Activation of Dimeric Calcium-sensing Receptor* Modulation of Interprotomer Relationships Is Important for Functional Reconstitution

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The extracellular calcium-sensing receptor (CaR) forms a disulfide-linked dimer through cysteine residues within its N-terminal extracellular domain (ECD). However, these disulfide linkages are dispensable for the formation of the dimeric CaR and for the functional reconstitution of two inactive CaRs. In this study, using molecular modeling, mutagenesis, and biochemical and biophysical analyses, we examined the importance of two leucine residues, Leu-112 and Leu-156, in the ECD of the CaR for the non-covalent dimerization and functional reconstitution. We found that the mutant receptor carrying L112S and L156S still exists mostly as a covalently linked dimer and has a significantly higher apparent affinity for calcium than the wild-type receptor. Thus, the detrimental effects of the combined mutations on the receptor activation. Moreover, these mutations are unlikely to have negative effects on the secondary structure of each protomer of the dimeric receptor. However, a combination of four mutations, L112S, L156S, C129S, and C131S, significantly reduces receptor dimerization and markedly inactivates the CaR. We also found that L112S and L156S mediate the non-covalent intermolecular interactions important for functional reconstitution. Because mutating either the two cysteines or the two leucines enhances the apparent ligand affinity of the CaR, it is likely that the changes in intermolecular relationships between two receptor protomers linked by these leucines and cysteines are essential for receptor activation. Moreover, these mutations are unlikely to have negative effects on the secondary structure of each protomer of the dimeric receptor. Therefore, the detrimental effects of the combined mutations on the function of the CaR further suggest that CaR dimerization through its ECD is essential for the formation of a functional tertiary structure of the CaR.

The extracellular calcium ([Ca2+]o)1-sensing receptor (CaR) plays a key role in the sensing of [Ca2+]o by homeostatic tissues such as parathyroid and kidney (1). The CaR is a G protein-coupled receptor that activates phospholipase C and mobilizes intracellular calcium in response to elevated concentrations of [Ca2+]o, other diveralent and trivalent inorganic cations, and organic polyamines. Recently, we have shown that the CaR forms a covalently linked dimer on the cell surface (2) and dimerizes even in the absence of any interprotomer disulfide linkage (3), suggesting that interprotomer interactions in addition to covalent disulfide bonds are critical for dimerization of the CaR. Through functional reconstitution of various pairs of inactivating mutant receptors, we demonstrated that non-covalent dimerization of the CaR is functionally important (3, 4). The CaR belongs to the subfamily C of G protein-coupled receptors, which share significant sequence and structural homology with metabotropic glutamate receptors (mGluRs). Like the mGluRs (5–7), the CaR interacts with its cationic agonist in its unusually large ECD (613 residues) (8, 9). The interprotomer disulfide linkages of both the CaR and mGluRs are formed only between dimeric ECD protomers (3, 10, 11). Although these disulfide linkages are dispensable for the dimerization and the functional reconstitution of the CaR (3), a collaborative involvement of two ECD protomers of the CaR is important in the sensing of [Ca2+]o (4). Thus, the structural integrity of the dimeric ECD of the CaR has to be maintained by non-covalent interprotomer interactions within the ECD in the absence of the disulfide linkages. In accordance, crystallographic studies suggest that the interprotomer disulfide bond in mGluR1 is unlikely to act as a structural scaffold to support the defined interprotomer relationships because of its location within the disordered segment. Instead, the dimeric ECD of mGluR1 possesses a well defined dimer interface, with its location apart from that of the cysteine mediating the interprotomer disulfide bond (12).

Studies of the possible sites of interprotomer interactions have suggested that dimerization of G protein-coupled receptors (GPCRs) could be mediated by hydrophobic interactions, involving associations of the extracellular domains (ECDs), transmembrane domains (TMDs), and/or C-terminal tails (C-tails) (13). The presence of conserved hydrophobic residues at the dimer interface of the ECD of mGluR1 suggests that hydrophobic interactions between the ECDs may play an important role in the dimerization of this receptor (12). The functional importance of these hydrophobic residues has been examined by Sato et al. (14), showing that one of hydrophobic residues at the dimer interface, Ile-120, was critical for setting the active state of the mGluR1. However, there is no study presently that shows the importance of Ile-120 and other hydrophobic residues at the dimer interface for the dimerization of mGluR1 nor that links the dimerization of mGluR1 to receptor activation. In this study, we elucidated the relations between receptor dimerization and activation of the CaR. We found that the CaR...
FIG. 1. Diagram of the CaR (A) and molecular modeling of the dimeric ECD of the CaR (B). A, the N terminus at the top is located extracellularly, called ECD. The C terminus at the bottom is located intracellularly, called the C-tail. Amino acid residues that are conserved in mGluR1a and the CaR are shown as filled circles, and the conserved cysteines are as red-filled dots. Arg-648 is located in the first intracellular
dimers through non-covalent interactions between two ECD protomers and that conserved Leu-112 and Leu-156 are important in mediating the non-covalent dimerization critical for the functional reconstitution. In addition, we found that the dimerization of the ECD via non-covalent as well as covalent interactions is essential to confine the CaR in an inactive conformation under non-stimulating conditions.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—We used the site-directed mutagenesis approach described by Kunkel (15) to produce mutated receptors in which the leucine residues at positions 112 and 156 on the putative dimer interface of the human CaR were mutated individually to serines. The wild type of Escherichia coli C2326 was transformed separately with mutagenesis cassettes 1 and 2, as described previously (16). To introduce mutation L112S into a mutant receptor containing C129S and C131S, we made another cassette 1 containing two Cys-to-Ser mutations. Uracil-containing, single-stranded (ss) DNA was produced by infecting the cells with the helper phage VC8M13 (Stratagene, La Jolla, CA). The ssDNA was then annealed to a mutagenesis primer that contained the desired nucleotide change encoding a single-point mutation flanked on both sides by wild-type sequences. The primer was subsequently extended around the entire ssDNA and ligated to generate closed circular heteroduplex DNA. DH5α-competent cells were transformed with these DNA heteroduplexes, and incorporation of the desired mutation was confirmed by sequencing the entire cassettes. The resultantly mutated cassette 1 or 2 containing the desired mutation was doubly digested with KpnI and XbaI and cloned into the full-length of the receptor in pcDNA3.

