The Venus’s-flytrap and Cysteine-rich Domains of the Human Ca²⁺ Receptor Are Not Linked by Disulfide Bonds*

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The extracellular N-terminal domain of the human Ca²⁺ receptor (hCaR) consists of a Venus’s-flytrap (VFT) domain and a cysteine-rich (Cys-rich) domain. We have shown earlier that the Cys-rich domain is critical for signal transmission from the VFT domain to the seven-transmembrane domain. The VFT domain contains 10 cysteines: two of them (Cys129 and Cys131) were identified as involved in intermolecular disulfide bonds necessary for homodimerization, and six others (Cys60, Cys101, Cys358–Cys395, and Cys437–Cys449) are predicted to form three intramolecular disulfide bonds. The Cys-rich domain contains nine cysteines, the involvement of which in disulfide bond formation has not been defined. In this work, we asked whether the remaining cysteines in the hCaR VFT, namely Cys236 and Cys482, form disulfide bond(s) with cysteines in the Cys-rich domain. We constructed mutant hCaRs with a unique tobacco etch virus (TEV) protease recognition site inserted between the VFT domain and the Cys-rich domain. These mutant hCaRs remain fully functional compared with the wild type hCaR. After TEV protease digestion of the mutant hCaR proteins, dimers of the VFT were identified on Western blot under nonreducing conditions. We concluded that there is no disulfide bond between the VFT and the Cys-rich domains in the hCaR.

The Ca²⁺ receptor (CaR) plays a central role in the regulation of [Ca²⁺]ₐ, homeostasis (for reviews, see Refs. 1 and 2). Activation of CaR by elevated levels of [Ca²⁺]ₐ stimulates phospholipase C via the G₄ subfamily of G-proteins, resulting in the increase of both phosphoinositide (PI) hydrolysis and the concentration of cytosolic calcium, [Ca²⁺]ₜ, The CaR mediates the inhibitory actions of [Ca²⁺]ₜ on parathyroid hormone secretion by the parathyroid gland and on Ca²⁺ reabsorption by the kidney.

The CaR is a member of family 3 of the G-protein-coupled receptor (GPCR) superfamily, which also includes metabotropic glutamate receptors (mGluRs) (3), γ-amino butyric acid type B receptors (GABA₉Rs) (4), some putative pheromone receptors (5), and some putative taste receptors (6). Their distinctively large extracellular domains (ECDs) consist of a “Venus’s-flytrap” (VFT) domain and a cysteine-rich (Cys-rich) domain with the exception of GABA₉Rs, which lack a Cys-rich domain. The three-dimensional structure of the VFT domain of mGluR1 has been determined recently by x-ray crystallography (7) showing a bilobed VFT-like structure.

The hCaR VFT contains 10 cysteines, while mGluR1 contains 9. mGluR1 forms homodimers involving an intramolecular disulfide bond through cysteine Cys₄⁰⁰ (8), whereas hCaR forms homodimers involving two intramolecular disulfide bonds through both Cys129 and Cys131 (9). The crystal structure of the mGluR1 VFT domain shows that it forms four intramolecular disulfide bonds. Based on amino acid sequence alignment, three homologous intramolecular disulfide bonds are predicted to form within the hCaR VFT, i.e. Cys⁶⁰–Cys¹⁰¹, Cys³⁵⁸–Cys³⁹⁵, and Cys⁴³⁷–Cys⁴⁴⁹ (7) (Fig. 1). The hCaR VFT has no cysteines corresponding to the disulfide-bonded pair Cys²⁸⁹–Cys²⁹¹ in mGluR1 VFT. Of the remaining two cysteines in the hCaR VFT, Cys²³⁶, which is conserved between the hCaR and mGluR1, is a free cysteine in mGluR1 VFT, whereas Cys⁴⁸² of hCaR has no counterpart in mGluR1. We reported previously that Cys²³⁶ was critical to the function of the hCaR, but Cys⁴⁸² was not (10).

