Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 2834–2839, March 1999
Cell Biology

Intermolecular interactions between dimeric calcium-sensing receptor monomers are important for its normal function

MEI BAI*, SUNITA TRIVEDI, OLGA KIFOR, STEPHEN J. QUINN, AND EDWARD M. BROWN

Endocrine-Hypertension Division, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA 02115

Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved January 14, 1999 (received for review November 13, 1998)

ABSTRACT **We recently demonstrated that the G protein-coupled, extracellular calcium-sensing receptor (CaR) forms disulfide-linked dimers. The functional significance of dimerization of this receptor was suggested by our earlier observations that CaRs carrying certain point mutations exert dominant negative effects on the function of the coexpressed wild-type receptor both *in vivo* and when cotransfected in human embryonic kidney cells. In this study, we explored the functional consequences of CaR dimerization. Coexpression in human embryonic kidney cells of specific pairs of mutant CaRs, each with reduced or absent activity because of distinct loss-of-function mutations, results in the formation of heterodimers and partially reconstitutes extracellular calcium-dependent signaling. Moreover, our results suggest that the CaR has at least two functionally separable domains. However, the presence of an abnormal domain in each mutant monomer substantially impairs the function of the CaR heterodimer, resulting in the reconstituted CaRs having characteristics distinct from those of the wild-type CaR. Our study suggests that intermolecular interactions within the dimeric CaR are important for the receptor's function.**

The extracellular calcium ($[Ca^{2+}]_o$)-sensing receptor (CaR) plays a central role in calcium homeostasis in humans and other mammals (1). The CaR belongs to a unique subfamily of G protein-coupled receptors (GPCRs) with an unusually large N-terminal, extracellular domain (ECD). Recent studies demonstrated that the CaR forms disulfide-linked dimers (2, 3).

The physiological importance of the CaR in determining the level at which $[Ca^{2+}]_o$ is set *in vivo* has been documented by the characterization of human syndromes resulting from activating or inactivating mutations of this receptor (4, 5). We have characterized a number of mutant CaRs bearing inactivating and activating mutations in transiently transfected human embryonic kidney (HEK) cells (6–11). Some of the point mutations in the CaR's ECD (i.e., G143E, R185Q, and E297K) or in its third intracellular loop (i3) (e.g., R795W) severely attenuate the CaR's activity (6). In addition, insertion of an Alu sequence into codon 877 (7) causes deletion of most of its intracellular C-terminal tail (C-tail) and inactivates the CaR. Two of these mutant CaRs (R185Q and R795W) exert dominant negative effects on the coexpressed wild-type CaR (wt) in cotransfected HEK cells (6). The latter finding suggested that the mutant CaR may negatively affect the coexpressed wt's function by forming heterodimers.

To characterize further the CaR's putative intermolecular interaction(s), we have now cotransfected two inactive CaRs having defects in different regions of the receptor, one with a loss-of-function mutation in its ECD and another with a loss-of-function mutation in its C-tail (7) or i3. We show that cotransfection of specific pairs of mutant CaRs reconstitutes $[Ca^{2+}]_o$-sensing capacity, as manifested by $[Ca^{2+}]_o$-elicited increases in cytosolic calcium ($[Ca^{2+}]_i$) and accumulation of inositol phosphates (IPs). Moreover, our biochemical studies provide unequivocal evidence that formation of heterodimers on the cell surface occurs and is likely to be the molecular basis for reconstitution of $[Ca^{2+}]_o$-dependent signaling capacities of these mutant CaRs.

MATERIALS AND METHODS

Construction of Flag-Tagged Mutant CaRs and Transient Transfection of Tagged as Well as Untagged CaRs in HEK Cells. The Flag, an epitope tag, was introduced into the third cassette of the mutant CaRs as described previously (7). The CaRs were transfected in HEK cells by using a DNA–Lipofectamine mixture (GIBCO) as described previously (6).

Detection of Cell Surface Forms of the CaRs. Before preparing whole-cell lysates, intact HEK cells transiently transfected with Flag-tagged and/or nontagged CaRs were labeled with ImmunoPure Sulfo-NHS-Biotin (Pierce) (3). The free thiol groups of the CaR in the intact cells were prevented from forming nonspecific disulfide bonds during protein preparation by including 100 mM of iodoacetamide in the lysis buffer. Flag-tagged CaRs were solubilized and immunoprecipitated with anti-Flag M2 mAb (VWR Scientific), resolved by SDS/PAGE, and blotted on nitrocellulose membranes. The immunopurified CaRs were detected with an avidin-horseradish peroxidase conjugate (Bio-Rad) to visualize the forms of the CaR expressed on the cell surface.

Measurement of $[Ca^{2+}]_i$ by Fluorimetry in Cell Populations. The CaR-transfected HEK cells on coverslips were loaded with fura-2/AM (Molecular Probes) and stimulated with increasing concentrations of $[Ca^{2+}]_o$. Increases in $[Ca^{2+}]_i$ were determined by measuring the emission ratio (340/380 excitation) at 510 ± 40 nm as before (6).

Determination of Total IPs. HEK cells prelabeled overnight with [^{3}H]myoinositol (25 μCi/well) (New England Nuclear) were incubated with varying concentrations of $[Ca^{2+}]_o$ for 30 min in the presence of 10 mM of LiCl (8). IPs were purified and quantified as described previously (8).

