Recent studies have shown that the G protein-coupled, extracellular calcium ([Ca$^{2+}$])$_o$-sensing receptor (CaR) forms disulfide-linked dimers through cysteine residues within its extracellular domain and that dimerization of the CaR has functional implications. In this study, we have investigated which of these disulfide linkages are essential for dimerization of the CaR and whether they are required for these functional interactions. Our results confirm the key roles of Cys$^{129}$ and Cys$^{131}$ in CaR dimerization. However, utilizing cross-linking of the CaR or immunoprecipitation of a non-FLAG-tagged CaR with a FLAG-tagged CaR using anti-FLAG antibody, we demonstrate that CaRs with or without these two cysteines form dimers on the cell surface to a similar extent. In addition, reconstitution of CaR-mediated signaling by cotransfection of two individually inactive mutant CaRs is nearly identical in the presence or absence of both Cys$^{129}$ and Cys$^{131}$, showing that covalent linkage of CaR dimers is not needed for functional interactions between CaR monomers. These findings suggest that the CaR has at least two distinct types of motifs mediating dimerization and functional interactions, i.e., covalent interactions involving intermolecular disulfide bonds and noncovalent, possibly hydrophobic, interactions.

The extracellular calcium ([Ca$^{2+}$])$_o$-sensing receptor (CaR) is a G protein-coupled receptor that plays a key role in mineral ion homeostasis by sensing small perturbations in the level of [Ca$^{2+}$]$_o$ and modulating the functions of parathyroid and kidney so as to restore [Ca$^{2+}$]$_o$ to its normal level. Like some G protein-coupled receptors (2, 3), recent studies have shown that the CaR on the surface of receptor-transfected cells forms disulfide-linked dimers (4) through cysteines within its extracellular domain (5, 6). However, one study suggested that two cysteines at positions 101 and 236 mediate intermolecular disulfide linkages (5), whereas another showed that two cysteines at positions 129 and 131 are involved in disulfide-linked dimerization (6). The resultant mutant receptors lacking either of these two cysteine residues were suggested to form monomers rather than dimers in transfected cells (5, 6). These putatively monomeric CaRs had no biological activity in one case (5) or exhibited increased sensitivity to [Ca$^{2+}$]$_o$ in the other (6). Thus there is currently no consensus as to the key cysteine residues within the CaR extracellular domain that mediate dimerization.

Our earlier studies indicated that the CaR may dimerize through noncovalent interactions between transmembrane domains in addition to doing so via covalent disulfide bonds within its extracellular domain (5, 6), as suggested by our studies on naturally occurring inactivating mutations causing familial hypocalciuric hypercalcemia (7, 8). We showed that under reducing conditions, most mutant receptors, including those with almost undetectable mature forms such as R66C and R680C, show substantial amounts of SDS-resistant disulfide forms in addition to monomeric forms in SDS-containing polyacrylamide gels. In contrast, P747frameshift, a single-base deletion in codon 747 resulting in a truncated receptor lacking the second extracellular loop and the remainder of the C terminus, including the fifth, sixth, and seventh transmembrane domains, shows only a single monomeric species and no detectable higher molecular weight forms. Interestingly, a consensus dimerization motif (9) for noncovalent hydrophobic interactions is present in the CaR within TM5, which is missing in the P747frameshift.

In the present studies, we have examined the roles of Cys$^{101}$, Cys$^{129}$, Cys$^{131}$, and Cys$^{236}$ in mediating disulfide-linked dimerization of the CaR and of covalent dimerization in the functional interactions between CaR monomers within the dimeric CaR. Mutating both Cys$^{129}$ and Cys$^{131}$ slightly reduces cell surface expression of the CaR and abolishes most of the sulf-hydryl-sensitive CaR dimerization on the cell surface. In contrast, mutating Cys$^{101}$ and Cys$^{236}$ severely reduces surface and overall expression of the receptor with no substantial conversion of dimers to monomers detected under nonreducing conditions. Furthermore, we show that the CaR still forms functional dimers even in the absence of these intermolecular disulfide linkages.

