 4.4 mM (Table 1). Immunoblot analysis and Endo-H treatment that qualitatively determined the cell surface levels of each receptor based on the intensity of the Endo-H resistant 150-kDa band (Fig. 5D) indicated that gain-of-function activities of L606V, L606I, and L606F mutants were not due to significantly higher cell surface expression of these receptors as compared with the wild type CaR, but indeed are due to the changes in signal transmission activities. We next evaluated the functional responses of the L606I, L606V, and L606A mutants in HeLa cells by transiently expressing the receptor constructs in these cells. Both L606I and L606V mutant receptors showed activating effects and L606A receptor showed a highly reduced response in PI assay (produced as a supplemental data). The findings confirm that signaling phenotypes of these mutant receptors are determined by structural changes within the receptors and not by other HEK293 cell-specific phenomenon.

DISCUSSION

The transmission of an activating signal from the VFTM recognition of agonist ligands in a family 3 GPCR presents a constraint not present in the major family 1 GPCR structures. Whereas studies of ECD-deleted CaR and mGluR5 receptor constructs have revealed 7TMD-contained activating site(s) for so-called allosteric ligands (15, 18), indicating that these receptors may retain the activation mechanism(s) found in the homologous 7TMD structures of the family 1 GPCRs, the intact structures must undergo additional conformational transitions involving the ECD structures. In this report, we examined the peptide linker region connecting the ECD to the 7TMD of a...
Role of Linker in CaR Activation

prototypical member of the family 3 GPCR, the CaR, to explore whether this sequence plays a role in the activation of the 7TMD upon binding of ligand in the extracellular VFTM of this receptor. Two general mechanisms have been proposed for the transmission of this signal illustrated in Fig. 1A: 1) through direct contacts of the ECD with exo-loops of the 7TMD with the linker acting solely to constrain the spatial separation of ECD and 7TMD or 2) through the peptide linker between the ECD and the 7TMD with the transmission of the signal dependent on the conformational transitions transmitted through the linker.

Results obtained from experiments with the GABA_B receptor in which a random-coiled sequence was introduced into the linker region suggested that the conformation of the linker was not essential to GABA_B receptor activation (11). Our results for the CaR would appear to be entirely discrepant implicating a more fundamental role for the CaR linker region. Either deletion of the linker sequence or replacing it with an unrelated sequence abolished cell surface expression of the CaR receptor constructs. Similarly, insertion of an additional sequence, including the homologous sequence from mGluR1, abrogated receptor processing and, hence, [Ca^{2+}]_o-mediated signaling. The one alteration that was well tolerated was replacement of the nine-amino acid CaR linker sequence with the corresponding sequence from mGluR1, resulting in a receptor with diminished cell surface expression but which retained [Ca^{2+}]_o-induced response via the VFTM. This result echoes findings from the exchange of ECD structures among family 3 GPCRs with successful expression and signaling of regulated chimeric receptors only obtained for CaR ECD/mGluR1 7TMD exchanges (19, 20).

Of the poorly or non-expressing constructs, the Gly-link9ins receptor bears particular scrutiny. Whereas the chimeric receptor Gly-link9ins with an insertion of a nine-amino acid long random coil Gly-peptide expressed poorly at the cell surface, by selecting a stably transformed clone of HEK293 that faced, by selecting a stably transformed clone of HEK293 that long random coil Gly-peptide expressed poorly at the cell surface receptor Gly-link9ins with an insertion of a nine-amino acid receptor bears particular scrutiny. Whereas the chimeric receptors only obtained for CaR ECD/mGluR1 7TMD successful expression and signaling of regulated chimeric the exchange of ECD structures among family 3 GPCRs with successful expression and signaling of regulated chimeric receptors only obtained for CaR ECD/mGluR1 7TMD exchanges (19, 20).

Of the poorly or non-expressing constructs, the Gly-link9ins receptor bears particular scrutiny. Whereas the chimeric receptor Gly-link9ins with an insertion of a nine-amino acid long random coil Gly-peptide expressed poorly at the cell surface, by selecting a stably transformed clone of HEK293 that expressed sufficient Gly-link9ins receptor, we found no response to [Ca^{2+}]_o but activation by the allosteric agonist NPS-R568 alone or in combination with Ca^{2+}. This phenotype mimics what we have previously reported for an ECD-deleted construct, T903-Rhoc, which revealed the 7TMD sites for two positive allosteric agonists NPS-R568 and Calindol and also for Ca^{2+} (15, 21). These results suggest that [Ca^{2+}]_o-activated VFTM of the Gly-link9ins mutant receptor did not transmit the activation signal to the 7TMD for G-protein signaling. These observations would seem to refute the role of the linker sequence as only a “tether” and they reveal a significant structural constraint upon this region of the CaR.

Interestingly, replacement of the nine residues of the CaR peptide linker with the mGluR1 linker sequence without changing linker length produced a receptor with very similar phenotype as the wild type CaR. Our examination of the four conserved amino acid residues within this sequence identified Leu^{606} as essential for CaR signaling. Replacing Leu with hydrophobic amino acids Val and Ile produced functional receptors at the cell surface with enhanced [Ca^{2+}]_o sensitivity, whereas Phe replacement at this position produced only a modestly activating phenotype. Identification of these activating mutations at Leu^{606} of the CaR eliminates a passive tether model and implies a critical role for the conformation of the linker region. In contrast to these activating mutations, transfection of HEK293 cells with a CaR construct with Ala substitution at this position produced cells with dramatically decreased surface-expressed receptors and impaired [Ca^{2+}]_o-induced signaling response. Whereas it seems likely that the L606A mutant failed to fold correctly and remained intracellularly trapped, we cannot rule out the alternative that this mutation generated a constitutively active CaR mutant form that is internalized rapidly leading to significant loss of receptors at the cell surface. It is interesting to note that a true constitutively active CaR mutant receptor has not been reported yet and it is quite possible that such CaR mutants may rapidly internalize and degrade or that their expression may be toxic to the cells.