Statistics. The mean EC_{50} values for wt or mutant CaRs were calculated as described previously (6).

RESULTS

Reconstitution of $[Ca^{2+}]_o$-Elicited $[Ca^{2+}]_i$ Responses in Cells Cotransfected with Two Inactive Mutant CaRs. HEK

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: $[Ca^{2+}]_o$, extracellular calcium; CaR, $[Ca^{2+}]_o$-sensing receptor; GPCR, G protein-coupled receptor; ECD, the N-terminal, extracellular domain; i3, the third intracellular loop; C-tail, the intracellular C-terminal tail; wt, the wild type CaR; $[Ca^{2+}]_i$, cytosolic calcium; IP, inositol phosphate; HEK cells, human embryonic kidney cells.

*To whom reprint requests should be addressed at: Endocrine Division, 221 Longwood Avenue, Boston, MA 02115. e-mail: MBai@bustoff.bwh.harvard.edu.

cells were transiently cotransfected with various pairs of mutant CaRs. As a control, cells also were transfected with wt or mutant CaRs alone. CaR-mediated biological responses first were examined by measuring $[Ca^{2+}]_o$-elicited increases in $[Ca^{2+}]_i$. As shown in Fig. 1, cells transfected with either G143E or A877Stop alone did not respond at all to increases in $[Ca^{2+}]_o$ as high as 60 mM. In contrast, cells cotransfected with G143E and A877Stop showed an initial rise in $[Ca^{2+}]_i$ at 10 mM $[Ca^{2+}]_o$ and achieved a maximal cumulative $[Ca^{2+}]_i$ response that was about 56% of that of wt at 60 mM $[Ca^{2+}]_o$ (Fig. 1*B*). The cotransfected CaRs exhibited an EC_{50} of 25.7 ± 1.0 mM (n = 9), much greater than that of wt, 4.1 ± 0.2 mM (n = 6). Similarly, cells cotransfected with A877Stop and another inactive mutant CaR (E297K) exhibited much greater responses than did cells transfected with E297K alone, showing an increase in the maximal cumulative $[Ca^{2+}]_i$ response from 17 to 63% of that of wt, with a slight reduction in EC_{50} from 22 ± 2 mM (n = 5) to 19.6 ± 1.4 mM (n = 4) (Fig. 1). However, cells cotransfected with G143E and E297K exhibited a maximal cumulative $[Ca^{2+}]_i$ response, 14% of that of wt, even slightly less than that of E297K transfected alone. The apparent Hill coefficients (2.2 and 2.1 for the cotransfections G143E/A877Stop and E297K/A877Stop, respectively) were much lower than that for wt (3.0). As a control, A877Stop had no effect on the function of the cotransfected wt (e.g., compare Fig. 1 *A* with *B*). When HEK cells were transfected with 1.25 μg of the individual cDNAs for single transfections or with 0.3125 μg of each cDNA for cotransfections, the concentration–response curves showed no significant differences from those obtained using 0.625 μg of each cDNA (data not shown). Therefore, 0.625 μg of each cDNA was used for all of the experiments described in this study.

Reconstitution of $[Ca^{2+}]_o$-Elicited Accumulation of IPs in Cotransfected Cells. To determine whether the reconstituted $[Ca^{2+}]_i$ responses in cells cotransfected with two mutant CaRs were a result of increased activation of phospholipase C, we measured $[Ca^{2+}]_o$-elicited accumulation of total IPs in HEK cells transiently transfected with the mutant CaRs. Fig. 2 shows that cells transfected with either G143E or A877Stop alone showed little accumulation of IPs with increases in $[Ca^{2+}]_o$ of up to 64 mM. Cells transfected with E297K showed a small increase in IPs at 64 mM $[Ca^{2+}]_o$, which was about 10% of that of wt when maximally stimulated at 32 mM $[Ca^{2+}]_o$ (Fig. 2*A*). The maximal $[Ca^{2+}]_o$-elicited increases in IPs in cells cotransfected with either A877Stop and G143E or A877Stop and E297K were about 30% of that of wt and were significantly greater than those in cells transfected with any one of the mutant CaRs alone. These experiments document that cotransfection of two individually inactive mutant CaRs can partially reconstitute $[Ca^{2+}]_o$-dependent signaling transduced via the phospholipase C signaling pathway.

Formation of Heterodimers by the Truncated and Full-Length Mutant CaRs. Our earlier studies showed that both full-length and truncated CaRs form homodimers on the cell surface (3). Moreover, the truncated CaR (i.e., A877Stop) and the full-length CaR (wt) form heterodimers in addition to their respective homodimers when cotransfected in HEK cells (3). If point mutations in the CaR's ECD do not interfere with dimerization, it is likely that both of the full-length inactive mutant CaRs would form their respective heterodimers with A877Stop. Under nonreducing conditions, the bands at 160 kDa for wt and mutants, G143E and E297K, that would have been anticipated for the full-length monomeric CaRs (3) were hardly visible (Fig. 3*A*). Instead, two major bands between 200 and 300 kDa as well as a minor band between 300 and 500 kDa were detected, likely corresponding to dimeric and trimeric forms of the CaR. These data indicate that the point mutations did not interfere with CaR dimerization.