**Experimental Procedures**

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the approach described by Kunkel (10) to produce mutated receptors in which one or two cysteine residues present in the extracellular domain of the human CaR were mutated to serine. The du-1 ung-1 strain of Escherichia coli, CJ236, was transformed separately with mutagenesis cassettes, as described previously (7). Uracil-containing, single-stranded DNA was produced by infecting the cells with the helper phage, VCSM13 (Stratagene, La Jolla, CA). The single-stranded DNA 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 single-stranded DNA and ligated to generate closed circular heteroduplex DNA. DH10B- or DH10B-compotent cells were transformed with these DNA heteroduplexes, and incorp...
poration of the desired mutations was confirmed by sequencing the mutated cassettes. The resultant mutated cassettes were cloned into the FLAG-tagged or nontagged receptor in pcDNA3 (Invitrogen), as described previously (7).

**Construction of Mutant CaRs with Double Mutations of C101S/C236S—**Cassette 1, carrying C101S, was doubly digested with BspEI and NheI, and the larger fragment obtained from the above digests was ligated to the smaller fragment resulting from digestion of the CaR carrying C236S with BspEI and NheI.

**Construction of C129S/C131S-containing CaRs with Inactivating Mutations—**Cassette 1 carrying C129S/C131S was doubly digested with KpnI and BspEI, and the smaller fragment obtained from the above digestion was ligated to the larger fragment resulting from digestion of the CaR carrying E297K or A877Stop with KpnI and BspEI.

**Transient Expression of CaRs in HEK293 Cells—**The DNA for transfection was prepared using the Midi Plasmid Kit (Qiagen). LipofectAMINE (Life Technologies, Inc.) was employed as a DNA carrier for transfection (11). The human embryonic kidney (HEK293) cells used for transient transfection were provided by NPS Pharmaceuticals, Inc. (Salt Lake City, UT) and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum (Hyclone). (Salt Lake City, UT) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone).

**Biotinylation of Cell Surface Forms of the CaR and Cross-linking of Multimeric Receptors—**Before preparing whole cell lysates, intact HEK293 cells transiently transfected with FLAG-tagged CaR were washed twice with phosphate-buffered saline and treated with 1 mg/mL Sulfo-NHS-Biotin (Pierce), a membrane-impermeant biotinylation reagent, at room temperature with constant agitation for 30 min to biotinylate the proteins on the cell surface. The reaction was then quenched by incubating the cells in 1 x Tris-HCl, pH 7.5, for 5 min. For cross-linking experiments, we added an appropriate amount of biotinylated CaR, substrate (BS'), a noncleavable, membrane-impermeant cross-linker, into the labeling solution with ImmunoPure Sulfo-NHS-Biotin (Pierce).

**Preparation of Whole Cell Lysates—**The surface-biotinylated and/or cross-linked HEK293 cells were rinsed twice with phosphate-buffered saline and solubilized with 1% Triton X-100, 0.5% Igepal CA-630, 150 mM NaCl, 10 mM Tris, pH 7.4, 2 mM EDTA, 1 mM EGTA, and protease inhibitor (1 µg/mL leupeptin, 1 µg/mL aprotinin). Pefabloc (Roche Diagnostics, Indianapolis, IN), 50 µg/mL calf pancreatic RNase A, 0.05 µg/mL peptatin (1 µg/mL immobiloprecipitation buffer) at room temperature. Insoluble material was removed by centrifuging the cell lysates at 15,000 rpm for 15 min at 4 °C. The supernatants were collected as total cell lysates. The protein concentration was determined using the Pierce BCA protein assay (Pierce).

**Immunoprecipitation of FLAG-tagged CaR—**To a microcentrifuge tube, 5 µg of anti-FLAG M2 monoclonal antibody, 400 µL of H2O, 500 µL of 2 x immobiloprecipitation buffer (see above), and 100 µL of total lysate containing ~250 or 500 µg of total proteins were added. The mixture was incubated at 4 °C for 1 h. To the mixture was then added 5 µL of an anti-mouse IgG (Sigma). The incubation was continued for an additional 90 min at 4 °C. To the mixture was subsequently added 50 µL of 10% protein A agarose (Life Technologies, Inc.) for a further 30-min incubation at 4 °C. The protein A-agarose was washed three times with 1 x immobiloprecipitation buffer, and the immune-reactive species were subsequently eluted in 45 or 60 µL of 2 x electrophoresis sample buffer at 65 °C for 30 min. In a given experiment, the same amount of total protein for each sample was used for immunoprecipitation and was eluted from the same volume of the SDS sample buffer. The receptor of interest was detected by Western analysis.

