Delineating a Ca\textsuperscript{2+} Binding Pocket within the Venus Flytrap Module of the Human Calcium-sensing Receptor*§

The Ca\textsuperscript{2+}-sensing receptor (CaSR) belongs to the class III G-protein-coupled receptors (GPCRs), which include receptors for pheromones, amino acids, sweeteners, and the neurotransmitters glutamate and \(\gamma\)-aminobutyric acid (GABA). These receptors are characterized by a long extracellular amino-terminal domain called a Venus flytrap module (VFTM) containing the ligand binding pocket. To elucidate the molecular determinants implicated in Ca\textsuperscript{2+} recognition by the CaSR VFTM, we developed a homology model of the human CaSR VFTM from the x-ray structure of the metabotropic glutamate receptor type 1 (mGluR1), and a phylogenetic analysis of 14 class III GPCR VFTMs. We identified critical amino acids delineating a Ca\textsuperscript{2+} binding pocket predicted to be adjacent to, but distinct from, a cavity reminiscent of the binding site described for amino acids in mGluRs, GABA-B receptor, and GPRC6a. Most interestingly, these Ca\textsuperscript{2+}-contacting residues are well conserved within class III GPCR VFTMs. Our model was validated by mutational and functional analysis, including the characterization of activating and inactivating mutations affecting a single amino acid, Glu-297, located within the proposed Ca\textsuperscript{2+} binding pocket of the CaSR and associated with autosomal dominant hypocalcemia and familial hypocalciuric hypercalcemia, respectively, genetic diseases characterized by perturbations in Ca\textsuperscript{2+} homeostasis. Altogether, these data define a Ca\textsuperscript{2+} binding pocket within the CaSR VFTM that may be conserved in several other class III GPCRs, thereby providing a molecular basis for extracellular Ca\textsuperscript{2+} sensing by these receptors.

The class III G-protein-coupled receptors (GPCRs)\textsuperscript{5} are activated by a variety of ligands, including calcium (Ca\textsuperscript{2+}), pheromones, \(\alpha\)-amino acids, diverse natural sweeteners, and the major neurotransmitters glutamate and \(\gamma\)-aminobutyric acid (GABA). These receptors are characterized by a large amino-terminal extracellular domain reminiscent of bacterial periplasmic binding proteins. This domain is formed by two lobes (LB1 and LB2) separated by a cavity delineating the ligand-binding site and called a Venus flytrap module (VFTM) (1, 2). The crystal structure of the glutamate-bound form of the extracellular domain of the metabotropic glutamate receptor type 1 (mGluR1) revealed key residues located at the interface of LB1 and LB2 and that were involved in glutamate binding (3). Homology modeling of other class III GPCRs, including mGluRs and GABA-B type 1 (GBR1) receptors, the recently deorphanized receptor for basic amino acids (GPRC6a) and its goldfish relative, and the sweet taste receptors T1R1, T1R2, and T1R3, have helped identify the ligand binding pocket for these receptor ligands and the receptor activation process (4–7).

The Ca\textsuperscript{2+}-sensing receptor (CaSR) expressed in the parathyroid glands senses minor changes in ionized plasma Ca\textsuperscript{2+} and by controlling parathyroid hormone secretion is the major molecular determinant of Ca\textsuperscript{2+} homeostasis (8, 9). Initially cloned from the parathyroid glands (10), the CaSR has been subsequently isolated from various tissues, including kidney and brain, where it is proposed to mediate diverse physiologic effects in response to variations in extracellular Ca\textsuperscript{2+} (11–13). The cloning of the CaSR made it possible to demonstrate directly that the CaSR is activated not only by Ca\textsuperscript{2+} but also by Mg\textsuperscript{2+} and other divalent cations (10, 14–16). Molecules aimed at modulating the activity of the CaSR and acting at the level of the transmembrane domains have been characterized (17, 18). Indeed, targeting the parathyroid CaSR by a positive allosteric modulator has been proposed recently for the treatment of secondary hyperparathyroidism linked to renal disease (19), and antagonizing the CaSR activity might be of benefit for treating female osteoporosis (17).