To determine whether full-length and truncated mutant CaRs form heteromultimers, we cotransfected the nontagged, truncated CaR with a Flag-tagged, full-length CaR or the Flag-tagged, truncated CaR with a nontagged, full-length mutant CaR and immunoprecipitated the tagged CaRs as described previously (3). Under reducing conditions, both the full-length and truncated mutant CaRs were converted to monomeric species with molecular masses of 160 and 120 kDa, respectively, on SDS/PAGE (Fig. 3*B*, lanes 1, 3, 5, and 8). In Fig. 3*B* (lanes 2 and 4), the nontagged CaR, A877Stop, was coimmunoprecipitated with the tagged, full-length mutant CaR, either G143E or E297K. Likewise, the nontagged, full-length mutant CaRs coimmunoprecipitated with the tagged A877Stop (Fig. 3*B*, lanes 9–10). When transfected alone, nontagged A877Stop (Fig. 3*B*, lane 7) and full-length mutant CaRs (data not shown) were not immunoprecipitated. There-

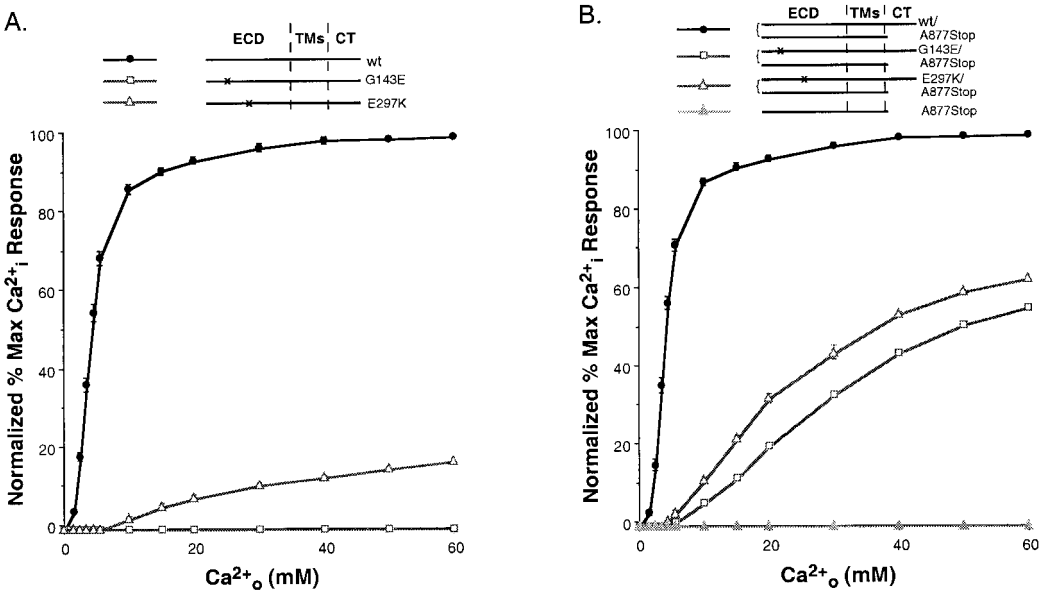

FIG. 1. Cotransfection of inactive CaRs reconstitutes CaR-mediated, $[Ca^{2+}]_o$-elicited $[Ca^{2+}]_i$ responses in HEK cells. Responses are normalized to the maximal cumulative $[Ca^{2+}]_i$ responses of cells transfected with wt alone for both *A* and *B*. (*A*) HEK cells were transfected with either wt or a mutant CaR, G143E, or E297K. (*B*) Cells were transfected with A877Stop or were cotransfected with A877Stop and the full-length wt (wt/A877Stop) or a mutant CaR, either G143E (G143E/A877Stop) or E297K (E297K/A877Stop). Points are mean values ± SEM) (n = 3–9).