Construction of CaR-fused YFP and CFPR—ECFP or EYFP was fused to the cytoplasmic tail of the CaR by replacing C-terminal 172 amino acid residues with these fluorescence proteins. We used PCR to amplify the coding region of the ECFP and EYFP with a BamHI site at the 5′-end and XbaI site at the 3′-end, using a primer pair, 2279 (5′-CGCGATATCTAGTGAAAGCGCCAG-3′) and 2280 (5′-GGCTTACT- GATTATTCTGACGTCGTCACA-3′). The PCR products containing ECFP or EYFP are digested with BamHI and XbaI and subcloned to the CaR in pcDNA3 through an internal BamHI site and an external XbaI at the 3′-end of the coding region of the CaR. The point mutations in the CaR that are generated by site-directed mutagenesis were moved into YFP- and CFPR-fused CaR by subcloning the mutation-containing cassettes, as described previously (16).

Transient Expression of Mutated CaR—HEK293—CaR cDNA was prepared with the Midi Plasmid Kit (QIagen). LipofectAMINE (Invitrogen) was used as a DNA carrier for transfection. The HEK293 cells used for transient transfection were provided by NPS Pharmaceuticals (Salt Lake City, UT) and were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (FBS) (HyClone). The DNA-liposome complex was prepared by mixing DNA and LipofectAMINE in OPTI-MEM I-reduced serum medium (Invitrogen) by incubating the mixture at room temperature for 30 min. The DNA-LipofectAMINE mixture was then diluted with OPTI-MEM I-reduced serum medium and added to 90% confluent HEK293 cells plated on 13.5 × 20.1-mm glass coverslips using 2.5 µg of DNA. After the cells were incubated for 5 h at 37 °C, an equivalent amount of OPTI-MEM I-reduced serum medium with 20% FBS was added to the medium to allow the transfection to take place, and the culture was refreshed with fresh Dulbecco’s modified Eagle’s medium with 10% FBS at 24 h after transfection. The expression [Ca2+]i-sensing receptor protein was assayed 48 h after the start of transfection. To co-express two receptors, 1.25 µg of each of the two cDNAs was mixed and used to transfect HEK293 cells.

Detection of Expressed CaR on the Cell Surface—Before whole cell lysates were prepared, intact HEK293 cells transiently transfected with FLAG-tagged CaR were labeled with 1 mM ImmunoPure N-hydroxysulfosuccinimidyl (Pierce), a membrane-impermeable biotinyla- tion reagent, as described previously (2). The surface-biotinylated HEK293 cells were solubilized, and the FLAG-tagged receptor was immunoprecipitated with FLAG M2 monoclonal antibody (Sigma). The immunopurified species were subsequently eluted and subjected to SDS-PAGE with a linear gradient of polyacrylamide (3–10%). The forms of the receptor present on the cell surface were detected with an avidin-horseradish peroxidase conjugate (Bio-Rad), followed by visualization of the biotinylated bands with an enhanced chemiluminescence (ECL) system (PerkinElmer Life Sciences). For cross-linking experiments, we added an appropriate amount of bis(sulfosuccinimidyl) suberate (BS3), a noncleavable, membrane-impermeable cross-linker (Pierce), into the labeling solution with ImmunoPure N-hydroxysulfosuccinimidobiotin.

Hydroxyl Radical and Fluorescence Resonance Energy Transfer in Fixed Cells—Cells singly or co-expressing CaR-fused CFP and YFP were fixed with 4% formaldehyde and observed with a Nikon Diaphot-300 epifluorescence microscope with a 100-W mercury lamp, a 60-A and ~1.4 numerical aperture Plan-ALPO objective, Nomarski optics, and the following filter sets (Omega Optical, Brattleboro, VT): CFP (440-nm/20-nm excitation filter, 455-nm longpass dichroic mirror, 480-nm/30-nm emission filter); YFP (500-nm/25-nm excitation, 525-nm dichroic, 545-nm/35-nm emission); and FRET (440-nm/20-nm excitation, 455-nm dichroic, 535-nm/25-nm emission). Images were captured with a liquid-cooled charge-coupled device camera (Photometrics, Tucson, AZ) equipped with a KAF-1400 chip, operated by the MetaMorph imaging system (Universal Imaging, West Chester, PA) and a Model D122 shutter driver (Uniblitz, Rochester, NY). The exposure time is 100 ms. We used a 100× objective lens to view the images and employed the image-processing technique called Threshold Exclusion to selectively quantify the fluorescence signals at the rim or surface of the cells that had fluorescent intensity much greater than that inside the cells.