We recently reported that the Cys-rich domain plays a critical role in signal transmission from the VFT domain to the 7 TM domain (11). The hCaR Cys-rich region contains nine highly conserved cysteines in a closely spaced (about 60 amino acids long) sequence (Fig. 1A). We reported previously that each of the nine cysteines in the Cys-rich domain in hCaR is critical for the receptor’s function (10). It is speculated that multiple intramolecular disulfide bonds are formed within this region, which may give rise to a tightly packed domain. However, the disulfides within the Cys-rich domain have not been characterized, and the mechanism by which an agonist signal is transmitted from the VFT domain through the Cys-rich domain to the 7 TM domain remains unknown. One possibility is that the hCaR VFT and Cys-rich domains are linked by disulfide bond(s) between Cys²³⁶ and/or Cys⁴⁸² in the VFT and one or more cysteines in the Cys-rich domain. If this were true, conformational changes in the VFT domain after agonist binding might directly cause the conformational changes of the Cys-rich domain through the disulfide bond(s) linkage. To test this hypothesis, we constructed mutant hCaRs with a tobacco etch virus (TEV) protease recognition site (for a review of TEV protease, see Ref. 12) inserted between Glu²³⁶ and Val⁴³⁷ and studied the products of TEV protease digestion of the mutant hCaRs.

MATERIALS AND METHODS

Construction of Mutant hCaRs—Site-directed mutagenesis was performed by using the Quickchange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), according to the manufacturer’s instructions. The mutagenic oligonucleotide primer pair for introducing six new residues (NLYFQG) between Glu²³⁶ and Val⁴³⁷ in the hCaR was
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5'-CCGGCTGGAAGAAGCCATGCAGG-3' and 5'-GGGCTGCCCTTCTCCTCCTCCTCAAAGGAAGGTGACCAATTCTCCTGAAAGTTCCACTCCACAGG-3'. The primer pair for introducing a 6-mer peptide sequence for six new residues (NLYFQG) between residues 214–235 of hCaR protein) and then with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD). For the His blot, a nitrocellulose membrane was incubated with rabbit polyclonal His-probe (Amersham Pharmacia Biotech). Blots shown in this paper were representatives from at least three independent experiments.

**RESULTS**

**Construction and Functional Assay of a Mutant hCaR with a Unique TEV Protease Recognition Site Inserted between Glu^{536} and Val^{537}**—Site-directed mutagenesis was applied to insert nucleotide sequence for six new residues (NLKFQG) between Glu^{536} and Val^{537} in the wild type (WT) hCaR, resulting in a unique seven-amino acid TEV protease recognition site (EN-LYFQG) (Fig. 1A). The mutant hCaR was termed hCaRT(EV).

**Immunoblotting Analysis**—Protein samples were resolved by SDS-PAGE under either nonreducing or reducing conditions (with the addition of 300 mM β-mercaptoethanol in sample buffer). The proteins on the gel were electrotransferred onto nitrocellulose membrane. For the ADD blot, the membrane was incubated with mouse monoclonal anti-hCaR antibody ADD (raised against a synthetic peptide corresponding to residues 214—235 of hCaR protein) and then with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD). For the His blot, a nitrocellulose membrane was incubated with rabbit polyclonal His-probe (Amersham Pharmacia Biotech). Blots shown in this paper were representatives from at least three independent experiments.