Naturally occurring activating and inactivating CaSR mutations are responsible for autosomal dominant hypocalcemia (ADH) and familial hypocalciuric hypercalcemia (FHH), genetic diseases linked to perturbations in Ca\textsuperscript{2+} homeostasis (9, 20). The CaSR VFTM has been shown to contain the Ca\textsuperscript{2+}-binding site (21, 22). Among the 13 residues shown to be involved in glutamate binding to the mGluR1 VFTM, 6 are identical or conservatively substituted in the human CaSR (see supplemental Tables 3 and 4), and the analysis of variants produced by site-directed mutagenesis has shown that three of these residues impair Ca\textsuperscript{2+} activation when changed to alanine (21, 23). However, the Ca\textsuperscript{2+}-binding amino acids delineating the Ca\textsuperscript{2+} binding pocket within the CaSR VFTM have not been identified, nor is it known if this Ca\textsuperscript{2+} binding cavity is conserved within class III receptors.

In this study, we describe a homology model of the VFTM of the human CaSR built from the x-ray structure of the rat mGluR1 and a

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The abbreviations used are: GPCR, G-protein-coupled receptor; CaSR, Calcium sensing receptor; GABA, \(\gamma\)-aminobutyric acid; VFTM, Venus flytrap module; mGluR1, metabotropic glutamate receptor type 1; LB, lobe; GBR1, GABA-B receptor type 1; T1R1, T1R2, T1R3, sweet taste receptors type 1, type 2, type 3; ADH, autosomal dominant hypocalcemia; FHH, familial hypocalciuric hypercalcemia; IP, inositol phosphate; WT, wild type.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 3–5.

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phylogenetic analysis of the ligand binding pocket of class III GPCRs. This model, which predicts the residues contributing to Ca\(^{2+}\) recognition, indicates that Ca\(^{2+}\) does not interact with amino acids homologous to those delineating the glutamate binding cavity but to a site adjacent to this cavity. We used mutational analysis, including the description and characterization of a novel mutation associated with ADH, and data from the literature to validate our model. In addition, our data are consistent with the presence of a conserved Ca\(^{2+}\) binding pocket within the VFTM of other class III GPCRs, adding a molecular basis for the previously observed effects of extracellular Ca\(^{2+}\) on the action of these receptors.

EXPERIMENTAL PROCEDURES

Identification of E297D Mutation in the CaSR Associated with ADH—Genomic DNA from the proband and family members was isolated from peripheral leukocytes using standard methods. CaSR exons 2–7 (proband) and exon 4 (all family members) and the corresponding intron-exon junctions were amplified using intronic primers (sequences available on request). PCR-amplified products were sequenced as described previously (24).

**FIGURE 1. Identification of the E297D activating mutation in the CaSR gene in patients affected with ADH.** A, three-generation pedigree of the family affected with ADH because of a heterozygous E297D mutation in the CaSR gene. B, DNA sequence of the CaSR gene. The index case (black arrow) and affected members harbored a heterozygous G to C substitution at codon 297, which would result in substitution of aspartic acid for glutamic acid (E297D) in the CaSR VFTM.

### TABLE ONE

| Receptor   | Binding site residues |
|------------|-----------------------|
| mGluR1     | Y74 R78 W110 S164 S165 S166 S186 T188 D208 Q211 Y236 F290 E292 G293 S317 D318 G319 R323 K409 |
| mGluR2     | R57 R61 S93 T144 S145 D146 A166 T168 D188 Q191 Y226 F269 R271 S272 S294 D295 G296 L300 K377 |
| mGluR3     | R67 R71 S103 T153 S154 S155 A175 T177 D197 Q200 Y225 F278 R280 S281 S303 D304 G305 Q309 K392 |
| mGluR4     | K74 R78 S110 G158 S159 S160 A180 T182 D202 Q205 Y230 F284 R286 S287 S311 D312 S313 K317 K405 |
| mGluR5     | R65 R68 W100 S151 S152 S153 S173 T175 D195 Q198 Y223 F277 R279 G280 S305 D306 S307 K312 K400 |
| mGluR6     | Q64 R68 S100 A153 S154 S155 A175 T177 D197 Q200 Y225 F279 R281 S282 S306 D307 S308 K312 K400 |
| mGluR7     | N64 R78 S110 G158 S159 S160 A180 T182 D202 Q205 Y230 F286 R288 S289 S313 D314 S315 K319 K407 |
| mGluR8     | K71 R75 S107 A155 S156 S160 A177 T179 D199 Q202 Y227 F281 R283 S284 S308 D309 S310 K314 R401 |
| GPRC6a     | S69 Q73 T104 Y148 S149 E150 E170 T172 D192 Q195 Y220 F277 R279 Q280 S302 D303 N304 A308 E408 |
| GBR1       | G184 C188 D221 C246 S247 S248 G268 S270 A290 H293 V318 L365 Y367 E368 A369 D398 N399 I403 G466 |
| CaSR       | R66 W70 N102 G146 S147 G148 A168 S170 D190 Q193 Y218 F270 S272 S276 E297 A298 S302 H413 |
| T1R1       | H71 L75 S107 S148 T149 N150 A170 S172 D192 Q195 Y220 F274 S276 R277 S300 E301 A302 S306 M383 |
| T1R2       | L67 L71 Y103 N143 E145 S165 L167 A187 H190 Y215 F275 P277 D278 S301 E302 D303 E382 |
| T1R3       | N68 W72 S104 S147 E148 G168 S170 D190 Q193 Y218 F274 S276 V277 S300 E301 A302 S306 H387 |