Our data then suggest that the analysis of the family 3 GPCR signaling must consider at least two distinct types of regulation based upon the receptor structures. Signaling of GABA_B receptor subunits appear to be mediated mostly by direct contacts between the ECD and 7TMD (11). These receptor subunits, in contrast to CaR and mGluR1, form heterodimers with the predominant dimeric interface encoded in an extended carboxy-terminal coiled-coil intracellular structure but the ECDs do not contain the NCD (6). CaR and mGluRs form homodimers structures with disulfide linkages in the extracellular ECD structures. Our data for the CaR strongly favors a peptide-linker mechanism of activation of this family 3 GPCR that may apply to other VFTM/NCD containing family 3 GPCRs. Our data cannot discriminate between a mechanism in which a direct interaction of the ligand-bound ECD with the exo-loops of the 7TMD requires a conformational rearrangement in the peptide linker during the activation process from one in which the conformational transition of the ECD produced upon ligand binding is transmitted by the linker region conformational rearrangement. The three-dimensional x-ray crystallographic structural analyses of the mGluR1 VFTM indicate that the closed conformation of this VFTM dimeric structure brings the carboxyl-terminal portions of the monomer VFTMs into closer apposition, which may produce the conformational rearrangement of the dimeric 7TMD structures via the linker regions (12). Whether this produces a torsional transmission of the conformation or specific binding contacts between the linker sequence and the ECD and/or 7TMD require more refined structures and/or models for the intact receptors. We believe these differences seen in the linker properties between the GABA_B receptor and the CaR in the activation processes may be applicable to other members of this receptor family and further research to test the generality of these results will be revealing.

Acknowledgments—We thank Drs. James Battey and Paul Randazzo for critical reading of the manuscript. We also thank Dr. Kenneth Jacobson for providing NPS-R568.

REFERENCES
1. Brown, E. M., and MacLeod, R. J. (2001) Physiol. Rev. 81, 239–297
2. Pin, J. P., Galvez, T., and Prezeau, L. (2003) Pharmacol. Ther. 98, 325–354
3. Ray, K., Hauschild, B. C., Steinbach, P. J., Goldsmith, P. K., Hauache, O.
and Spiegel, A. M. (1999) *J. Biol. Chem.* 274, 27642–27650
4. Ray, K., and Hauschild, B. C. (2000) *J. Biol. Chem.* 275, 34245–34251
5. Romano, C., Miller, J. K., Hyrc, K., Dikranian, S., Mennerick, S., Takeuchi, Y., Goldberg, M. P., and O’Malley, K. L. (2001) *Mol. Pharmacol.* 59, 46–53
6. Marshall, F. H., Jones, K. A., Kaupmann, K., and Bettler, B. (1999) *Trends Pharmacol. Sci.* 20, 396–399
7. Yu, L., Liang, S., Liu, X., He, Q., Studholme, D. J., and Wu, Q. (2004) *Trends Biochem. Sci.* 29, 458–461
8. Hu, J., Hauache, O., and Spiegel, A. M. (2000) *J. Biol. Chem.* 275, 16382–16389
9. Ray, K. (2001) *Int. Arch. BioSci.* 2001, 1027–1035
10. Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., and Pin, J. P. (2001) *EMBO J.* 20, 2152–2159
11. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 14643–14648
12. Kunishima, N., Shimada, H., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K. (2000) *Nature* 407, 971–977
13. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H. S., Pollack, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) *J. Biol. Chem.* 271, 19537–19545
14. Ray, K., Fan, G. F., Goldsmith, P. K., and Spiegel, A. M. (1997) *J. Biol. Chem.* 272, 31355–31361
15. Ray, K., and Northup, J. K. (2002) *J. Biol. Chem.* 277, 18908–18913
16. Petrel, C., Kessler, A., Dauban, P., Dodd, R. H., Rognan, D., and Ruat, M. (2004) *J. Biol. Chem.* 279, 18990–18997
17. Miedlich, S. U., Gama, L., Seuwen, K., Wolff, R. M., and Breitwieser, G. E. (2004) *J. Biol. Chem.* 279, 7254–7263
18. Goudet, C., Gaven, F., Kniazeff, J., Vol, C., Liu, J., Cohen-Gonsaud, M., Acher, F., Prezeau, L., and Pin, J. P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 378–383
19. Hammerland, L. G., Krapcho, K. J., Garrett, J. E., Alasti, N., Hung, B. C., Simin, R. T., Levinthal, C., Nemeth, E. F., and Fuller, F. H. (1999) *Mol. Pharmacol.* 55, 642–648
20. Hauache, O. M., Hu, J., Ray, K., Xie, R., Jacobson, K. A., and Spiegel, A. M. (2000) *Endocrinology* 141, 4156–4163
21. Ray, K., Tisdale, J., Dodd, R. H., Dauban, P., Ruat, M., and Northup, J. K. (2005) *J. Biol. Chem.* 280, 37013–37020

Role of Linker in CaR Activation