Because CFP and YFP are not an ideal pair of fluorophores due to overlapping emission and excitation spectra of the donor (CFP) and the acceptor (YFP), a certain amount of the CFP emission leaked through the FRET filter, and a certain amount of YFP was excited by the FRET excitation wavelength when cells were observed under the FRET filter set. To eliminate these experimental artifacts, the following correction was performed for each pair of CFP and YFP,

\[
\text{FRET} = I_{\text{FRET}} - C_{\text{YFP}} - C_{\text{CFP}} \quad (\text{Eq. 1})
\]

where \(I_{\text{FRET}}\) is the intensity of FRET in CFP- and YFP-co-expressed cells measured by the FRET filter set and \(C_{\text{YFP}}\) and \(C_{\text{CFP}}\) are the contributions from the emission of CFP and direct excitation of YFP, respectively (17). These two contributions could not be directly measured in cells co-expressing the YFP and CFP. However, they could be measured in cells expressing only CFP or YFP. The contributions of YFP and CFP to FRET in co-expressed cells are proportional to those in single-expressed cells (18).

\[
C_{\text{CFP}} = I_{\text{CFP}} \quad \text{in co-transfected cells with CFP filter} \quad (\text{Eq. 2})
\]

\[
C_{\text{YFP}} = I_{\text{YFP}} \quad \text{in co-transfected cells with CFP filter} \quad (\text{Eq. 3})
\]

In singly transfected cells, \(C_{\text{CFP}}I_{\text{CFP}}\) and \(C_{\text{YFP}}I_{\text{YFP}}\) were determined to be 0.61 and 0.38 for the wild-type CaR-fused CFP and YFP, 0.63 and

loop, and Ala-877 is located in the C-tail. The receptor has five putative protein kinase C sites; one of them in blue is functionally important. B, using the free form I of the ECD of dimeric mGlur1 as a template (1EWT), we generated the model of the CaR ECD by SWISS-MODEL, a fully automated protein structure homology-modeling server. The ECD was predicted to consist of two lobes, LB1 and LB2. In LB1, the cysteines, Cys-129 and Cys-131, forming interprotomer disulfide linkages are shown in yellow spheres, and the leucines, Leu-112 and Leu-156, at the dimer interface are shown in cyan spheres. The serine that is important for the potentiating the effect of an allosteric amino acid modulator, phenylalanine, is shown in gray spheres at the interface of LB1 and LB2. The C-terminal residue of the ECD is shown in purple spheres.
before being lysed in the presence of 100 mM iodoacetamide. After immunoprecipitation with FLAG antibody, elution with SDS sample buffer containing DTT and SDS-PAGE (3–10%), CaR surface expression was detected with avidin. The samples in lanes 1–6 are tagged R648Stop (lane 1), tagged C129S/C131S/R648Stop (lane 2), tagged C129S/C131S and non-tagged C129S/C131S/R648Stop (lane 3), non-tagged C129S/C131S and tagged C129S/C131S/R648Stop (lane 4), tagged C129S/C131S alone (lane 5), and tagged wild-type receptor alone (lane 6). The full-length and truncated receptors are indicated as F and T, respectively, on the right-hand side.

0.39 for the mutant CaR-fused CFP and YFP; and 0.64 and 0.36 for non-fused CFP and YFP, respectively. In co-transfected cells, we first measured the FRET with the FRET filter set, then the I_CFP with the YFP filter set, and finally the I_CFP with the CFP filter set. We used the equations above to calculate C_CFP and C_YFP in co-transfected cells and subsequently FRET. Because FRET intensity is a function of the expression level of CFP and YFP, it is not possible to compare FRET intensity between cells expressing different levels of CFP and YFP. To compare FRET between cells, we normalized the FRET signal to the expression of CFP and YFP that was proportional to I_CFP and I_YFP, respectively. The normalized FRET signals, FRET(I_CFP × I_YFP), in multiple cells were averaged and their standard errors of the mean (S.E.) were calculated.

Measurement of Ca2+, by Fluorometry in Cell Populations—HEK293 cells, which were plated on coverslips and transfected with CaR cDNAs, were loaded for 2 h at room temperature with Fura-2/AM (Molecular Probes) in 20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 0.1% (w/v) bovine serum albumin, and 0.1% dextran and washed once at 37 °C for 20 to 30 min with a buffer solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.1% dextran, and 0.1% bovine serum albumin). The coverslips were then placed diagonally in a thermostatted quartz cuvette that contained the buffer solution, using a modification of the technique employed previously (19). The CaR was activated by multiple additions of Ca2+ in incremental doses to reach the desired concentrations. Excitation monochrometers were centered at 340 nm and 380 nm, with emission light collected at 510 ± 20 nm through a wide-band emission filter. The 340/380 excitation ratio of emitted light was used as readout for Ca2+, as described previously (19). The activities of the wild-type and mutant CaRs were determined in response to increasing concentrations of Ca2+. The mean EC50 values for receptors were calculated from the EC50 values for all individual experiments and were expressed with S.E. as the index of dispersion. Each of the experiments presented in the results was performed at least three times.

RESULTS

Among the three crystal structures of the ECD of mGluR1 that have been determined (12), two crystal forms of mGluR1 ECD were obtained without glutamate; one exists as an open–open dimer (Protein Data Bank number 1EWT) and the other as an open-closed dimer (1EWV). Only one crystal form was obtained with glutamate (1EWS), similar to the open-closed form obtained without glutamate (1EWV). In all three crystal forms, each protomer consists of a lobe I (LB1) domain and a lobe II (LB2) domain. Only the LB1 domain provides the dimer interfaces. Fig. 1 shows a ribbon diagram of a model of the CaR ECD obtained by submitting a partial sequence of the CaR ECD to the SWISS-MODEL, a fully automated protein structure homology-modeling server, accessible via the ExPASy web server (20–22). Even though we submitted the sequence of the CaR ECD with all three crystal structures of mGluR1 as tem-