**Site-directed Mutagenesis**—Wild-type (WT) cDNA encoding the human CaSR inserted in pcDNA3.1/Hygro plasmid was a kind gift of E. F. Nemeth and P. Jacobson (NPS Pharmaceuticals Inc., Salt Lake City, UT). Missense mutations were introduced in the WT cDNA construct using the Quick Change Site-directed Mutagenesis kit (Stratagene) and were confirmed by complete sequencing of cDNA inserts (Genome Express, Meylan, France) (oligonucleotide sequences available on request).

**Cell Culture, Transient Transfection, and Western Blot Analysis**—Experiments were performed in HEK293 cells. Techniques used for cell culture, transient transfection by electroporation, and the detection of WT and mutant CaSRs expression by Western blot analysis have been described (25).

**[^3]HjIP Formation**—Cells were cultured in the presence of 0.5 μCi/well myo[^3]H]inositol (Amersham Biosciences) for 20 h. The measurement of[^3]H]IP accumulation was performed in freshly prepared buffer (125 mM NaCl; 4.0 mM KCl; 0.5 mM MgCl\(_2\); 20 mM Hepes; 0.1% d-glucose, pH 7.4) containing the indicated CaCl\(_2\) concentration (25). Two to five independent experiments were performed in duplicate or triplicate using the same conditions. The data were fitted to a sigmoid dose-response curve to determine EC\(_{50}\) values by using the GraphPAD Prism. Values for each curve were normalized to the maximal activation of each receptor, obtained by stimulation with 20 μM Ca\(^{2+}\). Significance was assayed by Excel 2000 Student’s t test.

**Homology Modeling of the Amino-terminal Domain of the Calcium-sensing Receptor**—A multiple alignment of 14 class III GPCR extracellular domains (shown in supplemental Table 3) was obtained with the T-Coffee program (26) Homology modeling of the human CaSR was performed with the SYBYL6.91 package (TRIPOS Associates Inc., St. Louis, MO) starting from monomer A of the mGluR1 x-ray structure (Protein Data Bank code 1ewk) (3). The insertion of residues in loops between helix A and strand B, helix B and strand D, and helix L and helix M (3) was accommodated by a knowledge-based loop search procedure as described previously (27). Further energy refinement of the model was achieved by a standard minimization protocol using the AMBER 8 program (University of California, San Francisco). Mapping of the Ca\(^{2+}\)-binding site was performed using the GRID version 20 software (28) using a Ca\(^{2+}\) probe and standard settings for the grid definition. The most energetically favored location of the calcium ion was then used to minimize (by 1,000 steps of steepest descent followed by 1,000 steps of conjugate gradient refinement) the receptor model in the pres-
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![Phylogenetic tree of class III GPCR ligand binding pocket](image)

**Results**

**Identification of E297D Mutation in the CaSR Associated with ADH**

The three generation pedigree of the family affected with ADH is shown in Fig. 1A. The index case was diagnosed during the 1st month of life because of severe hypocalcemic symptoms requiring calcium infusion. Biochemical features of the proband and family members at the time of diagnosis of hypocalcemia and identification of the activating mutation in the CaSR are shown in supplemental Table 5.

