Three Adjacent Serines in the Extracellular Domains of the CaR Are Required for L-Amino Acid-mediated Potentiation of Receptor Function*

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The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) is a key player in Ca\(^{2+}\) homeostasis. The activity of CaR can be potentiated by various L-amino acids. In this study, we examined whether conserved amino acid residues in the binding of glutamate to metabotropic glutamate receptors (mGluRs) also participate in the potentiation of the activity of CaR by l-phenylalanine. Ser-170 corresponding to Thr-188 in rat mGluR1a appears to be important for the modulating actions of phenylalanine. In the presence of phenylalanine, a mutant CaR with a single mutation S170A showed a significant decrease in its EC\(_{50}\) for stimulation by Ca\(^{2+}\) and a modest increase in its maximal activity. In addition, mutating Ser-169 and Ser-171 together with Ser-170 yielded a more complete block of the phenylalanine modulation than did the single mutation. The presence of the triple mutation, S169A/S170A/S171A, also eliminated phenylalanine potentiation of the activities of heterodimeric receptors in which one of the monomeric receptors had intact triple serines (A877STOP). The putative amino acid binding site of the CaR is probably close to or structurally dependent on the Ca\(^{2+}\) binding sites of the receptor, because mutant CaRs with mutations in the putative amino acid binding site exhibited severely reduced responses to Ca\(^{2+}\).

The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) plays a key role in mineral ion homeostasis by sensing small perturbations in the level of Ca\(^{2+}\) and modulating the functions of parathyroid and kidney so as to restore Ca\(^{2+}\) to its normal level. The CaR forms disulfide-linked dimers (1) and can be activated allosterically by L-amino acids in the presence of Ca\(^{2+}\) in the millimolar concentration range of 0.1–10 mM with a preference for aromatic and small aliphatic L-amino acids (2). Moreover, these amino acids stereoselectively enhance the sensitivity of the CaR to its agonists including Ca\(^{2+}\) and spermine. The CaR is widely distributed in the upper gastrointestinal tract including gastrin-secreting G cells and may mediate physiological responses including the release of gastric acid and pancreatic enzymes upon the ingestion of protein and free amino acids in the diet (3). In addition, the CaR may serve as a fundamental link between protein and Ca\(^{2+}\) metabolism. It has been documented that these two homeostatic systems are intimately related. For instance, a reduction in protein intake below the normal level results in secondary hyperparathyroidism in the context of normocalcemia (4), and high dietary protein intake induces elevated urinary calcium excretion (5, 6).

The CaR is a G protein-coupled receptor in the same subfamily C as the metabotropic glutamate receptors, mGluRs1–8 (7–9). The family C receptors all possess unusually large 500–600-residue extracellular amino-terminal domains (ECDs), possibly with a bilobed Venus flytrap structure similar to that found in bacterial periplasmic binding proteins for nutrients (10, 11). The family C receptors interact with their physiological agonists through their ECDs (11–13).

For the rat homolog of mGluR1a, the key residues involved in the binding of glutamate to its ECD have been identified through the determination of its crystal structure when it is complexed with glutamate (11). Ser-164, Ser-165, Thr-188, and Glu-292 form hydrogen bonds with the \(\alpha\)-carboxyl group in glutamate. Five residues, Ser-186, Thr-188, Asp-208, Tyr-236, and Asp-318, form additional hydrogen bonds with the \(\alpha\)-amino group of glutamate. Of these residues, Ser-165, Thr-188, Asp-208, Tyr-236, and Asp-318 in mGluR1a are conserved in the CaR, corresponding to Ser-147, Ser-170, Asp-190, Tyr-218, and Glu-297, respectively. The \(\gamma\)-carboxyl group in glutamate forms one hydrogen bond each with Lys-409 and Arg-323 in mGluR1a; however, these two positively charged amino acid residues are not conserved in the CaR. Mutations S165A and T188A have been reported to impair glutamate binding and resultant activation of mGluR1a (10), whereas the functional significance of other residues in the glutamate binding site of the mGluRs has not been reported.