FIG. 2. Co-immunoprecipitation of the C-terminal truncated receptor (R648Stop) with a full-length receptor, both of which have cysteine and leucine mutations that disrupt the covalent interactions between two protomers. HEK293 cells transfected singly or doubly with receptors either FLAG-tagged or non-tagged were biotinylated before being lysed in the presence of 100 mM iodoacetamide. After immunoprecipitation with FLAG antibody, elution with SDS sample buffer containing DTT and SDS-PAGE (3–10%), CaR surface expression was detected with avidin. The samples in lanes 1–6 are tagged R648Stop (lane 1), tagged C129S/C131S/R648Stop (lane 2), tagged C129S/C131S and non-tagged C129S/C131S/R648Stop (lane 3), non-tagged C129S/C131S and tagged C129S/C131S/R648Stop (lane 4), tagged C129S/C131S alone (lane 5), and tagged wild-type receptor alone (lane 6). The full-length and truncated receptors are indicated as F and T, respectively, on the right-hand side.

FIG. 3. Detection of surface expression of mutant receptors with cysteine and leucine mutations in various combinations. The cells transfected with FLAG-tagged wild-type or mutant receptors were biotinylated before being lysed in the presence of 100 mM iodoacetamide. After immunoprecipitation with FLAG antibody, elution with SDS sample buffer, and SDS-PAGE (3–10%), CaR surface expression was detected with avidin. The SDS sample buffer for samples in A contains DTT, whereas the sample buffer for samples in B contains no DTT. The receptors in A, lanes 1–8, are the wild-type receptor and mutant receptors carrying L112S, L156S, L112/L156S, C129S/C131S, L112/S/C129S/C131S, C129S/C131S/L156S, or L112/S/C129S/C131S/L156S. The receptors in B, lanes 1–3, are L112S, L156S, and L112/L156S. The monomeric, dimeric, and oligomeric CaRs are indicated as M, D, and O, respectively, on the right-hand side.

plates, the SWISS-MODEL successfully generated only one model for the CaR corresponding to the open-open conformer of mGluR1a (1EWT). The two conserved leucine residues, Leu-112 and Leu-156, are predicted at the dimer interfaces (Fig. 1). The Leu-156 residue in one protomer is in close contact with its counterpart in the other protomer, whereas the Leu-112 in one protomer is aligned spatially with its counterpart in the other protomer but is not in very close contact. Therefore, our model predicts that Leu-156 plays more important role than Leu-112 in mediating hydrophobic interprotomer interactions.

To demonstrate that the key hydrophobic dimerization motifs of the CaR are present in the ECD, we tested whether a CaR lacking both the cysteines that mediate interprotomer disulfide linkages and the TMs 2 to 7 could remain associated with a co-expressed full-length receptor during immunoprecipitation. Our previous studies have shown that the truncated receptor, R648Stop, is expressed on the surface but functionally inactive (23). Here, we further demonstrated that the truncated receptor, C129S/C131S/R648Stop, was also expressed on the cell surface but at a level significantly lower than those of R648Stop and the full-length receptors (Fig. 2, lanes 1, 2, 5, and 6). When co-transfected a FLAG-tagged receptor and a non-tagged receptor, we found that the non-tagged truncated receptor, C129S/C131S/R648Stop, could be readily co-immunoprecipitated with a tagged full-length receptor, C129S/C131S (Fig. 2, lane 3), and vice versa: the non-tagged full-length receptor, C129S/C131S, could be readily co-immunoprecipitated with a tagged truncated receptor, C129S/C131S/R648Stop (Fig. 2, lane 4), where the less-expressed, tagged, truncated receptor, C129S/C131S/R648Stop, was mostly in association with the non-tagged full-length receptor, C129S/C131S. In addition, the stability of the truncated receptor C129S/C131S/R648Stop is significantly increased when it was associated with the wild-type receptor (Fig. 2, lanes 2 and 3), suggesting that the expression level of the truncated receptor is dependent on the formation of homodimers and heterodimers. Our previous studies have shown that non-tagged receptors when transfected alone could not be immunoprecipitated by the FLAG M2 antibody (2). These results suggest that non-covalent dimerization motifs present in the ECD are strong enough to hold the dimeric receptor together through the process of immunoprecipitation.
Fig. 4. Co-immunoprecipitation of the receptors with cysteine and leucine mutations. Various tagged full-length receptors with leucine mutations were co-transfected with non-tagged receptors C-terminally truncated at 877 (A) and 648 (B). The co-transfected cells were biotinylated before being lysed in the presence of 100 mM iodoacetamide. After immunoprecipitation with FLAG antibody, elution with SDS sample buffer containing DTT and SDS-PAGE (3–10%), CaR surface expression was detected with avidin. In lanes 5–8, the truncated receptors have two cysteine mutations, C129S and C131S, disrupting the covalent interactions between two protomers. The full-length receptors are wild-type receptors in lanes 1 and 5 and mutant receptors carrying L112S in lanes 2 and 6, L156S in lanes 3 and 7, or L112S/L156S in lanes 4 and 8. The full-length and truncated receptors are indicated as F and T, respectively, on the right-hand side.

