A Double Mutation in the Extracellular Ca\textsuperscript{2+}-sensing Receptor’s Venus Flytrap Domain That Selectively Disables L-Amino Acid Sensing*

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The extracellular Ca\textsuperscript{2+}-sensing receptor is activated allosterically by L-amino acids, and recent molecular analysis indicates that amino acids are likely to bind in the receptor’s Venus flytrap domain. In the current study we set out to identify residues in the VFT domain that specifically support amino acid binding and/or amino acid-dependent receptor activation. Herein we describe two mutations of the Ca\textsuperscript{2+}-sensing receptor (CaR) Venus flytrap domain, T145A and S170T, that specifically impair amino acid sensing, leaving Ca\textsuperscript{2+} sensing intact, as determined by receptor-dependent activation of intracellular Ca\textsuperscript{2+} mobilization in fura-2-loaded HEK293 cells. With respect to the wild-type CaR, T145A and S170T exhibited reduced sensitivity to L-Phe, and T145A also exhibited markedly impaired L/D selectivity. When combined, the double mutant T145A/S170T exhibited normal or near-normal sensitivity to extracellular Ca\textsuperscript{2+} but was resistant to L-Phe at concentrations up to 100 mM. We conclude that T145A/S170T selectively disables L-amino acid sensing and that the Ca\textsuperscript{2+} and L-amino acid-sensing functions of the CaR can be dissociated.

This conclusion has recently been confirmed by chimeric receptor analysis showing that the CaR VFT domain, but none of its other domains, is required for L-amino acid sensing (8).

In the current work we set out to identify residues of the CaR VFT domain that are specifically required for L-amino acid binding and/or amino-dependent receptor activation. As a starting point we used an alignment of the VFT domains of the human CaR and rat metabotropic glutamate receptor type-1 (mGlu-1), for which high resolution crystal structures are available (9). A previous analysis of this type indicated that mutations of conserved binding domain residues in the CaR VFT domain interfered with Ca\textsuperscript{2+} and, in some cases, amino acid sensing (10). In an effort to distinguish between the requirements of amino acid and Ca\textsuperscript{2+} sensing, we have now reinvestigated the impact of conservative mutations on CaR residues Ser-147, Ser-170, Tyr-218, and Glu-297, which align to the key mGlu-1 glutamate binding residues Ser-165, Thr-188, Tyr-236, and Asp-318 respectively. In addition, we investigated the impact of site-directed mutagenesis on a closely associated CaR residue Thr-145, which might conceivably fulfill a role similar to that of mGlu-1 residue Ser-164, which contributes to the H-bond network that ligates the α-carboxyl group of the bound glutamate. This analysis led to the identification of two mutations, T145A and S170T, that selectively impaired amino acid sensing by the CaR, leaving sensing of extracellular Ca\textsuperscript{2+} ions intact. Combining these two mutations had the effect of disabling receptor sensitivity to the CaR-active amino acid L-Phe up to a concentration of 100 mM. These findings support the concept that the CaR L-amino acid binding site lies in its VFT domain and demonstrate that Ca\textsuperscript{2+} and amino acid sensing can be dissociated.

**EXPERIMENTAL PROCEDURES**

MATERIALS—pcDNA3.1(+) (Invitrogen) containing the wild-type human calcium-sensing receptor (cassette version (11)) was the kind gift of Dr. Mei Bai and Professor Edward Brown (Endocrine-Hypertension Division and Membrane Biology Program, Brigham and Women’s Hospital, Boston, MA).