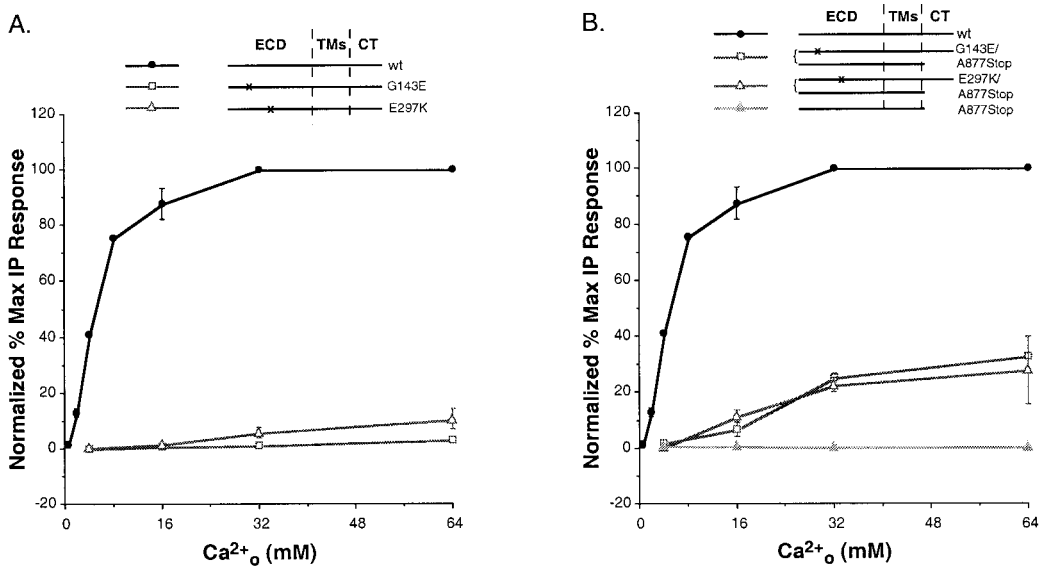

FIG. 2. Cotransfection of inactive CaRs reconstitutes CaR-mediated, $[Ca^{2+}]_o$-elicited IP responses in HEK cells. Responses are normalized to the maximal IP responses of cells transfected with wt alone. (*A*) HEK cells were transfected with either wt or a mutant CaR, G143E, or E297K. (*B*) Cells were transfected with A877Stop and wt or were cotransfected with A877Stop and a mutant CaR, G143E (G143E/A877Stop) or E297K (E297K/A877Stop). Points are mean values ± SEM ($n = 4$).

fore, the nontagged CaR in cotransfected cells was associated with the tagged CaR during the immunoprecipitation.

The intensities of the bands corresponding to the tagged, full-length mutant CaRs were much greater than that of nontagged A877Stop and vice versa, indicating that these mutant CaRs form their respective homomultimers in addition to heteromultimers. Slight but insignificant differences in the levels of surface expression of full-length mutant CaRs transfected alone vs. those that were cotransfected were found in individual experiments. Fig. 3*C* shows that under nonreducing conditions CaRs on the surface of cotransfected cells existed exclusively as high-molecular-mass species, mostly between 200 and 300 kDa, similar to those observed in cells singly transfected with individual CaRs, including the truncated CaR (A877Stop). Taken together, these results show that the cotransfected CaRs exist primarily as dimers on the cell surface and a significant fraction of them are heterodimers.

Enhancement of the $[Ca^{2+}]_o$-Sensing Capability of the Mutant CaR, R185Q, by Cotransfection with the Truncated CaR, A877Stop. R185Q is another mutation located within the CaR's ECD, which partially inactivates the CaR. Unlike G143E and E297K, the mutant CaR, R185Q, elicits significant increases in $[Ca^{2+}]_i$ and IPs in response to extremely high concentrations of $[Ca^{2+}]_o$ with a defined EC_{50} (6, 8). To demonstrate that heterodimerization with A877Stop has positive effects on the partially active R185Q, we cotransfected A877Stop and R185Q. As shown in Fig. 4, the mutant CaR, R185Q, elicited a maximal cumulative response of about 36% of that of wt with an elevated EC_{50} of 26 mM. Cotransfection of R185Q with A877Stop increased the maximal $[Ca^{2+}]_i$

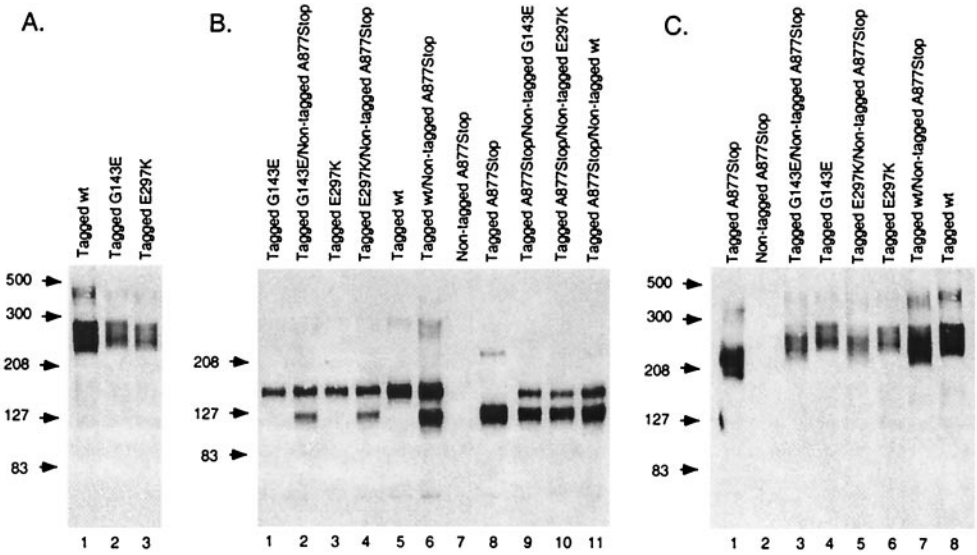

FIG. 3. (*A*) Detection of surface expression of dimeric mutant CaRs G143E and E297K. (*B* and *C*) Coimmunoprecipitation of A877Stop, a truncated CaR, and full-length mutant CaRs. HEK cells transfected with Flag-tagged CaRs or cotransfected with Flag-tagged and nontagged CaRs (as indicated in the figure) were biotinylated and lysed in the presence of 100 mM iodoacetamide. After immunoprecipitation with anti-Flag antibody, elution with SDS sample buffer without DTT (*A* and *C*) or with DTT (*B*) and SDS/PAGE (3–10%), CaR surface expression was detected with avidin.