To test whether the hydrophobic residues at the dimer interfaces of the ECD are important for forming CaR dimers, we mutated Leu-112 and Leu-156 in the CaR to hydrophilic serines. We examined the effects of these mutations on expression and dimerization of the CaR in the presence or absence of C129S and C131S (3). Fig. 3 shows that all the mutant receptors had cell-surface expression similar to the wild-type receptor and that the receptors carrying leucine mutations still mostly exist as disulfide-linked dimers. Only a trace amount of the mutant receptor carrying both L112S and L156S was detected as a monomer under non-reducing conditions (Fig. 3B, lane 3). Next, we co-transfected some of these mutant receptors with the CaRs C-terminally truncated at either 877 or 648. The truncated receptor, A877Stop, has seven TMs intact, whereas the other, R648Stop, has only TM 1. Both non-tagged truncated receptors were co-immunoprecipitated with the tagged wild-type receptor when the leucines and cysteines were intact (Fig. 4, lane 1). Likewise, both non-tagged truncated receptors, A877Stop and R648Stop, were co-immunoprecipitated with the tagged mutant receptors carrying single or double leucine mutations, L112S and L156S (Fig. 4, lanes 2–4), via disulfide-linkages (data not shown). In contrast, neither non-tagged truncated receptor was effectively co-immunoprecipitated with the mutant receptors, if these truncated receptors had the two cysteine mutations that disrupt the interprotomer disulfide linkages (Fig. 4, lanes 6–8). For instance, C129S/C131S/R648Stop was hardly co-immunoprecipitated with any receptor carrying single or double leucine mutations, whereas the co-immunoprecipitated C129S/C131S/A877Stop was significantly reduced. These data support the hypothesis that the Leu-112 and Leu-156-containing dimer interface in the ECD is the major site that mediates non-covalent dimerization via the ECD. The similar co-immunoprecipitations of R648Stop and C129S/C131S/R648Stop with the wild-type receptor (Fig. 4, lanes 1 and 5) suggest that the stability of these heterodimers is similar, although their corresponding homodimeric receptors had different stability (Fig. 2).

Nevertheless, the presence of both intact TMDs in full-length and truncated receptors resulted in reduced but detectable co-immunoprecipitation of co-transfected C129S/C131S/A877Stop (Fig. 4A, lanes 6–8), indicating that hydrophobic interactions between TMDs of the dimeric CaR also contribute to the dimerization of the CaR. The reduction in co-immunoprecipitated C129S/C131S/A877Stop suggests that the strengths of non-covalent interactions between TMDs are not adequate to hold all the dimeric receptor together through the process of immunoprecipitation. Next, we determined whether the dimerization of the CaR was significantly reduced when the receptor pair lost interactions mediated by the two cysteines and two leucines.

To capture the dimeric CaR while it was still on the surface of the intact cells, we performed a cross-linking experiment on the mutant receptor carrying two cysteine mutations and one or two leucine mutations. The cell-surface proteins were treated with a cross-linking reagent (BS3) prior to the lysis of the cells. The cross-linked dimeric CaR is expected to be resistant to reducing agents, such as DTT. Under reducing conditions, the amount of the dimer relative to the monomer increased in cells transfected with the CaR with or without mutations as the concentration of the cross-linker increased (Fig. 5A). However, the change in dimer/monomer ratio in cells...
transfected with the mutant receptor L112S/C129S/C131S/L156S was significantly less than that in cells transfected with the wild-type receptor and other mutant receptors (Fig. 5B). The apparently low surface detection in samples treated with high concentrations of BS3 was due to the fact that both the biotinylation reagent and BS3 were chemically linked to the same pool of lysine residues on the CaR. As a result, the more BS3 that was used, the less the signal that was detected for the receptor on the cell surface by avidin. Occasionally, an uneven protein loading may cause an irregular change in detection of total surface expression of the CaR. However, the changes in loading bear no consequence on the determination of the dimer/monomer ratio, which is the focus of this experiment. The SDS-resistant dimeric species of the mutant receptor carrying the quadruple mutations was slightly higher than that of the wild-type receptor in the absence of the cross-linker, suggesting that this mutant receptor is a little more easily denatured than the wild-type receptor under immunoprecipitating conditions.

To further assess the importance of the leucines in dimerization of the CaR, we used a biophysical technique called FRET to examine changes in CaR dimerization through determination of the proximity between the cytoplasmic tails of two protomers. Because the emission spectrum of CFP overlaps with excitation spectrum of YFP, we expect that excitation of CFP will yield emission of YFP or FRET signal if these two fluorophores are placed next to each other within 100 Å. The FRET signal is exquisitely sensitive to the distance of these two fluorophores, which can be used to monitor the changes in the distance between cytoplasmic tails that may reflect the extent of CaR dimerization, if CFP and YFP are fused to the C termini of the CaR. The CFP- and YFP-fused CaRs had similar function to that of the CaR (data not shown). We found that the cells co-transfected with CaR-fused YFP and CFP generated FRET signals significantly higher than those co-transfected with non-fused YFP and CFP (Table I and Fig. 6). Consistent with the results from our earlier co-immunoprecipitating and cross-linking experiments, the removal of the covalent and non-covalent interprotomer interactions between YFP- and CFP-fused CaRs by mutating the two leucines and cysteines in YFP- and CFP-fused CaRs, respectively, significantly reduced the FRET signal in co-transfected cells (p < 0.002). This decrease in the FRET signal further supports that the covalent and non-covalent interactions mediated by Cys-129, Cys-131, Leu-112, and Leu-156 are important for the extent of dimerization of the CaR. However, we cannot entirely rule out that the decrease in FRET is contributed by a simple increase in distance between the two cytoplasmic tails of the heterodimeric receptor.