HEK293 Cell Culture—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (GSL, Parkville, Victoria, Australia) and 0.5% (v/v) penicillin/streptomycin solution (Invitrogen) under standard conditions (5% CO\textsubscript{2}, 37 °C).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the Stratagene (La Jolla, CA) QuikChange™ kit according to the manufacturer’s instructions. Briefly, a pair of complementary primers of 25–35 bases was designed for each mutagenesis with the mutation placed at the middle of the primers (the sequences of the primers are available from authors on request). The template human CaR in pcDNA3.1(+) was amplified using Pfu DNA polymerase (Stratagene) with these primers for 12–16 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the template DNA with
DpnI (New England Biolabs) the amplified mutant DNA was transformed into Escherichia coli DH5α or XLI-Blue. The incorporation of the desired mutations and absence of other mutations were confirmed by automated DNA sequencing (Australian Genome Research Facility, Brisbane, Queensland, Australia).

Transient Transfection of Wild-type and Mutant Receptors in HEK293 Cells—Transient grade DNA was prepared using the High-speed™ Midi Kit (Qiagen, Melbourne, Victoria, Australia). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. When the cells reached 90–95% confluence, they were transfected with wild-type or mutant CaR constructs using Lipofectamine 2000™ (Invitrogen). A given amount of plasmid DNA was diluted in Dulbecco’s modified Eagle’s medium, mixed with diluted Lipofectamine 2000™, and allowed to complex at room temperature for 20 min before being added to HEK293 cells in plates. For fura-2 assays in the microscope format, transfections were performed in 6-well plates using 2–2.5 μg of DNA per well. For autoradiography assays (‘Detection of Changes in Cytoplasmic Free Ca2⁺ Concentration’), 150 ng of each expression construct was co-transfected with 150 ng of the pcÆQ reporter plasmid in 96-well plates as described previously (12).

FLAG Tagging of Human CaR Constructs—The human CaR in pcDNA3.1 was FLAG-tagged using the site-directed mutagenesis procedure using complementary mutagenic oligonucleotides that contained the FLAG peptide epitope (YKDDDDK) as an insert. The mutagenic primers were designed to insert the FLAG tag between CaR amino acid residues 371 and 372. This site was selected after Bai et al. (13) and lies in a loop of the VFT domain that does not contribute significantly to ligand binding or receptor activation (14). The sequencing of the mutagenic primers (with the complementary sequences encoding the FLAG tag in italics and underlined) was performed on an Applied Biosystems 310 DNA sequencer.

Western Blot Analysis of Wild-type and Mutant CaR Surface Expression in HEK293 Cells—HEK293 cells were cultured in 24-well plates and transiently transfected with a FLAG-tagged CaR construct using Lipofectamine 2000™ (Invitrogen) as described above. Proteins on the surface of the transfected cells were labeled with 50 μl of NHS-Biotin per 5 × 10⁶ cells for 30 min at 4 °C before lysis with a solution that contained 20 mM Tris-HCl, 150 mM NaCl, 25 mM NaF, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 50 mM β-glycerophosphate (all Sigma), protease inhibitors (Roche Applied Science), and 100 mM iodoacetamide (Sigma). The FLAG-tagged CaR was immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma) according to the manufacturer’s instructions. The immunopurified protein samples were then eluted in SDS sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue), heated to 65 °C for 30 min, and subjected to SDS-PAGE (4% stacking gel, 6% resolving gel). Equivalence of protein loading was checked using the DC protein assay (Bio-Rad). The proteins were then transferred to a nitrocellulose membrane by Western blotting using a Hoefer TE 70 Series SemiPhor semi-dry transfer apparatus (Amersham Biosciences). CaR proteins present on the cell surface (biotin labeled as above) were then detected using an avidin-horseradish peroxidase conjugate (Bio-Rad) followed by ECL Plus Western-blotting detection reagents (Amersham Biosciences). Films were developed using Eastman Kodak Co. (Cedex, Coburg, VIC) GBX developer and fixer reagents.