**Western Analysis of the Human CaR Expressed in Whole Cells and on the Cell Surface—**If not specified, 15 µL of eluted, immunoprecipitated sample was subjected to SDS-containing polyacrylamide gel electrophoresis (PAGE) (12) using a linear gradient of polyacrylamide (3–10%). The proteins on the gel were subsequently electrotransferred to a nitrocellulose membrane. After blocking with 5% milk, the forms of the receptor present on the cell surface were detected using an avidin-horsedradish peroxidase conjugate (Bio-Rad) followed by visualization of the biotinylated bands with an enhanced chemiluminescence (ECL) system (PerkinElmer Life Sciences). After removal of the avidin using the recommended procedure for stripping the blots (Amersham Pharmacia Biotech), all forms of the CaR on the same blot were detected using an anti-CaR antiserum (4641 or 4657, polyclonal antiserum raised against two peptides within the extracellular domain of the CaR, kindly provided by Drs. Forrest Fuller and Rachel Simin at NPS Pharmaceuticals, Salt Lake City, UT) followed by a secondary, horsedradish peroxidase-conjugated goat anti-rabbit antibody and then an ECL system (Amersham Pharmacia Biotech).

**Measurement of [Ca2+]o, by Fluorimetry in Cell Populations—**Sweeps with HEK293 cells previously transfected with the appropriate CaR cDNAs were loaded for 2 h at room temperature with fura-2/AM 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% bovine serum albumin, and 0.1% dextrose and then washed once with a bath solution containing 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% bovine serum albumin, and 0.1% dextrose and then washed once with a bath solution containing 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% bovine serum albumin) at 37 °C for 20 min. The coverslips were then placed diagonally in a thermostatted quartz cuvette containing the bath solution, using a modification of the technique employed previously in this laboratory (13). Extracellular calcium was increased stepwise to give the desired final concentrations with additions of Ca2+, in increments of 1 mM, which were followed by 5 mM increments after achieving a level of 5.5 mM [Ca2+]o. The fluorescence was measured at 510 ± 40 nm through a wide-band emission filter. The 340/380 excitation ratio of emitted light was used to calculate [Ca2+]o, as described previously (13).

**Statistics—**The mean EC50 values for the various wild type or mutant receptors determined in response to increasing concentrations of Ca2+ were calculated from the EC50 values for each of the individual experiments and were expressed as the S.E. as the index of dispersion. Comparison of the EC50 values was performed using analysis of variance or Duncan's multiple comparison test (14) (p < 0.05). Each of the experiments described above in the experimental protocols was generally performed at least three times.

**Results**

**Characterization of CaRs Carrying Single Cys to Ser Mutations in the Extracellular Domain—**To examine the role of cysteines in the formation of functional CaR dimers, we first looked at single Cys mutations of the CaR. We constructed CaRs in which one of nine cysteines in the extracellular domain (Cys60, Cys101, Cys129, Cys131, Cys236, Cys437, Cys449, Cys482, and Cys588) was mutated to Ser. Transient expression of these CaRs in HEK293 cells showed that the mutations have varied

| Mutations | EC50 [Ca2+]o | Maximal Ca2+ response |
|-----------|--------------|-----------------------|
| wt        | 3.0 ± 0.07*  | 70.0 ± 3.9 (7)**      |
| C129S     | 2.50 ± 0.11* | 36.0 ± 2.5 (7)**      |
| C131S     | 1.91 ± 0.05* | 54.2 ± 2.7 (7)**      |
| Doubleb   | 1.85 ± 0.36* | 91.7 ± 6.7 (7)**      |
| Tripleb   | 2.33 ± 0.20* | 37.3 ± 4.0 (8)*       |
| C437S     | 4.89 ± 0.24* | 99.0 ± 13.3 (5)       |
| C482S     | 6.79 ± 0.17* | 67.8 ± 3.3 (7)**      |
| Quinutpleb| 2.5 ± 0.16*  | 24.7 ± 2.5 (7)        |
| C60S      | 11.0 ± 0.6*  | 58.9 ± 3.4 (7)*       |
| C101S     | 10.3 ± 0.6*  | 36.6 ± 3.2 (8)*       |
| C236S     | ND           | ND                    |
| C101S/C236S| 5.81 ± 0.29*| 14.8 ± 2.2 (5)*       |
| C598S     | ND           | ND                    |

b Values are means ± S.E.; values marked with asterisks are significantly (p < 0.05) different from the wild type CaR. The number of the experiments is indicated in parentheses.

b Double refers to C129S/C131S, triple refers to C129S/C131S/C482S, and quintuple refers to C129S/C131S/C437S/C494S/C482S.