Previous studies of the CaR have shown that substitutions of amino acids such as Ser-147 and Ser-170, which correspond to Ser-165 and Thr-188 in mGluR1a, markedly reduce activation of the receptor by Ca\(^{2+}\) (12, 13). In addition, the mutations of Tyr-218 and Glu-297 in the CaR that correspond to Tyr-236 and Asp-318 in mGluR1a produce familial hypocalciuric hypercalcemia (FHH) in humans (14). Receptors harboring these two mutations are not responsive to physiological concentrations of Ca\(^{2+}\). Therefore, 4 of the 5 residues in the CaR conserved with...
the mGlus appear important for the responsiveness of the CaR to its physiological agonist Ca\(^{2+}\).

In the present studies, we tested the hypothesis that the CaR is modulated by \(\text{\textalpha}-\text{amino acids through a putative binding site similar to the glutamate binding site in the ECD of mGluR1a.}\) We examined the roles of these conserved amino acids in mediating the action of the \(\text{\textalpha}-\text{amino acid phenylalanine.}\) We found that one of the conserved residues, Ser-170, is critical for functional modulation of the CaR by phenylalanine.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Site-directed mutagenesis employed the approach described by Kunkel (10) to produce mutated receptors in which one or more serine residues at positions 147 and 169–171 as well as Asp-190 and Tyr-218 in the ECD of the human CaR were mutated to alanines. The dent-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). The single-stranded DNA was then annealed to a mutagenesis primer that contained the desired nucleotide change encoding single or multiple point mutations 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. DH5\(_\alpha\) or DH10B competent cells were transformed with these DNA heteroduplexes, and the incorporation of the desired mutations was confirmed by sequencing the mutated cassettes. The resultant mutated cassettes were cloned into the FLAG-tagged receptor in pcDNA3 (Invitrogen) as described previously (7).

**Site of Allosteric Activation of the CaR by L-Amino Acids**

**Detection of Expressed CaR**—Before whole cell lysates were prepared, intact HEK293 cells transiently transfected with FLAG-tagged CaR were labeled with 1 mM ImmunoPure Sulfo-NHS-Biotin (Pierce), a membrane-impermeable biotinylation reagent as described previously (1). The surface-biotinylated HEK293 cells were solubilized, and the FLAG-tagged receptor was immunoprecipitated with anti-FLAG M2 antibody (Sigma). The immunopurified species were subsequently eluted and subjected to SDS-PAGE (12) using a linear gradient of polyacrylamide (5–10%). 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).

**Measurement of Ca\(^{2+}\), by Fluroimetry in Cell Populations**—Cover slips 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 CaCl\(_2\), 1 mM MgSO\(_4\), 1 mM NaH\(_2\)PO\(_4\), 0.1% bovine serum albumin, and 0.1% dextrose and then washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.1% dextrose, 0.1% bovine serum albumin) at 37°C for 20 min. The coverslips were then placed diagonally in a thermostated quartz cuvette that contained the bath solution using a modification of the technique employed previously in this laboratory (13). Phenylalanine was added to the bath solution and preincubated for 50 s. Concentrated Ca\(^{2+}\), at 100 mM or 1 mM was added stepwise to an experimental solution containing 0.5 mM Ca\(^{2+}\) to achieve the following concentrations: 1.5, 2.5, 3.5, 4.5, 5.5, 10, 15, 20, 30, 40, and 50 mM. Because phenylalanine was not included in the concentrated Ca\(^{2+}\), solutions, the initial phenylalanine concentration was sequentially diluted by the 11 additions of Ca\(^{2+}\), from 9.9 to 9.13 mM. Excitation monochrometers were centered at 340 and 380 nm, and emission light was collected at 510 ± 10 nm through a wide band emission filter. The 340/380 excitation ratio of emitted light was used to estimate Ca\(^{2+}\) as described previously (13).

**Statistics**—The activities of the wild-type and mutant CaRs were determined in response to increasing concentrations of Ca\(^{2+}\), in the presence or absence of phenylalanine. The mean responses at various concentrations of Ca\(^{2+}\) were calculated from individual experiments and were expressed with the standard error of the mean (±S.E.) as the index of dispersion. The comparison of responses of various receptors at 50 mM Ca\(^{2+}\) was performed using ANOVA or Duncan’s multiple comparison test (\(p < 0.05\)) (8). Each of the experiments presented in the results was performed at least three times. To calculate the EC\(_{50}\) value and the maximal response, we used the MacFitCurve program and the Hill equation, F(X) = aFmax(1 + X/h) \(^\alpha\) where “F” is function, “a” is maximal response, “e” is EC\(_{50}\) and “h” is Hill coefficient to fit the experimental data.