Stable Expression of Wild-type and Mutant Receptors in HEK293 Cells—HEK293 cells were maintained in 25-cm² culture flasks and transfected with 8 μg of the wild-type or mutant CaR expression constructs using Lipofectamine 2000™ according to the manufacturer’s instructions (Invitrogen). After 24 h cells were transferred to 24-well plates and grown for a further 24 h. Selection of stable transfectants was then carried out in the presence of 50–100 μg/ml hygromycin (Invitrogen) or 100–400 μg/ml Geneticin (Invitrogen). Individual resistant clones were isolated 3 weeks later and screened by aquorin luminescence for activity and subsequently studied by microfluorimetry using fura-2.

Detection of Changes in Cytoplasmic Free Ca²⁺ Concentration—The aquorin luminescence assay used to screen for HEK293 cell clones that stably expressed the wild-type or mutant CaRs was performed as described previously using 96-well plates in a thermostat-controlled Wallac Victor² multilabel counter at 37 °C (8, 12). For analysis of amino acid- and Ca²⁺-dependent activation of the wild-type or mutant CaRs, changes in cytoplasmic free Ca²⁺ concentration were determined by microfluorimetry after loading with fura-2 AM. HEK293 cells that had been transfected with the wild type or one of the mutant CaR constructs were cultured on glass coverslips in Dulbecco’s modified Eagle’s medium, 10% foetal bovine serum in 6-well plates for 48 h. They were then loaded with fura-2 AM (5 μM, 37 °C) in physiological saline solution containing 1 mM CaCl₂ and 1 mg/ml bovine serum albumin. After 2 h the fura-2-containing solution was removed, and the cells were resuspended in physiological saline solution for 30 min at 37 °C. Fura-2 loaded cells were transferred into a superfusion chamber and placed in the light path of a Nikon Diaphot microscope. The control superfusion solution had the following composition: 125 mM NaCl, 4.0 mM KCl, 0.2 or 0.5 mM CaCl₂, 0.5 mM MgCl₂, 20 mM HEPES (NaOH), 0.1% D-glucose, pH 7.4. Excitation at alternating wavelengths (340 and 380 nm), detection of fluorescent light (F₀, peak 510 nm) and its digitized recording using Acqknowledge software for the Macintosh was performed as described previously (15). Data for cytoplasmic free Ca²⁺ concentration were expressed either as uncorrected excitation ratios (F₃₈₀/F₃₄₀) or as cumulative excitation ratio responses (∆ from base line).

Curve Fitting and Statistical Analysis—The Ca²⁺ mobilization data were expressed as concentration-response curves and fitted to the following form of the Hill equation: R = b + (a – b) × Cⁿ/(Cⁿ + C°), where R is the receptor response, a is the maximum response, b is the basal response, C is the extracellular Ca²⁺ concentration (in mM), n is the Hill coefficient. Estimates of the curve-fitting parameters were obtained using MacCurveFit 1.5 for Macintosh. Other data are routinely expressed as the means ± S.E. (number of experiments).

RESULTS

Sensitivity of Wild-type Calcium-sensing Receptor to α-Amino Acids—The wild-type CaR was stably expressed in HEK293 cells, and the cells were loaded with fura-2 and exposed to stepwise increments in Ca²⁺o concentration in the absence or presence of L-Phe or D-Phe at a concentration of 10 mM. L-Phe stereospecifically enhanced the sensitivity to extracellular Ca²⁺ (Fig. 1). In the absence of Phe, the EC₅₀ for Ca²⁺o was 4.9 ± 0.1 mM (n = 8). In the presence of 10 mM D-Phe, it was 4.6 ± 0.1 mM (n = 3), and in the presence of 10 mM L-Phe it was 3.4 ± 0.1 mM (n = 4).