To examine whether dimerization through the hydrophobic interaction between two ECD protomers is important for the function of the CaR, we measured \([\text{Ca}^{2+}]_i\)-elicited \([\text{Ca}^{2+}]_o\) responses of mutant receptors carrying L156S and/or L112S. We found that the mutant receptor carrying L112S had a subtle effect on the apparent ligand affinity of the CaR, as reflected by an increase of 0.71 mM in the \(EC_{50}\) (Fig. 7A). Conversely, mutation of Leu-156 substantially reduced the apparent ligand affinity of the CaR, as reflected by a much larger increase of 7.06 mM in the \(EC_{50}\) (Fig. 7A). However, neither of these two mutations had negative effects on the maximal response of the CaR, even though both leucines were found to play important roles in the non-covalent dimerization. In contrast, the receptor carrying double mutations, L112S and L156S, had a somewhat increased affinity for \([\text{Ca}^{2+}]_i\), reflected by an \(EC_{50}\) value lower than that of the wild-type receptor. The apparent difference in the maximal responses in Fig. 7A, at least in part, resulted from constant activation of the receptor by \([\text{Ca}^{2+}]_i\) in normal culture media at 1.8 mM that more substantially activates the mutant CaR with \(EC_{50}\) values lower than that of the wild-type receptor. In general, the lower the \(EC_{50}\), the less the apparent maximal response, most probably due to the depletion of intracellular calcium stores in the activated cells.

Previously, we have shown that the receptor carrying double mutations, C129S/C131S, had an even higher affinity for \([\text{Ca}^{2+}]_i\), (Fig. 7B) (3). Under normal culture conditions, this mutant receptor is almost half-maximally activated, which may explain why this receptor has an even lower maximal response. Even though the \(EC_{50}\) values for mutants containing triple mutations L112S/C129S/C131S or L156S/C129S/C131S were higher than that of the mutant containing double mutations, C129S/C131S, the maximal responses of the mutants containing an additional leucine mutation were further reduced (Fig. 7B). Because the additional Leu-to-Ser mutation could not induce more activation of the receptor under the culture conditions than C129S/C131S alone, the further reduction in the maximal response reflects a significant decline in the signal transduction of the C129S/C131S-containing receptor. However, a single leucine mutation was not enough to completely reverse the potentiating effect of C129S/C131S on the apparent affinity of the CaR for \([\text{Ca}^{2+}]_i\). As a result, mutant receptors in Fig. 7B remained more sensitive than the

| Constructs          | FRET ± S.D. | FRETN ± S.E. | p value |
|---------------------|-------------|--------------|---------|
| CaR-YFP             | 555.6 ± 204.6 | 59.8 ± 6.4  | <1 x 10^-7 (n = 11) |
| CaR-CFP             | 411.9 ± 268.6 | 33.8 ± 3.2  | <1 x 10^-7 (n = 11) |
| CaR-YFP(-2L)        | 167.1 ± 131.3 | 7.4 ± 0.7   | n = 11   |
| CaR-CFP(-2C)        |              |              |         |
| YFP                 |              |              |         |
| CFP                 |              |              |         |

**TABLE I**

**FRET and FRETN comparison of different co-transfections**

Values are means ± S.D. or S.E. The number of experiments is indicated in parenthesis. For the statistical analysis, each group of cells was compared with the group transfected with non-fused YFP and CFP using the standard t test provided in Microsoft Excel software.

![Fig. 6. Detection of changes in dimerization of the CaR using FRET when a pair of co-transfected receptors lost interactions through disulfide linkages and leucines at the dimer interface. ECFP or EYFP was fused to the cytoplasmic tail of the CaR. The FRET signals were measured in fixed cells that have been co-transfected with a pair of CaR-CFP and CaR-YFP, a pair of CaR-YFP containing L112S/ L156S [CaR-YFP(-2L)] and CaR-CFP containing C129S/C131S [CaR- CFP(-2C)], or a pair of non-fused CFP and YFP. See “Experimental Procedures” for data acquisition and processing. The result shown in this figure is a representative of two independent experiments. The height of each bar represents the mean FRET signal of multiple cells (n = 11). The S.E. is indicated with a vertical line at the top of each bar.**
wild-type receptor to \([\text{Ca}^{2+}]_o\), as reflected by their relatively lower EC50 values.

When two cysteine and two leucine mutations were introduced simultaneously into the CaR, the resultant mutant receptor had an EC50 value significantly higher than that of the wild-type receptor and had a prominent 70% reduction in its maximal response (Fig. 7C). Because the EC50 of the mutant receptor carrying quadruple mutations was higher than that of the wild-type receptor, the substantially lower maximal response was not an artifact of the experiment, because the mutant receptor could not be any more activated than the wild-type receptor under the culture conditions. The markedly reduced response displayed by the mutant receptor likely reflects the reduction in the formation of functional dimeric receptors and the ineffectiveness of the monomeric CaR. Note from the previous figure that the total expression of the mutant CaR is similar to that of the wild-type receptor.