Direct sequence analysis of the PCR-amplified CaSR exons led to the identification of a heterozygous G to C nucleotide substitution in exon 4 of the CaSR gene at position 889 of the CaSR cDNA sequence (Fig. 1B). This base change resulted in the substitution of glutamate for aspartate at position 297 (E297D) located in the extracellular domain. The E297D mutation was present in all affected family members and absent in others (data not shown). Most interestingly, a missense E297K mutation has been identified in subjects with FHH (30, 31).

**Phylogenetic Tree of the Ligand Binding Pocket of Family 3 GPCRs**

The Glu-297 residue is structurally homologous to an aspartate residue conserved in all mGluRs that is located within the mGluR VFTM (TABLE ONE and supplemental Table 4) and has been implicated in ligand binding and receptor activation (3, 4). This suggests that Glu-297 is part of the Ca\(^{2+}\) binding pocket of the CaSR. In an attempt to delineate the putative ligand binding pocket of this receptor, we first constructed a phylogenetic tree for 14 human class III GPCRs based on 19 residues (TABLE ONE) lining the glutamate binding cavity identified in the mGluR1 structure (3). This phylogenetic tree indicates that the CaSR-binding site is not closely related to that of any other receptors (Fig. 2.A), including the GPRC6a receptor recognizing basic amino acids (7).

**Molecular Modeling of the Ca\(^{2+}\)-binding Site of the CaSR**

We next developed a homology model of the extracellular domain of the human CaSR built from the x-ray structure of the mGluR1 and according to a multiple alignment of the above-mentioned receptor sequences (supplemental Table 4). Comparison of the molecular models of the human mGluR1 and CaSR ligand binding pockets shows that the overall three-dimensional structure of the CaSR amino-terminal tail is very similar to the x-ray structure of the rat mGluR1 described by Kunishima et al. (3) (Fig. 2, B and C). Only three insertions in loop regions occur (see “Experimental Procedures”). The multiple sequence alignment used to thread the CaSR coordinates onto the mGluR1 structure (TABLE ONE and supplemental Table 4) shows only a single difference with that published (3) and occurs just before the beginning of helix M at Lys-409, which is a glutamate-anchoring residue. We propose a shorter insertion of rat mGluR1 (Protein Data Bank code 1ewk) (3). C, CaSR Ca\(^{2+}\)-binding site was modeled from the rat mGluR1 structure. Coordinates of the calcium ion are displayed by a green ball. A water molecule (cyan ball) completes the pentagonal bipyramidal coordination of the calcium ion.

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**FIGURE 2. Phylogenetic tree of class III GPCR ligand binding pocket (A) and molecular modeling of the human mGluR1 (B) and CaSR (C) ligand binding pockets.**

A, neighbor-joining tree of human class III GPCRs. The 19 residues lining the binding pocket of human class III GPCRs (supplemental Table 4) were extracted to calculate a distance matrix with the MEGA2 software (29). A neighbor-joining tree was calculated out of 100 bootstrap replicas using the γ correction for estimating pairwise protein distances. Bootstrap values are indicated in italics. B and C, ligand binding pocket of mGluR1 (B) and CaSR (C). Receptor atoms are shown as capped sticks, whereas ligand atoms are shown in ball and sticks (white, receptor carbon atom; green, α-glutamate carbon atom; red, oxygen; blue, nitrogen). Main chain atoms of the receptor participating in H-bonds to the ligands are displayed in yellow. Residues from the ligand binding domains 1 and 2 (LB1 and LB2) are labeled at the Cα atoms in white and cyan, respectively. Water molecules mediating ligand binding are displayed as cyan balls. Pink dashed lines indicate electrostatic (H-bonds, ion coordination) intermolecular interactions. B, the amino-terminal mGluR1-binding site residues were taken from the glutamate-ligated x-ray structure of Ca\(^{2+}\) and within a box of 23,844 TIP3P water molecules, placed automatically with the leap module of AMBER 8.0.

**Neighbor-joining Tree**—19 residues lining the binding pocket of glutamate in the mGluR1 structure (3) were extracted from 14 human class III GPCRs, concatenated into ungapped sequences, and were used to calculate a distance matrix with the MEGA2 software (29). A neighbor-joining tree was calculated out of 100 bootstrap replicas using the γ correction for estimating pairwise protein distances.