Screen of Conservative Mutations in the CaR Venus Flytrap Domain—The following mGlu-1 ligand binding residues are readily aligned to residues in the CaR (the CaR residue is shown in parentheses): Ser-165 (Ser-147), Thr-188 (Ser-170), Thr-191 (Thr-187), Thr-193 (Ser-175), and Thr-196 (Ser-176). The following mGlu-1 ligand binding residues are also readily aligned to residues in the CaR (the CaR residue is shown in parentheses): Ser-164 (Ser-147), Thr-188 (Ser-170), Thr-191 (Thr-187), Thr-193 (Ser-175), and Thr-196 (Ser-176). The mGlu-1 ligand binding residues are shown in parentheses: Ser-165 (Ser-147), Thr-188 (Ser-170), Thr-191 (Thr-187), Thr-193 (Ser-175), and Thr-196 (Ser-176). The following mGlu-1 ligand binding residues are also readily aligned to residues in the CaR (the CaR residue is shown in parentheses): Ser-164 (Ser-147), Thr-188 (Ser-170), Thr-191 (Thr-187), Thr-193 (Ser-175), and Thr-196 (Ser-176).
to L-Phe. A conservative mutation of the critical residue Ser-170 i.e. S170T also appeared to exhibit near-normal sensitivity to Ca\(^{2+}\) but reduced sensitivity to L-Phe (Table I). On the basis of these results it was decided to perform further analysis on two mutants, T145A and S170T, as well as the double mutant T145A/S170T in an effort to identify an L-amino acid-insensitive CaR.

**T145A Exhibits Reduced Sensitivity to L-Phe**—The possibility that T145A also exhibited selective loss of sensitivity to L-Phe was explored further in HEK293 cells that were transiently transfected with S170T and then loaded with fura-2 as described above for T145A. S170T also exhibited reduced sensitivity to L-Phe (Fig. 2, A and C). In the absence of L-Phe, the EC\(_{50}\) for Ca\(^{2+}\) was 2.6 ± 0.1 mM; in its presence, the EC\(_{50}\) for Ca\(^{2+}\) fell modestly (all \(n = 4\)). At a concentration of 1 mM L-Phe, the EC\(_{50}\) for Ca\(^{2+}\) was 2.5 ± 0.1 mM. At concentrations of 3 and 10 mM L-Phe, the EC\(_{50}\) values for Ca\(^{2+}\) were 2.3 ± 0.2 and 2.2 ± 0.1 mM respectively. These data provide support for the idea that the L-Phe sensitivity of S170T is impaired when compared with wild type.

**L-Phe Sensitivity of the Double Mutant T145A/S170T Expressed Transiently in HEK293 Cells**—The data described above suggested that T145A and S170T exhibit impaired sensitivity to the CaR-active amino acid L-Phe. To further assess the significance of these effects, the double mutant T145A/S170T was synthesized and expressed transiently in HEK293 cells. Up to a concentration of 10 mM L-Phe, had no effect on the EC\(_{50}\) for Ca\(^{2+}\) in HEK293 cells that transiently expressed T145A/S170T (Fig. 2D). In the absence of L-Phe, the EC\(_{50}\) for Ca\(^{2+}\) was 2.6 ± 0.2 mM (n = 4). In the presence of 1, 3, and 10 mM L-Phe, the EC\(_{50}\) values for Ca\(^{2+}\) were, respectively, 2.7 ± 0.1 mM (n = 5), 2.8 ± 0.2 mM (n = 4), and 2.7 ± 0.2 mM (n = 7). These data indicate that the double mutant retains normal or near-normal Ca\(^{2+}\) sensitivity but exhibits markedly reduced sensitivity to L-Phe.

**Surface Expression of the Wild-type CaR, T145A, S170T, and the Double Mutant T145A/S170T**—All four constructs in pcDNA3.1 exhibited similar EC\(_{50}\) values for Ca\(^{2+}\) and maximal responses when expressed transiently in HEK293 cells (Fig. 2). These results suggest that the level of CaR surface expression was similar for all four receptors and that functional differences between the various receptors arose from differences in amino acid binding or signal transmission. However, to exclude the possibility that functional differences might have arisen from differences in surface expression, HEK293 cells were transiently transfected with pcDNA3.1 constructs for all four receptors that had been surface expressed with biotin then solubilized. FLAG-tagged CaR proteins were immunoprecipitated using an anti-FLAG antibody then resublimized and subjected to SDS-PAGE and Western blotting. The blots were developed using an avidin-horseradish peroxidase conjugate and reagents suitable for
MacCurveFit 1.5 for Macintosh.