Next we tested whether these leucine residues at the putative dimer interfaces play important roles in the reconstitution of CaR signaling in the cells co-transfected with inactive receptors. We introduced two leucine mutations, L112S and L156S, and/or two cysteine mutations, C129S and C131S, into a mutant receptor carrying R185Q, and the surface expression of these mutant receptors was detected. Only L112S/C129S/C131S/L156S/R185Q significantly reduced the expression of the CaR on the cell surface (Fig. 8A) but not significantly more than previously characterized mutations, such as G143E and E293K (4). Our previous studies showed that co-transfection of two inactive receptors, one carrying an inactivating mutation in the ECD, such as G143E, R185Q, or E293K, and the other having an activating C-tail truncation at 877, reconstituted CaR signaling to 60% of the maximal response of the wild-type receptor regardless of the functional activity of the mutant homodimer (4). It appears that the mutations in the ECD mutants and the absence of the interprotomer disulfide linkages (3, 4) do not affect the maximal responses of the reconstituted receptors, i.e. about 60% of that of the wild-type receptor, if the intracellular domains of resultant heterodimeric receptors are identical. Consistent with our earlier finding that the disulfide linkages are not essential for the functional reconstitution (3), the maximal response of the mutant receptor carrying C129S/C131S/L156S/R185Q was reconstituted to 60% of that of the wild-type receptor when co-transfected with A877Stop (Fig. 8C). In contrast, the responses of the mutant receptor carrying L112S/L156S/R185Q or L112S/C129S/C131S/L156S/R185Q cannot be restored to 60% of the maximal response of the wild-type receptor when co-transfected with the same truncated receptor, A877Stop (Fig. 8, D and E). Some modest reconstitution observed in Fig. 8 (D and E) indicates that only a limited functional heterodimerization had occurred between these full-length receptors and the truncated receptor, A877Stop. Similarly, co-transfection of the truncated receptor, A877Stop, with mutant receptors, C129S/C131S/L156S and L112S/C129S/C131S/L156S, did not reconstitute CaR signaling (Fig. 9). Together these data suggest that dimerization through hydrophobic interactions between two ECDs is important for functional reconstitution of CaR signaling in co-transfected cells.

**DISCUSSION**

The CaR exists principally as a disulfide-linked dimer on the cell surface (2). However, our earlier studies suggest that additional non-covalent interprotomer linkages are important for functional reconstitution of inactive receptors (3, 4). In this study, we have shown that Leu-112 and Leu-156 in the ECD of the CaR are the key residues mediating these functionally important non-covalent interprotomer linkages.

**FIG. 7. The functional impact of the mutations in the putative dimer interacting sites.** HEK293 cells transfected with the wild-type (wt) and mutant receptors including the one carrying quadruple mutations, L112S/C129S/C131S/L156S (-2L/-2C), were loaded with Fura-2. Changes in the emission ratio (340/380 excitation) were measured to assess \([\text{Ca}^{2+}]_o\)-evoked \([\text{Ca}^{2+}]_i\) response. Responses were normalized to the maximal cumulative \([\text{Ca}^{2+}]_i\) response of cells transfected with the wild-type receptor alone. Points are mean values ± S.E. (n = 4–19). Error bars in some cases are smaller than the symbols.
Using three-dimensional structures of the ECD of mGluR1a as templates for molecular modeling, we found that the ECD of the CaR shares a significant structural homology with only one of three conformers, the ligand-unbound open-open form (1EWT), but not with the ligand-unbound or -bound open-closed forms (1EWV and 1EWK) (12). The open-closed form of mGluR1 that is free of the ligand is essentially the same as the form that binds to the ligand, but distinct from the open-open form. Therefore, the ligand-unbound mGluR1 is believed to be in a dynamic equilibrium between the activated state and the...
Consistent with the importance of Leu-112 and Leu-156 in the formation of the CaR dimer, our molecular modeling predicts that they are located at the dimer interfaces in the ECD of the CaR, having Leu-112 right above Leu-156. In addition to Leu-112 and Leu-156, it is predicted that two more hydrophobic residues, Val-58 and Leu-159, are at the dimer interface. Instead of pairing with another hydrophobic residue, Val-58 in one protomer is paired with positively charged Lys-119 in the other, suggesting that Val-58 is unlikely to play any role in stabilizing the interprotomer hydrophobic interactions. Instead, the presence of Val-58 above Leu-112 may keep Leu-112 in one protomer apart from Leu-112 in the other protomer and prevent the protomers from interacting with one another too intensely through hydrophobic interactions. As predicted, the mutation L112S had very little negative effect on the function of the CaR, unlike its corresponding residue Ile-120 that is critical for activation of mGluR1. The fourth hydrophobic residue, Leu-159, in one protomer is right below Leu-156 and in close contact with another Leu-156 in the other protomer, suggesting that Leu-159 participates in interprotomer hydrophobic interactions. We believe that the mutation L156S also significantly weakens the hydrophobic interaction mediated by Leu-159. This may explain why mutating Leu-156 had a more detrimental impact on the function of the CaR than Leu-112.

Previously, we have shown that the formation of the interprotomer bond through hydrophobic dimer interfaces is independent of the formation of the interprotomer disulfide linkages (3). Now, inversely, we have demonstrated that the formation of the interprotomer disulfide linkages is hardly dependent on the formation of hydrophobic interactions through the two leucines at the dimer interface (Fig. 3B). Therefore, simultaneous substitutions of cysteines at 129 and 131 and leucines at 112 and 156 were required to significantly reduce the formation of the dimeric CaR as suggested by crosslinking and FRET experiments. Because the double mutations, C129S/C131S or L112S/L156S, significantly increase the sensitivity of the CaR to its agonist, the marked inactivation of the CaR carrying all four mutations is likely due to the reduction in the formation of the dimer that is capable of activating G proteins. Therefore, CaR dimerization through its ECD is essential for the formation of a functional tertiary structure of the CaR.

Even though mutating one of the cysteines or leucines, Cys-129, Cys-131, Leu-112, or Leu-156, does not affect the expression and dimerization of the CaR, the functional impacts are very different. For instance, the single substitution of Cys-129 or Cys-131 significantly increases the receptor sensitivity to [Ca^{2+}]_i (3). In contrast, the single substitution of Leu-112 or Leu-156 with a serine significantly reduces the sensitivity of the receptor to [Ca^{2+}]_i. Instead of having an additive effect, the double mutations, L112S/L156S, reverse the negative impacts of single mutations on the sensitivity of the CaR, in which case they increase the receptor sensitivity. This functional reversal suggests that the key contact residues at the putative dimer interface could be hydrophilic and the receptor that has a hydrophilic dimer interface is still functional as long as the dimeric receptor possesses the interprotomer disulfide linkages. However, the change from hydrophobic to hydrophilic interaction appears to increase the sensitivity of the CaR to its ligand. Therefore, the presence of non-covalent motifs as well as covalent motifs in the ECD is important for the configuration of the CaR to have a proper sensitivity to physiologically relevant concentrations of calcium.