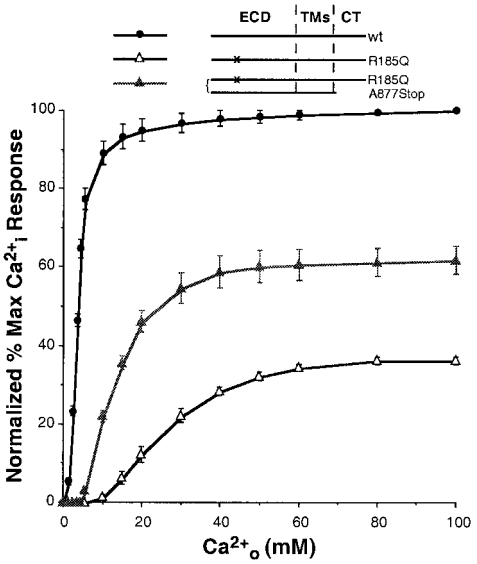

FIG. 4. Enhancement of the $[Ca^{2+}]_o$-sensing capacity of the mutant CaR, R185Q, after its cotransfection with the truncated CaR, A877Stop. $[Ca^{2+}]_o$-elicited $[Ca^{2+}]_i$ responses in HEK cells transfected with R185Q or cotransfected with R185Q and A877Stop were measured and normalized to the maximal cumulative $[Ca^{2+}]_i$ responses of cells transfected with wt alone. Points are mean values ± SEM (n = 4).

response from 36 to 62% of that of wt and reduced the EC_{50} from 26 mM to 13 mM (Fig. 4) as a result of increased activation of phospholipase C (as assessed by accumulation of IPs; data not shown). The apparent Hill coefficients for R185Q and R185Q/A877Stop were the same, 2.8, and were slightly less than for wt, 3.0. A877Stop could be coimmunoprecipitated with the cotransfected R185Q and did not significantly change surface expression of the cotransfected R185Q compared with that of singly transfected R185Q (data not shown). Taken together, these results strongly suggest that the heterodimeric CaRs (R185Q/A877Stop) were likely to be the signal-transducing species producing the initial $[Ca^{2+}]_i$ response when $[Ca^{2+}]_o$ was increased from 5.5 to 10 mM in the cotransfected cells, because no significant $[Ca^{2+}]_i$ response was observed in cells transfected with R185Q or A877Stop alone in response to a similar change in $[Ca^{2+}]_o$ (Fig. 4).

Impact of the Arginine Residue at Position 795 in i3 on Signal Transduction by Cotransfected CaRs. A point mutation, R795W, in i3 severely attenuates the activity of the resultant mutant CaR (Fig. 5*A*). Nevertheless, this mutation has no effect on the surface expression of the CaR (Fig. 6*A*, lanes 3 and 5). Cotransfection of this mutant CaR, R795W, and A877Stop, which has the normal i3, produced a very limited augmentation of overall $[Ca^{2+}]_o$-dependent intracellular signaling (Fig. 5*A*), even though the two mutant CaRs formed a substantial quantity of heterodimeric receptor (R795W/A877Stop, Fig. 6*A*, lane 2 and 4). This result raises the possibility that i3 and the intact C-tail are part of an integral domain involved in downstream signaling.

The mutant CaR, R795W, was cotransfected with another full-length mutant CaR, G143E, which has both the normal i3 and an intact C-tail. Alternatively, we cotransfected a mutant CaR carrying two mutations, R795W&A877Stop (a tail-truncated CaR carrying the point mutation in i3) with the full-length mutant CaR, G143E. The truncated CaR, R795W&A877Stop, is completely inactive (data not shown). Cotransfections of G143E with either R795W or R795W &A877Stop reconstituted $[Ca^{2+}]_o$-dependent receptor signaling in very similar ways (Fig. 5*B*). The maximal response in cells cotransfected with either G143E/R795W or G143E/R795W&A877Stop was about two-thirds of that in cells cotransfected with G143E/A877Stop. Furthermore, both cotransfections produced EC_{50} values at least 10 mM higher than that observed in the cotransfection of G143E and A877Stop. Thus, the mutation R795W affects $[Ca^{2+}]_o$-dependent signal transduction. In cells cotransfected with G143E/R795W&A877Stop or G143E/A877Stop, similar amounts of the truncated CaRs, R795W&A877Stop or A877Stop, were coimmunoprecipitated with the full-length mutant CaR, G143E (Fig. 6*B*, lanes 3 and 4). Taken together, these results show that possessing both the normal i3 and the intact C-tail within one of monomeric subunits of the heterodimeric CaR is important in $[Ca^{2+}]_o$ sensing and downstream signaling.