Microscope and exposed to various extracellular Ca²⁺ described under “Experimental Procedures.” The cells on glass coverslips were then transferred into the superfusion chamber of a fluorescence experiments for each construct. They were converted to cumulative excitation ratios (Δ from baseline), and curve-fitting was performed using MacCurveFit for Macintosh.

Behavior of T145A, S170T, and T145A/S170T Stably Expressed in HEK293 Cells—A second clone of HEK293 cells that stably expressed the wild-type CaR (distinct from the clone used in the experiments shown in Fig. 1) together with clones of HEK293 cells that stably expressed T145A, S170T, or the double mutant T145A/S170T were examined for their sensitivity and stereoselectivity to L-Phe. T145A exhibited reduced sensitivity to L-Phe when compared with the wild-type CaR (see Fig. 4, A and B). In addition, T145A exhibited L/D selectivity (Fig. 4B). To further investigate a possible loss of L/D selectivity, HEK293 cells that stably expressed either the wild-type CaR or T145A were exposed to stepwise increments in Ca²⁺ concentration in the absence or presence of the following pairs of aromatic amino acids: L-Phe/D-Phe, L-Trp/D-Trp, and L-His/D-His. In all three cases T145A exhibited loss of L/D selectivity with similar results for all three amino acids when compared with control (not shown).

S170T also exhibited reduced sensitivity to L-Phe, although L/D selectivity was preserved (Fig. 4C). Next, the T145A/S170T double mutant was investigated. These cells retained normal or near-normal sensitivity to extracellular Ca²⁺ (Fig. 4D). However, unlike control cells that stably expressed the wild-type CaR (Figs. 1A and 4A), cells that stably expressed the double mutant were completely resistant to L-Phe as well as D-Phe up to 10 mM (Fig. 4). In a separate series of experiments, L-Phe at a concentration of 100 mM had no effect on the Ca²⁺ sensitivity of T145A/S170T (n = 4; data not shown). In these experiments in the absence of L-Phe the EC₅₀ for Ca²⁺ was 3.1 ± 0.3 mM. In the presence of 100 mM L-Phe, the EC₅₀ for Ca²⁺ was 3.4 ± 0.3 mM.

**TABLE I**

| Mutant/wild-type | EC₅₀ for Ca²⁺ | Maximal response | Hill coefficient |
|------------------|--------------|-----------------|-----------------|
|                  | Control | L-Phe 10 | Control | L-Phe 10 | Control | L-Phe 10 |
| Wild type        | 3.8 ± 0.1  | 2.8 ± 0.2  | 0.25 ± 0.01  | 0.25 ± 0.01  | 3.0 ± 0.3  | 2.2 ± 0.4  |
| T145A            | 5.9 ± 0.9  | 4.8 ± 0.4  | 0.24 ± 0.03  | 0.24 ± 0.02  | 1.9 ± 0.3  | 2.4 ± 0.4  |
| S170T            | 24.5 ± 3.6 | 12.2 ± 0.3  | 0.27 ± 0.03  | 0.22 ± 0.01  | 1.4 ± 0.2  | 2.3 ± 0.1  |
| S170T            | 24.4 ± 10.3| 20.7 ± 5.0  | 0.10 ± 0.02  | 0.09 ± 0.01  | 0.8 ± 0.1  | 0.8 ± 0.1  |
| T145A/S170T      | 3.3 ± 0.1  | 2.8 ± 0.2  | 0.24 ± 0.01  | 0.24 ± 0.01  | 2.5 ± 0.2  | 2.0 ± 0.3  |
| T218F            | 19.4 ± 4.7 | 14.9 ± 2.8  | 0.16 ± 0.02  | 0.26 ± 0.03  | 1.3 ± 0.2  | 1.5 ± 0.3  |
| E297Q            | 24.3 ± 6.8 | 9.8 ± 0.6   | 0.33 ± 0.07  | 0.26 ± 0.07  | 1.9 ± 0.6  | 2.2 ± 0.3  |