* ND, not detectable.
HEK293 cells were transfected with the relevant FLAG-tagged wt or mutant CaRs. Proteins on the cell surface were treated with Sulfo-NHS-Biotin before lysing the cells in the presence of 100 mM iodoacetamide. The same amount of total proteins, 500 μg, were immunoprecipitated with anti-FLAG antibody in each case. The immunopurified protein samples were eluted with 60 μl of SDS sample buffer containing 100 mM dithiothreitol (DTT), and 15 μl of each was subjected to SDS-PAGE (3–10%). The surface expression of the CaRs was detected with avidin (panel A). Both surface and intracellular forms of the CaRs were then detected with an anti-CaR antibody (4641) after removal of the avidin (panel B). The samples in lanes 1–7 are the following: lane 1, wt; lane 2, C129S; lane 3, C131S; lane 4, C129S/C131S; lane 5, C437S; lane 6, C449S; and lane 7, C482S. The monomeric species N-glycosylated with complex carbohydrates or high mannose are indicated as C and H, respectively, on the right hand side of the figure and are marked with arrows.

Effects on [Ca\(^{2+}\)]\(_{\text{ion}}\)-elicited responses (Table 1). Two of the mutant receptors, C129S and C131S, become substantially more sensitive to [Ca\(^{2+}\)]\(_{\text{ion}}\), with EC\(_{50}\) values that are 0.6 and 1.2 mM lower than the EC\(_{50}\) of wt, respectively, but with significant reductions in their maximal responses, which are 77 ± 4% and 54 ± 3% of that of wt, respectively. One of the mutations, C482S, does not significantly affect receptor function. Others become less sensitive to [Ca\(^{2+}\)]\(_{\text{ion}}\), than wt or are completely inactive (Table 1). Most of the mutant receptors that have decreased sensitivities to [Ca\(^{2+}\)]\(_{\text{ion}}\), also exhibit reduced maximal responses. The mutant receptors containing C236S and C598S have no detectable activity.

Consistent with the results of a previous study (15), one group of mutant receptors carrying C129S, C131S, C437S, C449S, or C482S (Group I mutations in Fig. 1), exhibit nearly normal levels of expression on the cell surface and in whole cell lysates (Fig. 1, lanes 2, 3, and 5–7). Of these receptors, C437S and C482S (Fig. 1A, lanes 5 and 7) have slightly higher cell surface expression than wt (Fig. 1A, lane 1), whereas C129S, C131S, and C449S (Fig. 1A, lanes 2, 3, and 6) have somewhat lower cell surface expression than wt.

In contrast, another group of mutant receptors carrying C60S, C101S, C236S, or C598S (Group II mutations in Fig. 2), have marked reductions in their surface expression (Fig. 2, lanes 3–6), as well as those in lanes 1 and 2. The low apparent immunoreactivity of C236S toward the anti-CaR antibody, 4641, in Fig. 2, lane 5, is likely due to the fact that Cys\(^{236}\) is the last amino acid in the peptide (Ala\(^{214}\)-Cys\(^{236}\)) against which 4641 was raised and may be important for the binding of 4641 to the receptor. As shown below, C236S has a similar level of overall expression to that of C598S when it was detected with another anti-CaR antibody, 4637, which was raised against a peptide consisting of amino acids 344–358 in the extracellular domain of the CaR. Whereas C598S (Fig. 2B, lane 2) had a much lower mature form than wt, two other mutant receptors, C60S and C101S, were even lower (Fig. 2B, lanes 3 and 4). Note that twice the amount of eluted, immunoprecipitated samples were loaded in Fig. 2, lanes 3 and 4, as in Fig. 2, lanes 1 and 2. The mature forms of these mutant receptors are N-glycosylated with complex carbohydrates, corresponding to the upper bands of the doublets between 127 and 200 kDa (7).