**RESULTS**

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amino acid-binding site but of smaller dimensions than that observed lining the glutamate binding cavity, the multiple sequence alignment is homologous to the glutamate binding cavity. Rather, the Ca$^{2+}$ residues for a ligand (Fig. 2, C) contacting residues, we measured PI hydrolysis as a function of extra-cellular Ca$^{2+}$ concentration in HEK293 cells transfected with the WT receptor and CaSR constructs harboring the naturally occurring E297D and E297K mutations or the artificial Q193A, F270A, and S296A mutations. The functional characterization of the E297D mutation identified in the family described above indicated that this receptor shows a left-shifted concentration-response curve to Ca$^{2+}$ compared with the WT receptor (EC$_{50}$ = 2.70 ± 0.30 mM versus 4.30 ± 0.20 mM, mean ± S.E., n = 4, p < 0.001) (Fig. 3A and TABLE TWO). In contrast, the E297K receptor was accompanied by a complete loss of Ca$^{2+}$ sensitivity (Fig. 3A and TABLE THREE). The three Q193A, F270A, and S296A mutants demonstrated a right-shift in Ca$^{2+}$ sensitivity compared with the WT receptor (Fig. 3A and TABLE THREE). The expression of WT and mutant receptors was demonstrated by Western blot analysis using the 141Ab antiserum directed against the carboxyl-terminal domain of the human CaSR that we have characterized recently (25) (Fig. 3B). Under reducing conditions, two main polypeptides of 150 and 130 kDa were identified in membrane preparations from HEK293 cells transfected with the WT or with the mutant receptors. These results are in agreement with those published by Bai et al. (35) showing that the polypeptide of higher molecular weight corresponds to N-linked glycosylated

### TABLE TWO

| Amino acids lining the putative Ca$^{2+}$-binding site of human class III GPCRs |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Receptor       | S165            | T188            | D208            | Q211            | Y236            | F290            | S317            | D318            |
| mGluR1         | mGluR2          | mGluR3          | mGluR4          | mGluR5          | mGluR6          | mGluR7          | mGluR8          | GPCR5a          |
| S149            | T149            | T144            | T147            | S154            | T157            | T159            | S156            | T172            |
| S170            | T170            | T172            | T174            | T175            | T177            | T178            | T179            | T172            |
| D190            | D200            | D202            | D203            | D195            | D197            | A202            | D192            | D192            |
| Gln-193, Phe-200, Tyr-218, and Ser-147) through water interactions. A pentagonal bipyramidal coordination of metal is proposed, as is observed in many calcium-binding proteins (32). Our present model identifies the presence of a Ca$^{2+}$ binding pocket with the identification of Glu-193, Phe-270, Ser-296, and Glu-297 as novel potential residues involved in Ca$^{2+}$ binding. The residues involved in this putative Ca$^{2+}$-binding site are all conserved in T1R3 and all but one in T1R1 (Ser-147 in CaSR substituted by Thr-149 in T1R1) (TABLE TWO). They are also conserved in mGluRs and GPRC6a except for two residues (Ser-170 and Glu-297 in CaSR substituted by Thr and Asp, respectively, in mGluRs and GPRC6a). The lowest homology is found with T1R2 and GBR1 (TABLE TWO).

| Functional Characterization of the Putative Ligand Contacting Residues—Four of the putative ligand contacting residues, Ser-147, Ser-170, Asp-190, and Tyr-218, have been shown previously to impair human CaSR activation when mutated to alanine (23) (TABLE THREE).