**FIG. 2.** The effects of various concentrations of L-Phe on extracellular Ca²⁺-sensitivity in HEK293 cells that transiently expressed the wild-type or mutant CaRs. HEK293 cells were transiently transfected with the wild-type CaR or mutant CaRs (B–D) using Lipo-fectamine 2000 as described under “Experimental Procedures.” After 48 h HEK293 cells were loaded with fura-2 and exposed to various extracellular Ca²⁺ concentrations in the absence or presence of L-Phe at 1, 3, and 10 mM. A, wild type; B, T145A; C, S170T; D, T145A/S170T. Open circles, control; open triangles, 1 mM L-Phe; open squares, 3 mM L-Phe; filled squares, 10 mM L-Phe. The data have been expressed as percentages of the maximum responses referenced to the wild-type maximum responses obtained in parallel experiments.

**TABLE II**

| Mutant/wild-type | Maximal response | Hill coefficient |
|------------------|-----------------|-----------------|
|                  | Control | L-Phe 10 | Control | L-Phe 10 |
| Wild type        | 3.8 ± 0.1  | 2.8 ± 0.2  | 3.0 ± 0.3  | 2.2 ± 0.4  |
| T145A            | 5.9 ± 0.9  | 4.8 ± 0.4  | 1.9 ± 0.3  | 2.4 ± 0.4  |
| S170T            | 24.5 ± 3.6 | 12.2 ± 0.3  | 1.4 ± 0.2  | 2.3 ± 0.1  |
| S170T            | 24.4 ± 10.3| 20.7 ± 5.0  | 0.8 ± 0.1  | 0.8 ± 0.1  |
| T145A/S170T      | 3.3 ± 0.1  | 2.8 ± 0.2  | 2.5 ± 0.2  | 2.0 ± 0.3  |
| T218F            | 19.4 ± 4.7 | 14.9 ± 2.8  | 1.3 ± 0.2  | 1.5 ± 0.3  |
| E297Q            | 24.3 ± 6.8 | 9.8 ± 0.6   | 1.9 ± 0.6  | 2.2 ± 0.3  |

**DISCUSSION**

The data described in the current work indicate that the mutants T145A and S170T in the CaR VFT domain impair L-amino acid sensing, leaving Ca²⁺ sensing intact. Furthermore, the double mutant T145A/S170T selectively disabled L-amino acid sensing. The results indicate that these effects arise from a selective disturbance of receptor function. Furthermore, surface expression was similar for all four CaR species that were fully characterized including wild-type, T145A, S170T, and T145A/S170T (Fig. 3), indicating that the disturb-
ance of amino acid sensing did not arise from a change in cell surface expression.

Previous analysis of conserved residues in the CaR VFT domain indicated that Ser-170 and possibly two adjacent serines, Ser-169 and Ser-171, play key roles in amino acid sensing (10). However, dissociating amino acids from Ca$^{2+}$, sensing was only possible when the S169A/S170A/S171A triple mutant was co-expressed with a C-terminally truncated wild-type receptor, A877Stop, that restored Ca$^{2+}$, but not amino acid sensing (10). These findings together with a recent analysis of chimeric CaR/mGlu-1 receptors indicate that the l-amino acid binding site lies in the CaR VFT domain (8). Therefore, in the current study we decided to re-investigate whether an amino acid-insensitive mutant that retained normal Ca$^{2+}$, sensing might be synthesized using site-directed mutagenesis of residues in the CaR VFT domain. This analysis led to the identification of T145A and S170T as candidate mutations with the desired properties (Table I, Figs. 2 and 4) and led to the conclusion that Ca$^{2+}$, and amino acid sensing can be dissociated.