The functional requirement for having the same hydrophobicities at positions 112 and 156 suggests that activation of the CaR involves the interprotomer interaction between these res-

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* M. Bai, unpublished observation.
idues, perhaps analogous to mGluR1α. It has been shown that the two corresponding residues, Ile-120 and Leu-174, on one LB1 domain of mGluR1α rotate about 70° around a rotation axis passing between them, as the conformation of mGluR1α changes from the open-open form to the open-closed form. The hydrophobic interactions mediated only by these two residues are preserved in these two conformers, except for an interchange in the contacting partners of these two residues. It is possible that the contacts of Leu-112 and Leu-156 in one protomer with those in the other are also being changed upon activation. That is, in the inactive form, Leu-112 and Leu-156 in one protomer are aligned with the corresponding residues in the other, and in the active form, Leu-112 and Leu-156 in one protomer are aligned with Leu-156 and Leu-112 in the other, respectively. The importance of such rotation in activation of the dimeric CaR is further strengthened by the fact that the activity of the CaR can be enhanced by disrupting either type of interprotomer bond between two ECDs of the dimeric receptor. In other words, a reduction in the spatial confinement of one protomer in relation to the other may facilitate receptor activation via an easier rotation around the axis passing between Leu-112 and Leu-156.

Our co-immunoprecipitating, cross-linking, and FRET experiments suggest that the CaR has other non-covalent dimerization motifs in addition to that mediated by Leu-112 and Leu-156. However, our co-immunoprecipitation studies suggest that the hydrophobic interprotomer interactions between the TMDs are not as strong as those between the ECDs. The receptor with quadruple mutations, L112S/C129S/C131S/L156S, also tends to become denatured more easily than the wild-type receptor. If the agonist binding indeed induces the rotation of one protomer versus the other around the axis between Leu-112 and Leu-156, we expect that the functional effect of disrupting the dimerization motifs in the TMD and the C-tail will promote the activity of the dimeric CaR.

In conclusion, we have clearly demonstrated that the leucines in the putative dimer interface are critical for the non-covalent dimerization and functional reconstitution of the CaR and that the dimerization of the CaR through its ECD is essential for the formation of an active receptor. The positive functional consequence of interrupting either one of two types of interprotomer interactions in the ECD suggests that the presence of both hydrophobic interactions and disulfide linkages in the ECD is required to confine the CaR in an inactive configuration under basal conditions. These results also suggest that changes in interprotomer relationships constitute a critical step for CaR activation.

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REFERENCES
1. Duncan, D. B. (1955) *Biometrics* 11, 1–42
2. Bai, M., Trivedi, S., and Brown, E. M. (1998) *J. Biol. Chem.* 273, 23605–23610
3. Zhang, X., Sun, S., Quinn, S. J., Brown, E. M., and Bai, M. (2001) *J. Biol. Chem.* 276, 5316–5322
4. Bai, M., Trivedi, S., Kifor, O., Quinn, S. J., and Brown, E. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2834–2839
5. Nakashima, S. (1992) *Science* 256, 597–603
6. Pin, J. P., and Duvoisin, R. (1995) *Neuropharmacology* 34, 1–26
7. Nakashima, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S., and Okada, M. (1998) *Brain Res. Rev.* 26, 230–235
8. 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
9. Brauner-Osborne, H., Jensen, A. A., Sheppard, P. O., O’Hara, P., and Krogsgaard-Larsen, P. (1999) *J. Biol. Chem.* 274, 18382–18386
10. Ray, K., Hauschild, B. C., Steinbach, P. J., Goldsmith, P. K., Haasche, O., and Spiegel, A. M. (1999) *J. Biol. Chem.* 274, 27642–27650
11. Tsuji, Y., Shimada, Y., Takeshita, T., Kajimura, N., Nomura, S., Sekiyama, N., Otomo, J., Usukura, J., Nakashima, S., and Jingami, H. (2000) *J. Biol. Chem.* 275, 28144–28151
12. Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakashima, S., Jingami, H., and Morikawa, K. (2000) *Nature* 407, 971–977
13. Biss, C. D., Jordan, B. A., Gomes, I., and Devi, L. A. (2001) *Pharmacol. Ther.* 92, 71–87
14. Sato, T., Shimada, Y., Nagasawa, N., Nakashima, S., and Jingami, H. (2003) *J. Biol. Chem.* 278, 4314–4321
15. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 488–492
16. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H. S., Pollak, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) *J. Biol. Chem.* 271, 19537–19545
17. Jin, T., Yue, L., and Li, J. (2001) *J. Biol. Chem.* 276, 12879–12884
18. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) *Biophys. J.* 74, 2702–2713
19. Fajtova, V. T., Quinn, S. J., and Brown, E. M. (1999) *Am. J. Physiol.* 261, E151–E158
20. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* 18, 2714–2723
21. Peitsch, M. C. (1996) *Bio/Technology* 13, 655–660
22. Peitsch, M. C. (1995) *Biochem. Soc. Trans.* 24, 274–279
23. Yamauchi, M., Sugimoto, T., Yamaguchi, T., Yano, S., Wang, J., Bai, M., Brown, E. M., and Chihara, K. (2002) *J. Bone Miner. Res.* 17, 2174–2182
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