Under nonreducing conditions, all of the mutant receptors with a single point mutation, behaving like wt, were present mostly as dimers or higher multimeric forms on SDS-PAGE (Fig. 3 and 4) when we detect either only the surface forms or all forms of the receptor. However, longer exposure reveals trace amounts of monomeric CaRs for some of the mutant receptors (e.g. C129S, C131S, and C482) on blots stained for cell surface receptor proteins (Fig. 3A, lanes 2, 3, and 6). In contrast to previous studies with C60S, C101S, and C236S (5, 15), no significant amounts of the monomeric species of these three mutant receptors were detected either on the cell surface or in the whole cell lysates (Fig. 4, lanes 2–4). As shown in Fig. 4B, lanes 4 and 5, C236S, has a similar level of overall expression to that of C598S when it was detected with 4637. Thus dimerization of wt appears to involve more than one intermolecular disulfide bond, necessitating mutation of two or more cysteines to identify those forming intermolecular disulfide bonds.

Construction and Characterization of Mutant CaRs with More than One Cys to Ser Mutation in the Extracellular Domain—To identify cysteines forming intermolecular disulfide bonds, we constructed mutant receptors with two or more mutations of cysteines to serines but focused on those that had no detrimental effects on either receptor expression or function when studied as single mutations. We found that the mutant receptor, C129S/C131S, had a substantial level of expression on the cell surface (Fig. 1A, lane 4), although it was slightly lower than those of the mutant receptors with C129S or C131S alone (Fig. 1A, lanes 2 and 3). The activity of the mutant receptor, C129S/C131S, was also affected more than those of the two singly mutated receptors, C129S and C131S (Table 1). C129S/C131S became even more sensitive to [Ca\(^{2+}\)]\(_{\text{ion}}\), with a lower EC\(_{50}\) than that of C129S and similar to that of C131S. The cumulative maximal response of C129S/C131S was significantly lower than those of the singly mutated receptors.

Under nonreducing conditions, a substantial amount of monomeric C129S/C131S was detected when assessed by SDS-PAGE (Fig. 5, lane 2). Detection of the cell surface form of this receptor (Fig. 5A, lane 2) revealed that the monomeric receptor is the major species, unlike wt (Fig. 5A, lane 1). In contrast, reprobing of the same blot with an anti-receptor antibody, detecting both cell surface and intracellular forms of the receptor (Fig. 5B, lane 2), showed that the monomeric form of the
receptor is a minor species. Thus, whereas a large fraction of the mutant receptor, C129S/C131S, no longer forms intermolecular disulfide bonds on the cell surface, the majority of intracellular receptor species either still forms intermolecular disulfide bonds or is resistant to dissociation of dimers to monomers during SDS-PAGE despite the lack of intermolecular disulfide linkages.

When we examined mutant receptors carrying triple, quadruple, or quintuple mutations, we found that, like C129S/C131S (Fig. 5A, lane 2), substantial amounts of receptors containing both C129S and C131S no longer form intermolecular disulfide-linked dimers on the cell surface (Fig. 5A, lanes 3 and 4). However, the major species in the whole cell lysates are still multimers (Fig. 5B, lanes 3 and 4). Other receptors with multiple Cys to Ser mutations, containing either Cys129 or Cys131, still form exclusively disulfide-linked dimers on the cell surface and in whole cell lysates (data not shown).

We also examined the properties of the mutant receptor with the two mutations, C101S and C236S (Figs. 2 and 4, lane 6). In contrast to a previously published study (5), the overall expression level of C101S/C236S (Fig. 4B, lane 6) is equivalent to that of C101S (Fig. 4B, lane 3) and much less than that of C236S (Fig. 4B, lane 4). In addition, we found the surface expression level of C101S/C236S (Fig. 2A, lane 6), although detectable, much less than the mutant receptor carrying either mutation alone (Fig. 2A, lanes 4 and 5). Under nonreducing conditions, there are no detectable mature monomeric species of the CaR in whole cell lysates even though we detected a trace amount of its immature form when the blot for Fig. 4B was exposed for a much longer period of time (data not shown). This mutant receptor does show slight activity (Table I).