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**DISCUSSION**

A detailed characterization of the structure of the hCaR is critical for understanding the mechanism of receptor activation by $[\text{Ca}^{2+}]_{o}$, and the basis for activation and inactivation of the hCaR by naturally occurring mutations (1, 2). Substantial evidence suggests that $[\text{Ca}^{2+}]_{o}$ binds to the VFT domain of the...
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hCaR (11, 16, 17). Our current structural information for the hCaR VFT comes largely from site-directed mutagenesis studies and from homology modeling based on the three-dimensional structure of the recently crystallized mGluR1 VFT (7). Our site-directed mutagenesis data (9) showed that both Cys\(^{129}\) and Cys\(^{311}\) are involved in intermolecular disulfide bonds. A homology model based on the mGluR1 VFT crystal structure predicts that Cys\(^{60}\)-Cys\(^{101}\), Cys\(^{358}\)-Cys\(^{390}\), and Cys\(^{437}\)-Cys\(^{449}\) are involved in intramolecular disulfide bonds within the hCaR VFT. Cys\(^{236}\) and Cys\(^{462}\) are the remaining cysteines in the hCaR VFT that have not been accounted for. It is unclear whether they are free cysteines in the hCaR. The Cys-rich domain in the hCaR ECD contains nine highly conserved cysteines in an \(-\)60-amino acid-long sequence. Multiple intramolecular disulfide bonds are speculated to form within this region, but none of the disulfides has been defined yet. Given the odd number of cysteines in this domain, at least one of them should be free or disulfide-linked to a cysteine in other domains.

We recently reported that the Cys-rich domain in the hCaR is critical for signal transmission from the VFT to the 7 TM domains (11). But a key question remains unanswered: how are the VFT and Cys-rich domains structurally and functionally coordinated during agonist binding and activation of the receptor? The crystal structure determined for the mGluR1 VFT domain does not address this question, because the Cys-rich domain was not included in the mGluR1 construct expressed and crystallized (7). One possible mechanism for communication between the VFT and Cys-rich domains could involve disulfide linkage. Conformational changes of the VFT after agonist binding would then cause conformational changes in the Cys-rich domain through disulfide(s) linkage between the two domains.

It is noteworthy that the hCaR has two cysteines (Cys\(^{677}\) and Cys\(^{765}\)) in the extracellular loops 1 and 2 of the 7 TM domain. However, they are less likely to pair with any cysteines in the ECD, because the two conserved cysteines in extracellular loops 1 and 2 are known to be linked by a disulfide in most GPCRs including bovine rhodopsin, the thyrotropin-releasing hormone receptor, the thromboxane receptor, the GnRH receptor, and many others (18).

To determine whether the VFT is linked to the Cys-rich domain by disulfide bond(s), we inserted a unique TEV protease recognition site between the two domains. To date, it remains unclear where the exact boundary is between the two domains. A construct of the mGluR1 VFT made by Tsuji and co-workers (7, 8) that ends at Ser\(^{522}\), two amino acids ahead of the Cys-rich domain, the TEV protease digestion product of hCaR(TEV-HIS) shows that the His antibody recognizes the site inserted in the hCaR(TEV) should remain as a holoprotein under nonreducing conditions. If the VFT is disulfide-linked to the Cys-rich domain, the TEV protease digestion product of hCaR(TEV-HIS) shows that the His antibody recognizes the protein with a C-terminal His tag but not the VFT released from the holoprotein, confirming that the hCaR VFT is not disulfide-linked to the Cys-rich domain. The absence of disulfide linkage between the VFT and other domains of the receptor might be common among family 3 members of GPCR, as GABA\(_{A}\) receptors lack a Cys-rich domain altogether.

In summary, our results exclude the existence of a disulfide bond between the hCaR VFT and Cys-rich domains. We cannot, however, exclude noncovalent interactions between these two domains, and indeed such interactions could be important in the mechanism of activation of the receptor. We attempted to determine whether the hCaR VFT and Cys-rich domains are associated by noncovalent interactions by analyzing the products of TEV protease digestion under native gel conditions. Unfortunately, perhaps because of its high degree of glycosylation, the hCaR is very poorly resolved under native gel conditions, making it difficult to draw definitive conclusions using this method. Further study of the structure of both the VFT and Cys-rich domains will help to reveal the mechanism by which the signal of Ca\(^{2+}\)-induced conformational changes in the VFT is transmitted through the Cys-rich domain to the 7 TM domain, resulting in CaR activation.

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