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### TABLE THREE

| Summary of the effects of various CaSR mutations affecting residues delineating the Ca$^{2+}$-binding site |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Receptor | Maximal response (as percent of that observed for the WT receptor in the same experiment) | Ca$^{2+}$, EC$_{50}$ |
| WT | 100 ± 2 | 4.3 ± 0.2 |
| S147A | 47 ± 1 | 18.0 ± 0.4 |
| S170A | 38 ± 1 | 23.1 ± 0.5 |
| D190A | 45 ± 1 | 22.1 ± 0.4 |
| Q193A | 102 ± 7 | 7.6 ± 0.7$^a$ |
| Y218A | 27 ± 2 | 38 ± 3.2 |
| F270A | 66 ± 5$^a$ | 6.1 ± 0.3 |
| S296A | 76 ± 9$^a$ | 5.4 ± 0.3$^a$ |
| E297D | 94 ± 5 | 2.7 ± 0.3$^a$ |
| E297K | 19 ± 2$^a$ | <10$^a$ |

$^a$ Results are from Zhang et al. (23) and represent measurement of intracellular Ca$^{2+}$ imaging. Values are normalized to the response of the WT receptor at 50 mM Ca$^{2+}$.

$^p < 0.001$ compared with the WT.
receptors expressed at the cell surface, whereas the lower molecular weight polypeptides represent intracellular mannose-modified receptors. In all cases, dimers migrating above 200 kDa were observed. The expression pattern of the various receptors was similar as assessed by immunoblot and was comparable with the one obtained from receptors harboring mutation at the level of the transmembrane domain and impairing recognition to calcilytics and calcimimetics (18, 25). Thus, taken together, our in vitro data and those from the literature (21, 23) demonstrate the importance of the eight putative ligand-contacting residues for CaSR activation. Furthermore, our data demonstrate the involvement of E297D and E297K mutations in ADH and FHH, respectively.

Analysis of the Interaction Area of Ca²⁺ with WT, E297D, and E297K CaSR—In order to understand further the effect of E297D and E297K mutation on Ca²⁺ binding, we determined the most favorable interaction area of Ca²⁺ with WT and E297D and E297K mutant receptors based on our model. For the WT and E297D mutant, the most favorable interaction area is displayed at a similar interaction energy of ∼37.5 kcal·mol⁻¹. For the E297K mutant (C), the Lys-297 ammonium side chain prevents Ca²⁺ binding, and its interaction energy with amino acids located in the Ca²⁺ binding pocket is only ∼11 kcal·mol⁻¹ and presumably not sufficient for initiating receptor activation. White arrows pinpoint the residue 297 side chain. A–C, right, diagrams of closed forms of the dimeric WT and mutant CaSR VFTMs are shown. The VFTMs are formed by two lobes (LB1 and LB2) and are presented in a closed position with Ca²⁺ bound (A and B) or not (C) to the module. In the E297K mutant, the rotation that is thought to occur around an axis through the VFTMs dimer interface (54) presumably does not occur, thus preventing receptor activation as indicated by the dotted arrow in C and the plain arrows in A and B.

DISCUSSION

In the present work, we identify a set of residues delineating a Ca²⁺ binding pocket within the VFTM of the human CaSR. This binding pocket is adjacent to, but distinct from, a cavity reminiscent of an amino acid-binding site involved in amino acid recognition in mGluRs, GR1 and GPRC6a. It is conserved within the VFTM of several class III GPCRs, thereby providing a molecular basis for the effects of Ca²⁺ on these receptors. We validate the model and provide direct clinical evidence for the physiological relevance of the VFTMs to class III GPCRs by characterizing pathogenic activating and inactivating mutations associated with a single amino acid of the CaSR located within the proposed Ca²⁺ binding pocket.

The construction of a three-dimensional model of the CaSR VFTM based on the mGluR1 crystal structure (3), and on the alignment of CaSR amino-terminal amino acid sequence with that of class III human GPCRs, allowed us to predict that the Ca²⁺-binding site of the CaSR comprises eight residues located within the cleft of the two putative VFTM lobes. Two sets of observations validate our model. First, functional studies, as shown here and by others (21, 23), demonstrate the importance of these residues for activation of the CaSR by Ca²⁺. Second, our model predicts that the Ca²⁺-binding site of the CaSR is centered on the acidic residue Glu-297. We report and characterize a novel activating mutation in the CaSR in a family affected with ADH, resulting from the conservative E297D substitution. A counterpart loss of function mutation, E297K, had been reported in patients with FHH (30, 31). Most interestingly, a glutamate residue is found at the homologous position in the human T1R1, T1R2, and T1R3 (and in a set of mouse vomeronasal receptors V2R2) (36), whereas an aspartate residue is found in all mGluRs (Asp-318 and Asp-309 in mGluR1 and mGluR8, respectively). GPRC6a and GR1. Asp-318 in mGluR1 has been demonstrated to be part of the ligand binding pocket (3), and introducing the D309E substitution in mGluR8 results in a decrease in glutamate-induced stimulation of IP production in transfected cells and in a conversion of antagonists into agonists (4). Therefore, it appears that this residue plays a pivotal role in determining ligand affinity for this family of receptors and is implicated in proper receptor activation.