**RESULTS**

Fig. 1. Sequence alignment of segments of the CaR and mGlur1a. These segments are in the extracellular domain of each receptor. The aligned identical residues are shaded in the figure. The five conserved residues in the putative amino acid binding site are marked with filled dots. The three non-conserved residues located in the glutamate binding site in the mGlur1a are marked with open dots.
as 50 mM. In contrast, the wild-type receptor reached its maximal response at 20 mM Ca\(^{2+}\). Moreover, the responses of these two mutants at 50 mM Ca\(^{2+}\) were approximately 13 and 22%, respectively, of that of the wild-type receptor. Pretreatment with 10 mM phenylalanine increased the magnitude of the high Ca\(^{2+}\) (50 mM) elicited responses of these mutant receptors by 2.3- and 3.5-fold, respectively, for Y218S and E297K (Fig. 2 and Table I). Likewise, the activity of the mutant receptor carrying R185Q, another FHH mutation located outside of the putative amino acid binding site, was also significantly increased by pretreatment of phenylalanine (Fig. 2D and Table I). These results suggest that Glu-297 and Tyr-218 are not crucial for the actions of phenylalanine on the CaR.

To examine whether other conserved residues in the glutamate binding site, namely Ser-147, Ser-170, and Asp-190, play roles in the modulation of the CaR by phenylalanine and to better test Tyr-218, these residues were mutated to alanines, and the resultant mutant receptors were tested to determine their responsiveness to phenylalanine. As shown in Fig. 3, all of these mutations substantially attenuated CaR-mediated Ca\(^{2+}\) responses. 10 mM phenylalanine significantly increased the activation of the CaR in each case with S170A showing the smallest increase (Fig. 3B). In addition, S147A, D190A, and Y218A each exhibited a significant increase in its sensitivity to Ca\(^{2+}\) as indicated by a decline in the EC\(_{50}\) value in the presence of L-Phe (Table I). However, there was no significant decrease in the EC\(_{50}\) for the S170A substitution. These results suggested that replacement of serine at position 170 significantly compromised the interaction of the CaR with phenylalanine.

In the CaR, Ser-170 is flanked by two additional serines at positions 169 and 171 with Ser-171 also being conserved in mGluR1a. Consistent with a previous report (9), the mutation S171A significantly reduced the affinity of the CaR to Ca\(^{2+}\) but not its maximal response, whereas the mutation S169A only slightly decreased the maximal response (Table I). Phenylalanine potentiated both S169A and S171A (Table I). We tested whether multiple alanine substitutions of serines at positions 169 and 171 in addition to S170A would further reduce the responsiveness of the CaR to phenylalanine when combined as double or triple mutations. S170A/S171A, S169A/S170A, and S169A/S170A/S171A each further attenuated the
CaR-mediated Ca$^{2+}$ responses (Fig. 4 and Table I). These combinations of mutations markedly reduced the responsiveness of the receptor to 10 mM phenylalanine (Fig. 4 and Table I). In fact, the activity of S169A/S170A/S171A is slightly lower in the presence of phenylalanine than in its absence (Fig. 4C). Our results suggest that these three serines cooperatively interact with phenylalanine.

Like many other ECD mutants such as E297K, the responsiveness of S169A/S170A/S171A to Ca$^{2+}$, could be reconstituted by co-transfecting with the inactive truncated receptor A877Stop (Fig. 5 and Table I), suggesting that the intracellular domains of the triple serine mutant were fully functional. However, the heterodimer formed in cells co-transfected with S169A/S170A/S171A and A877Stop did not respond to 10 mM phenylalanine (Fig. 5B and Table I), suggesting that the mutant receptor containing these three serines replaced by alanines has a dominant negative effect on the responsiveness to phenylalanine of its dimeric partner. In contrast, the heterodimer formed in cells co-transfected with E297K and A877Stop responded to 10 mM phenylalanine (Fig. 5A and Table I).