Consideration of the crystal structure of the ligand-bound form of the mGlu-1 VFT domain (9) together with the predicted homologies between the human CaR and rat mGlu-1 structures (10) suggest the hypothesis that the mutants T145A and S170T impair amino acid sensing by interfering with amino acid binding. For example, Thr-145 in the CaR VFT domain may perform a similar function to Ser-164 in the rat mGlu-1 VFT domain. In the mGlu-1 crystal structure, Ser-164 ligation a water molecule that, in turn, ligation one of the α-carboxyl oxygens of the bound glutamate (9). If Thr-145 serves a similar function in the CaR, loss of its side chain (as in T145A) could impair amino acid binding without disturbing amino acid-independent receptor activation i.e. without disturbing Ca$^{2+}$, sensing. In addition to disturbing amino acid sensing, T145A also interfered with l/d selectivity for CaR-active amino acids (Fig. 4B; Table II). This finding suggests that the side chain of Thr-145 provides one of the three key points of contact required for stereoisomer selectivity, perhaps by imposing a local water structure as in the case of mGlu-1 residue Ser-164 (9).

Ser-170, on the other hand, aligns to Thr-188 in the rat mGlu-1 VFT domain. In the mGlu-1 crystal structure, Thr-188 supports glutamate binding by providing an H-bond from its side-chain hydroxyl to the α-amino group of the ligated glutamate (9). However, unlike Thr-145, Ser-170 is also required for amino acid-independent CaR activation since S170A exhibited grossly impaired Ca$^{2+}$, -dependent receptor activation (Table I) as reported previously (10, 16). Furthermore, l-amino acids did not restore Ca$^{2+}$, -dependent function of S170A (Table I; 10), providing support for a role of Ser-170 in amino acid binding. For this reason the more conservative mutation S170T, which retains a side-chain hydroxyl, was selected for further analysis. As described above, S170T exhibited normal or near-normal Ca$^{2+}$, sensing and impaired l-amino acid sensing (Table I, Figs. 2 and 4). However S170T, unlike T145A, did not disturb l/d selectivity. This suggests that S170T has a more modest impact on amino acid binding/sensing and is compensated by the presence of the side-chain hydroxyl of the threonine at

![Fig. 3. Surface expression of FLAG-tagged wild-type and mutant CaRs.](image)

**FIG. 3.** Surface expression of FLAG-tagged wild-type and mutant CaRs. HEK293 cells were transiently transfected with FLAG-tagged wild-type and mutant CaRs in 24-well plates as described under "Experimental Procedures." After 2 days the cells were labeled with sulfo-NHS-biotin and then lysed in the presence of 100 mM iodoacetamide to prevent disulfide bond formation. The CaRs were then immuno-precipitated from the lysates using anti-FLAG antibody, suspended in SDS-PAGE sample buffer, and assayed for protein content. Equivalent amounts of protein were loaded into each well for SDS-PAGE. After SDS-PAGE (6% acrylamide resolving gel), Western blotting and detection of biotin-labeled proteins were performed using avidin-horseradish peroxidase and enhanced chemiluminescence as described under "Experimental Procedures." The arrows indicate the molecular masses of markers in kDa. Lane 1, the wild-type CaR; lane 2, T145A; lane 3, S170T; lane 4, T145A/S170T. The lowest molecular weight form observed (around 160 kDa) is likely to represent monomers of the mature complex carbohydrate form of the CaR (11). In addition, higher molecular weight species including presumably dimers and higher order oligomers were also detected.