Thus Cys129 and Cys131 appear essential for formation of intermolecular disulfide bonds in most of the cell surface forms of the CaR, similar to the result of Ray et al. (6). However, we still detect a substantial amount of dimeric C129S/C131S on the cell surface (Fig. 5A, lane 2), even though it is a minor species relative to its monomeric counterpart. Next we examined whether the mutant receptor, C129S/C131S, which appears as a monomeric receptor in SDS-containing buffer without any dithiothreitol, may exist on the cell surface as noncovalently linked dimers. We utilized cross-linking of cell surface receptors and coimmunoprecipitation of cotransfected receptors as described previously (4).

Demonstration of Dimer Formation of the CaR on the Cell Surface in the Absence of Intermolecular Disulfide Bonds—To determine whether the mutant receptor, C129S/C131S, forms cell surface dimers, we stabilized any preexisting dimeric or higher multimeric forms of the receptor by covalently linking them with BS3, a noncleavable, membrane impermeant cross-linker, and surface-labeled the cells with Sulfo-NHS-Biotin. The surface-biotinylated and cross-linked cells were then lysed in the presence of iodoacetamide. The FLAG-tagged C129S/C131S and wt were then immunoprecipitated, and the immunopurified CaRs were eluted with DTT-containing SDS sample buffer. Increasing the concentration of cross-linker causes a progressive rise in the ratio of dimer to monomer for both wt (Fig. 6, lanes 1–3) and C129S/C131S (Fig. 6, lanes 4–6). In the absence of the cross-linker, the surface form of the mutant receptor is mostly detected as monomer (Fig. 6, lane 4), whereas the dimeric species becomes the major form of the receptor with 5 mM BS3 (Fig. 6, lane 6). This result suggests that the dimeric receptor is still the principal form present on
the cell surface for C129S/C131S. In Fig. 6, twice as much volume of the immunoprecipitated samples in *lanes 1* and *4* were loaded in *lanes 2* and *5*, whereas four times as much in *lanes 1* and *4* were loaded in *lanes 3* and *6*. This increased loading is necessary because the inclusion of BS3 reduces biotinylation for surface detection since both sulfo-NHS-Biotin and BS3 form covalent bonds with the same pool of primary amines on the receptor.

To further demonstrate that C129S/C131S forms noncovalent dimers on the cell surface, we cotransfected a truncated C129S/C131S-containing receptor with the full-length C129S/C131S-containing receptor, one of which is FLAG-tagged, and immunoprecipitated with anti-FLAG antibody (Fig. 7). If the nontagged and tagged receptors formed heterodimers, we would be able to coimmunoprecipitate the nontagged receptor with the tagged receptor. Since the monomeric full-length and cytoplasmic tail-truncated receptors can be resolved under reducing conditions on SDS-PAGE because of their differences in size, it should be possible to determine the relative amounts of the tagged and nontagged receptors on the cell surface using avidin as a probe for surface-biotinylated receptors on the blot.

Fig. 7A, *lane 7*, shows that the nontagged truncated receptor, C129S/C131S/A877Stop, was coimmunoprecipitated in cotransfected cells with the FLAG-tagged full-length receptor, C129S/C131S, to a similar extent as the nontagged receptor, A877Stop, when it was cotransfected with FLAG-tagged wt (Fig. 7A, *lane 2*). If one of the molecular partners in cotransfected cells has C129S/C131S and the other does not, they apparently still associate with one another equally well. For instance, nontagged C129S/C131S/A877Stop can be coimmunoprecipitated with tagged wt (Fig. 7A, *lane 3*), and nontagged wt can be coimmunoprecipitated with tagged C129S/C131S/A877Stop (Fig. 7A, *lane 4*). As a control, none of the nontagged receptors isolated from singly transfected cells could be immunoprecipitated by anti-FLAG antibody (data not shown).