We also examined two additional mutant receptors, G143E and G549R, which do not respond to Ca$^{2+}$ at concentrations as high as 50 mM (Table I). Like R185Q, these mutations are in the ECD but not in the putative amino acid binding site. Both residues are conserved in mGluR1a with the residue corresponding to Gly-143 located close to the glutamate binding site in mGluR1a, whereas the residue corresponding to Gly-549 is not. This latter residue was excluded from the preparation of mGluR1a used for x-ray structural determination by Kunishima et al. (11), suggesting that this residue in mGluR1a is not essential for glutamate binding. However, these two mutant receptors, G143E and G549R, remained totally inactive even in the presence of 10 mM phenylalanine (Table I). It appears that Ca$^{2+}$ responsiveness may be required for the action of phenylalanine on the receptor.

We next tested whether phenylalanine responsiveness could be reconstituted by co-transfecting these inactive ECD mutants with an inactive cytoplasmic tail-truncated receptor A877Stop containing a normal ECD. As shown in Fig. 6, A and B, high Ca$^{2+}_{\text{o}}$ elicited substantial Ca$^{2+}$ responses in cells co-transfected either with G143E and A877Stop (G143E&A877Stop) or with G549R and A877Stop (G549R&A877Stop). 10 mM phenylalanine had only a minimal effect on these heterodimers. The maximal responses were slightly increased, whereas their EC$_{50}$ values were slightly decreased (Table I). Therefore, in these two types of heterodimeric receptors, phenylalanine responsiveness is poorly restored. As a result, phenylalanine responsiveness of both monomers may be required for the action of phenylalanine on the resultant heterodimer.

Besides the mutations in the ECD, we also examined the effect of R795W, an FHII mutation in the third intracellular loop, on phenylalanine-mediated potentiation of the CaR. As reported in our previous study (14), the mutant receptor R795W has substantially attenuated Ca$^{2+}$ responses (Fig. 7A and Table I). The mutation presumably blocks the receptor from activating its cognate G proteins since its activity can be reconstituted by co-transfecting with inactive receptors having mutations in the ECD. Consistently, the mutant receptor was not significantly more active in the presence of 10 mM phenylalanine than in its absence. To determine whether R795W would facilitate phenylalanine modulation when present as one of the monomeric subunits in a heterodimeric receptor, we co-transfected R795W with E297K (responsive to phenylalanine) or with G143E (unresponsive to phenylalanine). Co-transfection of R795W and E297K or R795W and G143E each substantially reconstituted receptor responsiveness to Ca$^{2+}$. In addition, E297K & R795W responded to phenylalanine with a substantial increase in maximal response and a 30% decrease in EC$_{50}$ (Fig. 7B and Table I). These results suggest that R795W may participate in the binding of Ca$^{2+}$, as well as the binding of phenylalanine. In contrast, R795W & G143E (Fig. 7C) did not respond to phenylalanine. Again, G143E showed a dominant negative effect on the response of the CaR.
resultant heterodimeric receptor to phenylalanine in co-transfected cells.

Western analysis showed that the mutations in the receptors constructed in this study had little effect on the cell surface expression of the receptor with the exception of S169A/S170A/S171A in which a reduction in surface expression was observed (Fig. 8A). However, the reduction in receptor expression is not the cause for the significant reduction in the response of S169A/S170A/S171A to Ca\(^{2+}\)/H\(_{11001}\) and phenylalanine, because S169A/S170A/S171A in cells co-transfected with A877Stop had a 4.5-fold higher Ca\(^{2+}\)/H\(_{11001}\) response at 50 mM Ca\(^{2+}\)/H\(_{11001}\) than that in singly transfected cells. As shown in Fig. 8B, the receptor in co-transfected cells had a level of expression similar to that in singly transfected cells.

**DISCUSSION**

The finding that the CaR can function as a stereoselective receptor for aromatic and other L-amino acids draws it closer functionally to the mGluRs than previously considered (2). The mGluRs are activated by the negatively charged amino acid glutamate at concentrations in the submillimolar range and can also be activated by millimolar concentrations of Ca\(^{2+}\)/H\(_{11001}\) (16). The CaR can be activated by Ca\(^{2+}\)/H\(_{11001}\) and L-amino acids (2); however, it requires the presence of greater than one millimolar concentrations of Ca\(^{2+}\)/H\(_{11001}\) in the bath solution for L-amino acids to be effective. In addition, the CaR responds to a large number of L-amino acids, exhibiting a preference for aromatic L-amino acids but with substantially lower affinities than those found with the mGluRs for glutamate.