![Fig. 4. Effects of l-Phe and d-Phe on wild-type and mutant CaRs stably expressed in HEK293 cells.](image)

**FIG. 4.** Effects of l-Phe and d-Phe on wild-type and mutant CaRs stably expressed in HEK293 cells. Stable transfectants for the wild-type CaR and T145A, S170T, and the double mutant T145A/S170T were derived by antibiotic selection as described under "Experimental Procedures." As required, cells were subcultured onto coverslips, loaded with fura-2, and studied by microfluorimetry. The cells were exposed to various extracellular Ca$^{2+}$ concentrations in the absence (open circles) or presence of 10 mM d-Phe (open triangles) or 10 mM l-Phe (filled triangles). A, wild type; B, T145A; C, S170T; D, T145A/S170T. The data have been expressed as percentages of the maximum response obtained in each individual experiment.
TABLE II
Comparison between the effects of various L- and D-amino acids on the Ca\(^{2+}\) sensitivity of the wild-type CaR or T145A mutant stably expressed in HEK293 cells

| Treatment       | EC_{50} for Ca\(^{2+}\) (mM) |
|-----------------|--------------------------------|
| Control         | Wild type                     | T145A                        |
|                 | 4.1 ± 0.2 (6)                  | 5.8 ± 0.2 (7)                |
| L-Phe           | 2.7 ± 0.1 (6)                  | 4.1 ± 0.4 (6)                |
| D-Phe           | 4.2 ± 0.1 (6)                  | 4.6 ± 0.2 (6)                |
| L-Trp           | 3.3 ± 0.4 (3)                  | 4.1 ± 0.2 (3)                |
| D-Trp           | 4.6 ± 0.1 (3)                  | 4.3 ± 0.3 (3)                |
| L-His           | 2.8 ± 0.4 (3)                  | 4.0 ± 0.3 (3)                |
| D-His           | 4.4 ± 0.2 (4)                  | 4.2 ± 0.3 (3)                |

The data are EC_{50} values for Ca\(^{2+}\) (in mM) obtained in the absence (control) or presence of various L- or D-amino acids (all 10 mM). HEK293 cells that stably expressed either the wild-type CaR or T145A mutant were plated onto coverslips. After the cells reached confluence, they were loaded with fura-2 and analyzed by microfluorimetry as described under “Experimental Procedures.” Cells were exposed to stepwise increments in Ca\(^{2+}\) concentration in the absence or presence of the amino acids shown. The data obtained were converted to cumulative excitation ratios (Δ from base line), and curve-fitting was performed using MacCurveFit L.5 for Macintosh. No significant differences were observed between the Hill coefficients and maximal responses for the various treatments.

The basis of this difference is unclear. The stably expressed CaR may exhibit enhanced amino acid sensitivity with respect to the transiently expressed CaR as a result of differences in surface expression, as described for another family 3 receptor, mGlur-5 (18). Alternatively, it might arise from differences in CaR-dependent expression of key signaling proteins.

The physiological significance of L-amino acid sensing by the CaR is not yet fully elucidated. However, recent work on normal human parathyroid cells indicates that L-amino acids regulate PTH secretion (19) and the CaR may also mediate the effect of high dietary protein intake on urinary calcium excretion (for review, see Ref. 20). Thus, the CaR appears to provide a molecular link between protein and calcium metabolism and may also act more generally as an amino acid sensor in the control of digestion, absorption, appetite, and whole body metabolism (for review, see Ref. 21). Identifying mutations that selectively disable CaR-dependent amino acid sensing offers a possible approach to establishing its full physiological significance e.g., via analysis of tissue-wide function in a knock-in mouse.

In conclusion, mutational analysis of conserved binding residues in the CaR VFT domain has led to the identification of two mutants, T145A and S170T, which selectively impair L-amino acid sensing, leaving extracellular Ca\(^{2+}\) sensing intact. Furthermore, the double mutant T145A/S170T selectively disables amino acid sensing. The results indicate that Ca\(^{2+}\) and L-amino acid sensing can be dissociated, and the double mutant may be suitable for further analysis of the physiological significance of amino acid sensing.

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