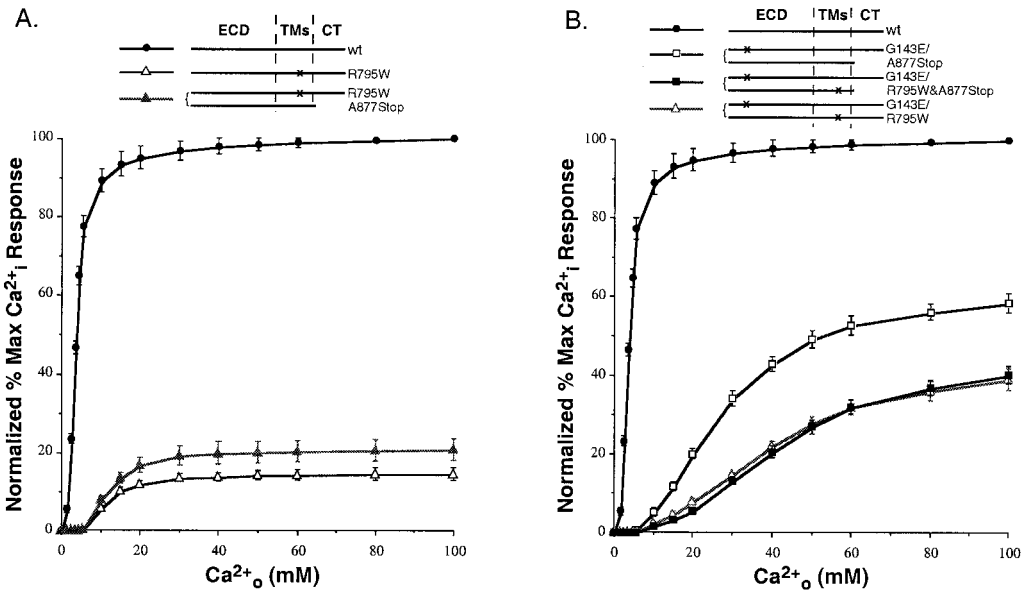

FIG. 5. Effects of the point mutation R795W within i3 on the function of heterodimeric CaRs. (*A*) $[Ca^{2+}]_o$-elicited $[Ca^{2+}]_i$ responses in HEK cells transfected with R795W or cotransfected with R795W and A877Stop were measured. (*B*) $[Ca^{2+}]_o$-elicited $[Ca^{2+}]_i$ responses in HEK cells cotransfected with G143E and a CaR bearing a mutation within i3 and/or the tail, such as A877Stop, R795W, and A877Stop&R795W, were measured. All the measurements were normalized to the maximal cumulative $[Ca^{2+}]_i$ responses of cells transfected with wt alone. Points are mean values ± SEM (n = 4).

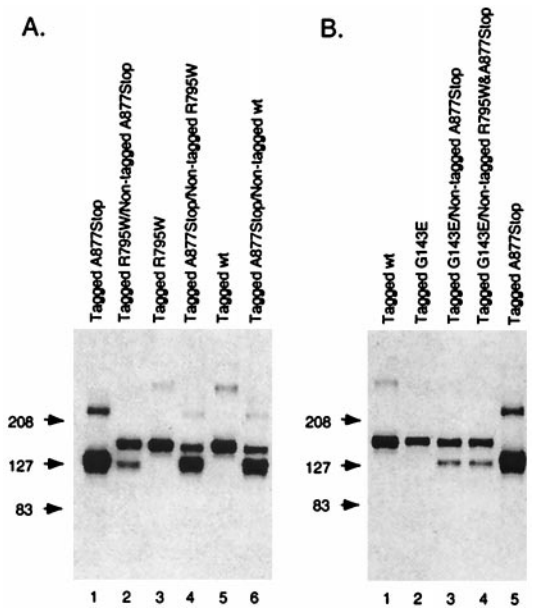

FIG. 6. Coimmunoprecipitation of the mutant CaR, R795W, with the truncated CaR, A877Stop (*A*), and an inactive truncated CaR (A877Stop or R795W&A877Stop) with the inactive full-length mutant CaR, G143E (*B*). HEK cells transfected or cotransfected with Flag-tagged and nontagged CaRs (as indicated in the figure) were biotinylated before cell lysis in the presence of 100 mM iodoacetamide. After immunoprecipitation with anti-Flag antibody, elution with SDS sample buffer with DTT, and SDS/PAGE (3–10%), CaR surface expression was detected with avidin.

Moreover, an inactivating mutation in the i3 of either monomer has a negative effect on overall signal transduction.

DISCUSSION

The CaR principally exists as a dimer on the cell surface of CaR-transfected HEK cells, which is covalently linked by disulfide bonds (3). The presence of covalently bound dimeric forms of the CaR also has been described recently in detergent extracts prepared from the inner medulla of the rat kidney, which endogenously expresses the receptor (2). Dimerization of other GPCRs, including the muscarinic receptor (12), β_2-adrenergic receptor (13, 14), glucagon receptor (15), δ-opioid receptor (16), and metabotropic glutamate receptors (17), γ-aminobutyric acid type B ($GABA_B$) receptors, has been reported (18–20). The functional implications of dimerization have not been established fully for most GPCRs including the CaR, except that the structurally related $GABA_B$ receptors recently have been shown to form functional heterodimers among their various subtypes (18–20). In this study, we demonstrate that coexpressed mutant CaRs function as a dimeric unit.

In HEK cells, coexpression of particular pairs of inactive mutant CaRs led to reconstitution of significant $[Ca^{2+}]_o$-sensing and intracellular signaling capabilities. For instance, coexpression of two CaRs carrying mutations within the ECD of one and within i3 and/or C-tail of the other resulted in substantial recovery of CaR-dependent intracellular signaling, as assessed by measurements of $[Ca^{2+}]_i$ and IPs, relative to the individual mutant CaRs, which were completely inactive or severely attenuated in their activities. However, the apparent affinities of the various coexpressed mutant CaRs for $[Ca^{2+}]_o$ (Fig. 1 and 4) were not only much less than those of wt but were also substantially different from one another, despite similar maximal activities. Moreover, the apparent cooperativities (as reflected by the Hill coefficients) of some pairs (i.e., G143E/A877stop and E297K/A877stop) differed substantially from those of other pairs with comparable maximal activities (e.g., R185Q/A877stop). Therefore, the functional reconstitutions are unlikely to have resulted from homologous recombination of the cotransfected cDNAs.