In contrast to the results observed with cotransfection of A877Stop and wt (Fig. 7B, *lane 2*), a substantial amount of the coimmunoprecipitated complex, consisting of one or both of the C129S/C131S-containing receptors, can be dissociated by the addition of SDS sample buffer alone in the absence of reducing agents (Fig. 7B, *lanes 3, 4*, and 7). For instance, the coimmunoprecipitated complex of FLAG-tagged wt and nontagged C129S/C131S/A877Stop can be detected as a monomeric species in a 1:1 ratio (Fig. 7B, *lane 3*) under nonreducing conditions, even though most of the FLAG-tagged wt (Fig. 7B, *lane 3*) in the cotransfected cells is still detected as disulfide-linked homodimers, similar to that seen for wt transfected alone (Fig. 7B, *lane 1*). In other words, the monomeric species of wt observed in Fig. 7B, *lanes 3* and 4 is actually associated noncovalently with C129S/C131S/A877Stop before the treatment with SDS sample buffer. The inclusion of iodoacetamide in the lysis buffer prevents the monomeric wt from associating with itself through disulfide linkages, specifically or nonspecifically, after it is dissociated from C129S/C131S/A877Stop in the SDS-sample buffer. Thus, our results suggest that the CaR can form dimers through interactions other than intermolecular disulfide bonds.

**Stabilization of oligomers of the CaR on the cell surface using a noncleavable, membrane-impermeant cross-linker.** HEK293 cells were transfected with FLAG-tagged wt (*lanes 1–3*) or C129S/C131S (*lanes 4–6*). Proteins on the cell surface were treated with both Sulfo-NHS-Biotin and varying concentrations of BS3, a cross-linker, before lysing the cells. The CaR was then immunoprecipitated with anti-FLAG antibody from the same amount of total proteins. The immunopurified protein samples were eluted with DTT-containing SDS buffer and subjected to SDS-PAGE (3–10%). Surface expression of the CaR was detected with avidin. The concentrations of BS3 were 0 mM for the samples in *lanes 1* and *4, 1* mM for those in *lanes 2* and *5*, and *5* mM for those in *lanes 3* and *6*, respectively. The monomeric and dimeric CaRs are indicated as *M* and *D*, respectively, on the right hand side of the figure and are marked with *arrows*.

**Coimmunoprecipitation of non-tagged and FLAG-tagged receptors with cysteine mutations.** HEK293 cells transfected singly or doubly with receptors either carrying or not carrying the double mutations, C129S and C131S, were biotinylated before lysing the cells in the presence of 100 mM iodoacetamide. After immunoprecipitation with anti-FLAG antibody and elution with SDS sample buffer containing DTT (panel A) or no DTT (panel B) and SDS-PAGE (3–10%), CaR surface expression was detected with avidin. The samples in *lanes 1–7* are tagged wt alone (*lane 1*), tagged wt and nontagged A877Stop (*lane 2*), tagged wt and nontagged C129S/C131S/A877Stop (*lane 3*), non-tagged wt and tagged C129S/C131S/A877Stop (*lane 4*), tagged C129S/C131S/A877Stop alone (*lane 5*), tagged C129S/C131S alone (*lane 6*), and tagged C129S/C131S and nontagged C129S/C131S/A877Stop (*lane 7*). The monomeric species of the full-length and truncated receptors are indicated as *F* and *T*, respectively, on the right hand side of the figure and are marked with *arrows*.
functions of the individual mutant receptors when transfected by themselves (Fig. 8, B and C; data are not shown for mutant receptors carrying A877Stop, which are completely inactive). Cotransfection of C129S/C131S/E297K with C129S/C131S/A877Stop reconstituted CaR-mediated signaling to an extent almost identical to that observed in cells cotransfected with A877Stop and E297K (Fig. 8). Furthermore, Fig. 9 shows that C129S/C131S/E297K and C129S/C131S/A877Stop readily form heterodimers when cotransfected. For instance, nontagged C129S/C131S/A877Stop can be coimmunoprecipitated with FLAG-tagged C129S/C131S/E297K to an extent similar to that observed with nontagged A877Stop and tagged E297K (Fig. 9A, lanes 3 and 4). However, a substantial amount of C129S/C131S/A877Stop and C129S/C131S/E297K are not associated with one another through disulfide linkages (Fig. 9B, lanes 4 and 5), unlike A877Stop/E297K (Fig. 9B, lanes 3). As a control, the nontagged C129S/C131S/E297K and C129S/C131S/A877Stop cannot be immunoprecipitated when transfected alone (Fig. 9, lanes 7 and 8).