The glutamate binding sites in the mGluRs have been located in their ECDs (11, 17, 18). Five residues (Ser-165, Thr-188, Asp-208, Tyr-236, and Asp-318) in rat mGluR1a, which are in direct contact with \(-\alpha\)-amino and \(-\alpha\)-carboxyl groups in glutamate, are conserved in the CaR (Ser-147, Ser-170, Asp-190, Tyr-218, and Glu-297) (11). We found that one of them, Ser-170 (corresponding to Thr-188), is critical for mediating functional modulation of the CaR by phenylalanine. With added phenylalanine, the CaR with a single mutation S170A shows no significant change in its EC\(_{50}\) for...
Ca\(^{2+}\) stimulation and less than a 1.6-fold increase in its response at 50 mM Ca\(^{2+}\). In addition, double (S169A/S170A and S170A/S171A) and triple mutations (S169A/S170A/S171A), which include S170A, demonstrated little change in the activity of the CaR in the presence of phenylalanine. These results suggest that modulatory l-amino acids may interact with the CaR in a location similar to the binding site of glutamate in the mGluRs.

Unlike Ser-170, the four other conserved residues, Ser-147, Asp-190, Tyr-218, and Glu-297, do not appear to be important for phenylalanine binding, because single residue replacements do not reduce phenylalanine modulation of the CaR. However, we have not examined mutational combinations of these residues. It is possible that a single mutation may not result in a substantial change in receptor function despite its involvement in phenylalanine binding. The studies of the mGluRs do not provide a guide to the interpretation of our studies on receptor function. Most residues in the glutamate binding sites of the mGluRs, which have been identified by the crystal structure of mGluR-glutamate complexes, have not been functionally tested to understand the impact that single mutations have on agonist binding. The putative amino acid binding site of the CaR may lie in close proximity to key Ca\(^{2+}\) binding sites in its ECD, because alteration of the activity of the CaR in the presence of phenylalanine.

FIG. 5. Substitution of triple serines at positions 169 to 171 in one of monomeric subunits within a heterodimeric complex blocks phenylalanine-mediated functional potentiation of the heterodimer. In HEK293 cells, a cytoplasmic tail-truncated receptor A877Stop was co-transfected with one of the ECD mutants E297K (A) and S169A/S170A/S171A (B). As described in Fig. 1, Ca\(^{2+}\)-evoked Ca\(^{2+}\) responses were measured in the presence (triangle) or absence (circle) of 10 mM phenylalanine and normalized to the maximal cumulative Ca\(^{2+}\) responses of the wild-type receptor in untreated cells. Each point is the mean value of the number of measurements indicated in Table I. Means ±S.E. are indicated with vertical bars through each point. Some error bars are smaller than the symbol. The data were fitted using MacCurveFit as described under “Experimental Procedures.”
numerous residues in this region substantially attenuates
the response of the receptor to Ca$^{2+}$/H11001. For instance, mutant
receptors carrying the substitutions S147A, S170A, D190A,
Y218S, Y218A, and E297K exhibit no responses to physiolog-
ical concentrations of Ca$^{2+}$/H11001 (under 5 mM), and the apparent
affinities of the receptors for Ca$^{2+}$/H11001 are decreased by more
than 6-fold.

In this study, we found that phenylalanine potentiates the
maximal responses of many mutant receptors but does not do
so for the wild-type receptor. In some cases, phenylalanine not
only increases the maximal responses of the mutant receptors
but also decreases their EC$_{50}$s. For instance, S147A has its
maximal response increased by 1.75-fold and has its EC$_{50}$
decreased by 0.55-fold. In the case of other mutants, phenylala-
nine only increases the maximal responses without affecting
the EC$_{50}$s. For instance, S170A has its maximal response in-
creased by 1.6-fold with its EC$_{50}$ essentially unchanged. Inter-

FIG. 6. Phenylalanine-mediated functional potentiation of
heterodimeric receptors formed in cells co-transfected with the
mutant receptors G143E and A877Stop or G549R and A877Stop.
HEK293 cells were co-transfected with the G143E and A877Stop (A) or
G549R and A877Stop (B). As described in Fig. 1, Ca$^{2+}$/evoked Ca$^{2+}$/
responses were measured in the presence (triangle) or absence (circle) of
10 mM phenylalanine and normalized to the maximal cumulative Ca$^{2+}$/
responses of the wild-type receptor in cells not treated with phenylala-
nine. Each point is the mean value of the number of measurements
indicated in Table I. Means ±S.E. are indicated with vertical bars
through each point. Some error bars are smaller than the symbol. The
data were fitted using MacCurveFit as described under “Experimental
Procedures.”