Furthermore, heterodimerization in cells cotransfected with CaRs bearing mutations within its ECD or C-tail was demonstrated directly by coimmunoprecipitating nontagged CaRs, each carrying one of the mutations, with the tagged CaR carrying the other. Analysis of surface expression of all of the mutant CaRs indicated similar levels of expression of any given mutant CaR in single and double transfections, except that the truncated CaR (A877Stop) had less surface expression in cotransfected cells than in singly transfected cells. Moreover, the extent of dimerization for mutant CaRs in the various singly and cotransfected cells also appeared similar. Therefore, heterodimers must be the principal form of the receptor contributing to the reconstituted $[Ca^{2+}]_o$ sensing and signal transduction in these cotransfected cells.

Coexpression of two mutant CaRs each with a different mutation in its ECD (e.g., G143E and E297K) did not result in recovery of intracellular signaling. Likewise, coexpression of a CaR with a mutation in i3 and another with a truncated C-tail led to no substantial recovery of function (Fig. 5*A*). In contrast, coexpression of a CaR containing a mutation in its ECD with another CaR bearing mutations in i3 and/or C-tail usually resulted in apparent gain of function (Figs. 1*B* and 5*B*). In the other words, for heterodimers containing either the truncated mutant or the i3 mutant, intracellular signaling must occur through the i3/C-tail domain of the coexpressed CaR containing a mutation in its ECD (Fig. 5*B*). Like other GPCRs (21–26), our results suggest that the CaR comprises at least two separable functional domains, one comprising the ECD and the other including the i3 and C-tail of the CaR. Possessing at least one set of intact wild-type domains in the heterodimeric complex appears to be critical for functional reconstitution. One possibility for this type of functional complementation is through domain swapping, as proposed by Gouldson and Reynolds (27).

Heterodimers made up of one CaR with a normal i3 and the other with an abnormal i3 are not as biologically active as those containing CaRs with two normal i3 segments (Fig. 5*B*), even though one of the mutant CaRs has a normal i3 and an intact C-tail (i.e., G143E). Therefore, it is likely that the abnormal i3 interacts intermolecularly with the normal i3, thereby adversely affecting signal transduction. Efficient signal transduction does not appear to require a dimeric full-length tail because coexpression of the truncated mutant with wt led to activation of cytosolic calcium responses similar to those of wt. Furthermore, cotransfection of G143E and R795W, having two intact C-tails, yielded a concentration–response curve very similar to that of G143E and R795W&A877Stop, which lacks one C-tail.

With heterodimers made up of one CaR containing a mutation in the ECD and another carrying mutations in i3/C-tail, there was typically an increase in the maximal degree of stimulation of $[Ca^{2+}]_i$ and IPs as well as a decrease in EC_{50}, suggesting an increase in ligand affinity (e.g., R185Q/A877Stop vs. R185Q). However, the EC_{50} values for cotransfections of A877Stop and various ECD mutants are significantly different from one another. Therefore, it is less likely that the wild-type ECD of the CaR with a mutation in its C-tail or i3 senses $[Ca^{2+}]_o$ as a monomer and subsequently couples to the normal i3/C-tail domains of CaRs with ECD mutations. Instead, the heterodimeric ECDs likely act as a unit, leading to EC_{50} values that are determined, in part, by the particular ECD mutant used in the cotransfection. In addition, the presence of an abnormal ECD in the heterodimeric CaRs affects the apparent cooperativity for the CaR's binding of $[Ca^{2+}]_o$ and subsequent activation of various intracellular signaling events.

The functional consequences of the inter- and intramolecular interactions between CaR monomers documented here in the heterodimeric complex are apparently much more extensive than those observed with other GPCRs, such as V2 vasopressin (22) and muscarinic receptor (24, 25). For instance, several inactive vasopressin receptors carrying single mutations in i3 or the sixth transmembrane segment could be rescued by cotransfection with a C-terminal V2 receptor peptide spanning the sequence where the various mutations occurred. In most cases, the sensitivities of the reconstituted receptors to the agonist arginine vasopressin were similar to those of the wild-type receptor. It appears that these mutated segments have little impact on the ligand-binding properties of the reconstituted receptors. This apparent discrepancy between the results observed with the CaR and the V2 vasopressin receptor may originate from the fundamental differences in their structural topologies, in particular, the sizes of their ECDs and the domains involved in agonist binding. As reported earlier, the CaR possesses a much larger ECD (consisting of more than 600 aa) (1) than that of the V2 vasopressin receptor (containing about 40 aa) (22). The $[Ca^{2+}]_o$-binding sites are believed to reside in the CaR's large ECD (28) whereas agonist binding to the V2 vasopressin receptor most probably involves residues near the extracellular ends of several transmembrane domains and within extracellular loops (29). In addition, the dimeric CaR monomers are linked intermolecularly by disulfide bonds possibly through cysteines in its ECD (30), most of which are conserved with metabotropic glutamate receptors (1). These intermolecular disulfide bonds may be sufficiently rigid to enable the structural topography and $[Ca^{2+}]_o$ sensing of one ECD and i3 to be altered by its dimeric partner.