**DISCUSSION**

The CaR principally exists as a dimer on the cell surface of CaR-transfected HEK293 cells, which is covalently linked by disulfide bonds (4). In previous attempts to identify the cysteines in the receptor extracellular domain responsible for CaR dimerization, two groups obtained discrepant results. Both groups identified two cysteines in the N-terminal extracellular domain that may be involved in intermolecular disulfide linkages, similarly to mGlur5 (2). However, one group proposed that Cys129 and Cys396 form intermolecular disulfide linkages, whereas the other showed that Cys129 and Cys131 mediate intermolecular disulfide linkages.

In the present studies, we initially focused on cysteines that are not very critical for receptor expression on the cell surface (15), assuming that the cysteines that participate in the intermolecular disulfide bonds are not critical for receptor protein folding. We found that mutating Cys129 and Cys131, individually or in combination, has little effect on receptor protein expression. Consistent with the results of the study performed by Ray et al. (6), we found that the presence of the double mutations, C129S/C131S, eliminated most intermolecular disulfide linkages in the receptor proteins expressed on the cell surface. In contrast to the other study (5), however, we found that the double mutations, C101S and C296S, which interfere substantially with receptor expression when present either individually or in combination, do not disrupt disulfide-mediated dimerization.

The dimerization of many other G protein-coupled receptors, including the β2-adrenergic receptor (9), the dopamine D2 receptor (17), the δ opioid receptor (3, 18), and the bradykinin B2 receptor (19), are not mediated by intermolecular disulfide bonds. For instance, constitutive dimerization of the β2-adrenergic and the dopamine D receptors occurs via their transmembrane regions, whereas agonist-induced dimerization of the bradykinin B2 receptor requires noncovalent interactions of the N terminus of the receptor. In this study, we have shown both biochemically and functionally that the removal of the intermolecular disulfide linkages normally present in the CaR does not affect its dimerization. As shown in Fig. 7A, lanes 2 and 7, pairs of CaRs with the double mutations, C129S and C131S, can associate with one another to an extent similar to those in which Cys129 and Cys131 are intact, even though the majority of the receptor species are no longer linked with one another through intermolecular disulfide bonds (Fig. 7B, lanes 3, 4, and 7). Functionally, coexpression of the inactive mutant CaRs, E297K and A877Stop, in which these double cysteine mutations were present also led to reconstitution of significant [Ca2+]i-sensing and intracellular signaling capability, which was indistinguishable from that of cotransfected CaRs harboring E297K and A877Stop in which Cys129 and Cys131 were intact.
One reason why others (6) did not find association of their truncated receptor, TM1 mutant, with C129S/C131S may be that the missing part in the TM1 mutant is likely essential for noncovalent association of dimeric receptors. This suggests that the CaR may form dimers through one or more noncovalent dimerization motifs present within the last six transmembrane domains and the cytoplasmic tail. Our current studies suggest that the cytoplasmic tail of amino acids 877 to 1078 is not essential for the noncovalent associations between our truncated and full-length receptors. Nevertheless, further studies are required to determine the structural domains mediating the noncovalent interactions with a focus on the region between TM5 and TM7, where putative hydrophobic dimerization domains exist.

We found that C129S, C131S, and C129S/C131S are all much more sensitive to $[\text{Ca}^{2+}]_i$ than wt. In addition, they all have reduced maximal responses in comparison to wt. C129S and C131S still form mostly intermolecular disulfide-linked dimers, suggesting that Cys129 and Cys131 are not only important for formation of intermolecular disulfide linkages but also for the normal function of the receptor even when the structural constraints conferred by formation of covalent bonds with another CaR molecule are still present. Consistent with the results of previous studies on several of the mutations causing autosomal dominant hypocalcemia, those mutations that are located in the vicinity of Cys129 and Cys131, such as E127A and F128L, significantly increase the sensitivity of the CaR to $[\text{Ca}^{2+}]_i$, even though they do not disrupt the formation of intermolecular disulfide bonds as suggested by Ray et al. (6).

In conclusion, our results support the involvement of Cys129 and Cys131 in sulfydryl-sensitive dimerization of the CaR and suggest that the region containing these two cysteines is important for the normal function of the CaR. However, the CaR still forms dimers, even in the absence of the intermolecular disulfide linkages mediated by Cys129 and Cys131. Furthermore, CaRs lacking these disulfide linkages are still capable of the intermolecular interactions required for functional reconstitution of individually inactive mutant receptors, such as E297K and A877Stop.

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