FIG. 7. The mutation R795W in the third intracellular loop of
the CaR blocks phenylalanine-mediated functional potentiation
when transfected alone or co-transfected with one of ECD mu-
tants. In HEK293 cells, R795W was transfected alone (A) or co-trans-
fected with one of the ECD mutants, G143E (B) and E297K (C). As
described in Fig. 1, Ca$^{2+}$/evoked Ca$^{2+}$/ responses were measured in the
presence (triangle) or absence (circle) of 10 mM phenylalanine and
normalized to the maximal cumulative Ca$^{2+}$/ responses of the wild-type
receptor in untreated cells. Each point is the mean value of the number
of measurements indicated in Table I. Means ±S.E. are indicated with
vertical bars through each point. Some error bars are smaller than the
symbol. The data were fitted using MacCurveFit as described under
“Experimental Procedures.” The plot in the inset is the same as that in
the corresponding figure but in its full scale.
estingly, the potentiating effects of phenylalanine on the maximal response and EC_{50} of a mutant CaR appear to be independent, depending on the particular mutation examined. However, at present we have no molecular explanation for this phenomenon.

With the notable exception of Ser-170, the residues that support phenylalanine-mediated potentiation of the CaR appear to be distinct from those that directly contact glutamate in mGluRs. For instance, Gly-143 in the CaR corresponds Gly-161 in mGluR1a, which is situated in loop 1 close to the glutamate binding site but not part of this site. In the CaR, the G143E substitution has been identified in individuals with FHH, and in vitro studies show that a CaR harboring this mutation is completely inactive in response to Ca^{2+}. (14). The glycine residue has no side chain per se and is unlikely to directly interact with Ca^{2+} or l-amino acids. However, glycine residues are often important for the overall structure of a protein as they introduce structural breaks in a-helices or b-sheets. Thus, the replacement of a glycine residue could severely disturb the structure of the protein and affect its stability. Indeed, we found that G143E is expressed at much lower levels on the cell surface than the wild-type receptor (14, 19), and this mutation has diminished responsiveness to phenylalanine. Thus, one mechanism for diminishing the modulation of the receptor by phenylalanine could be through indirect disturbance of the structure of the amino acid binding site in the receptor.

Our previous studies have shown that the CaR exists mainly as a dimer on the cell surface of the CaR-transfected HEK293 cells and that this dimer is covalently linked by disulfide bonds (4). Importantly, we have previously demonstrated the presence of domain complementation in certain heterodimeric receptors that can exhibit substantial recovery of function when the dimer is formed (4). We recently have shown that domain complementation is closely linked to the presence of many different mutations in the CaR (2). The glycine residue could severely disturb the structure of the protein and affect its stability. Indeed, we found that G143E is expressed at much lower levels on the cell surface than the wild-type receptor (14, 19), and this mutation has diminished responsiveness to phenylalanine. Thus, one mechanism for diminishing the modulation of the receptor by phenylalanine could be through indirect disturbance of the structure of the amino acid binding site in the receptor.

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In conclusion, phenylalanine binds to the CaR in its ECD. At least one residue, Ser-170, homologous to a conserved residue in the glutamate binding site of mGluR1a, appears critical for this CaR modulation by phenylalanine. Both monomeric subunits of the dimeric receptor appear to be required for the full phenylalanine effect on the activation of the CaR by its physiological agonist, Ca^{2+}. Phenylalanine retains its ability to potentiate receptor activation in the presence of many different mutations in the CaR, as well as Ca^{2+} sensitivity for many FHH-inducing mutations. Thus, plasma amino acid levels might limit the severity of some FHH phenotypes in vivo.

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