In conclusion, our results support the hypothesis that the formation of heterodimers between two inactive mutant CaRs is the mechanism underlying reconstitution of the CaR's signaling function in cotransfected cells. In addition, abnormal functional domains within a heterodimeric CaR complex adversely affect the function of normal domains via intermolecular interactions that might contribute to the pathogenesis of some pathological conditions involving the CaR, such as familial hypocalciuric hypercalcemia. Furthermore, intermolecular interactions within the homodimeric wt likely play an important role in its function.

We thank Drs. J. Macleod and Y. Yang for critical review of our manuscript. Generous support for this work was provided by National Institutes of Health grants (DK09436 and DK54934 to M.B.; DK48330, DK41415, and DK52005 to E.M.B.; and HL42120 to S.Q.) and by The St. Giles Foundation (to E.M.B.).

1. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J. & Hebert, S. C. (1993) *Nature (London)* **366,** 575–580.
2. Ward, D. T., Brown, E. M. & Harris, H. W. (1998) *J. Biol. Chem.* **273,** 14476–14483.
3. Bai, M., Trivedi, S. & Brown, E. M. (1998) *J. Biol. Chem.* **273,** 23605–23610.
4. Pollak, M. R., Brown, E. M., Chou, Y. H., Hebert, S. C., Marx, S. J., Steinmann, B., Levi, T., Seidman, C. E. & Seidman, J. G. (1993) *Cell* **75,** 1297–1303.
5. Pollak, M. R., Brown, E. M., Estep, H. L., McLaine, P. N., Kifor, O., Park, J., Hebert, S. C., Seidman, C. E. & Seidman, J. G. (1994) *Nat. Genet.* **8,** 303–307.
6. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H. S., Pollak, M. R., Krapcho, K., Hebert, S. C. & Brown, E. M. (1996) *J. Biol. Chem.* **271,** 19537–19545.
7. Bai, M., Janicic, N., Trivedi, S., Quinn, S. J., Cole, D. E. C., Brown, E. M. & Hendy, G. N. (1997) *J. Clin. Invest.* **99,** 1917–1925.
8. Bai, M., Pearce, S. H., Kifor, O., Trivedi, S., Stauffer, U. G., Thakker, R. V., Brown, E. M. & Steinmann, B. (1997) *J. Clin. Invest.* **99,** 88–96.
9. Pearce, S. H., Bai, M., Quinn, S. J., Kifor, O., Brown, E. M. & Thakker, R. V. (1996) *J. Clin. Invest.* **98,** 1860–1866.
10. Pearce, S. H., Williamson, C., Kifor, O., Bai, M., Coulthard, M. G., Davies, M., Lewis-Barned, N., McCredie, D., Powell, H., Kendall-Taylor, P., *et al.* (1996) *New Engl. J. Med.* **335,** 1115–1122.
11. Watanabe, T., Bai, M., Lane, C. R., Matsumoto, S., Minamitani, K., Minagawa, M., Niimi, H., Brown, E. M. & Yasuda, T. (1998) *J. Clin. Endocrinol. Metab.* **83,** 2497–2502.
12. Avissar, S., Amitai, G. & Sokolovsky, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 156–159.
13. Venter, J. C. & Fraser, C. M. (1983) *Fed. Proc.* **42,** 273–278.
14. Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C. & Bouvier, M. (1996) *J. Biol. Chem.* **271,** 16384–16392.
15. Herberg, J. T., Codina, J., Rich, K. A., Rojas, F. J. & Iyengar, R. (1984) *J. Biol. Chem.* **259,** 9285–9294.
16. Cvejic, S. & Devi, L. A. (1997) *J. Biol. Chem.* **272,** 26959–26964.
17. Romano, C., Yang, W. L. & O'Malley, K. L. (1996) *J. Biol. Chem.* **271,** 28612–28616.
18. Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., Yao, W.-J., Johnson, M., Gunwaldsen, C., Huang, L.-Y., *et al.* (1998) *Nature (London)* **396,** 674–679.
19. Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., *et al.* (1998) *Nature (London)* **396,** 683–687.
20. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M. & Marshall, F. H. (1998) *Nature (London)* **396,** 679–682.
21. Schoneberg, T., Liu, J. & Wess, J. (1995) *J. Biol. Chem.* **270,** 18000–18006.
22. Schoneberg, T., Yun, J., Wenkert, D. & Wess, J. (1996) *EMBO J.* **15,** 1283–1291.
23. Ridge, K. D., Lee, S. S. & Yao, L. L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3204–3208.
24. Maggio, R., Vogel, Z. & Wess, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3103–3107.
25. Maggio, R., Vogel, Z. & Wess, J. (1993) *FEBS Lett.* **319,** 195–200.
26. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
27. Gouldson, P. R. & Reynolds, C. A. (1997) *Biochem. Soc. Trans.* **25,** 1066–1071.
28. Nemeth, E. F. (1996) in *Calcium Receptors as Novel Drug Targets*, eds. Bilezikian, J. P., Raisz, L. G. & Rodan, G. A. (Academic, San Diego), pp. 1019–1035.
29. Berthold, M. & Bartfai, T. (1997) *Neurochem. Res.* **22,** 1023–1031.
30. Fan, G. F., Ray, K., Zhao, X. M., Goldsmith, P. K. & Spiegel, A. M. (1998) *FEBS Lett.* **436,** 353–356.