With respect to the known structure of the glutamate-binding site in the mGluR1, the main distinct features of our CaSR model are observed.
at the putative α-amino acid-binding site where all residues interacting with the carboxylate side chain of glutamate in mGluR1 are not conserved in CaSR (Tyr-74 to Arg-66, Arg-323 to Ser-302, and Lys-409 to His-413) (Fig. 2, B and C, and TABLE ONE). In contrast, the second part of the α-amino acid-binding site is conserved or conservatively substituted, and all residues have been shown to be ligand-binding residues in the mGluR1, with the exception of Gin-211 (Gin-193 in the CaSR) (3). The presence of Arg-66 and Ser-302 in the CaSR (replacing Tyr-74 and Arg-323 in mGluR1) would prevent the binding of acidic molecules such as glutamate. In this regard, aromatic α-amino acids such as L-phenylalanine have been proposed to potentiate the effects of Ca\(^{2+}\) on the CaSR (37). However, we have not been successful in demonstrating such modulation of the WT human CaSR response to Ca\(^{2+}\) under our standard experimental conditions (data not shown). Two residues interacting with the glutamate carboxylate main chain via a water molecule in mGluR1 have been changed in CaSR (Glu-292 to Ser-272 and Ser-164 to Gly-146). Finally, the Asp-318 (mGluR1) to Gln-297 (CaSR) substitution would disfavor the ionic interaction with the ammonium main chain moiety of an α-amino acid. It therefore appears unlikely that α-amino acids bind to the bicalix-binding site of CaSR in a manner analogous to the interaction between glutamic acid and the metabotropic receptors.

Based on our model, residues delineating the Ca\(^{2+}\)-binding site within the VFTM of the CaSR are all conserved in T1R3 and all but one conserved in T1R1. This model suggests that activation of these receptors might be also modulated by Ca\(^{2+}\). Most interestingly, T1R3 is necessary for the functional expression of T1R1 and T1R2. T1R1/T1R3 heterodimers have been proposed to serve as α-amino acid sensors and to respond to the umami taste stimulus L-glutamate, whereas T1R2/T1R3 heterodimers have been designated as sweet receptors (38–40). These receptors have been hypothesized to be involved in sugar sensing on the tongue and possibly in other tissues such as the intestine (41) where they could be involved in a taste-sensing mechanism present in the gastrointestinal tract (42). A working model for the sweet and umami taste receptors proposes that L-glutamate and aspartame interact with the amino-terminal extracellular domain of T1R1 and T1R2, respectively (5). Further functional experiments are required to identify whether such responses are modulated by Ca\(^{2+}\), acting either on T1R1, T1R2, T1R3, or all three heteromers.

Most interestingly, all residues predicted to be located in the close vicinity of the Ca\(^{2+}\)-binding site and whose mutations affect Ca\(^{2+}\) binding (Ser-147, Ser-170, Asp-190, Ser-296, and Glu-297), as demonstrated here (Fig. 3 and TABLE TWO) and in previous reports (21, 23), are conserved or conservatively substituted in all mGluRs and most class III GPCRs (TABLE ONE). This may explain why the activity of the related mGluRs, GRB1 and GPRC6a, are Ca\(^{2+}\)-dependent (43–45) and why this dependence is sensitive to single point mutations affecting amino acids adjacent to the Ca\(^{2+}\)-binding site (S166D for mGluR1 and S269A for GRB1) (44, 46). The effect of Ca\(^{2+}\) acting at the level of the VFTM on the GRB1 occurs at a relatively high affinity (around 40 μM) (44), whereas the activity of the CaSR is modulated by changes in the Ca\(^{2+}\) concentrations occurring in the millimolar range. Most interestingly, the mouse GPRC6a response to cysteine and histidine is potentiated by Ca\(^{2+}\) (45). These results raise the possibility that Ca\(^{2+}\) interacts with the putative Ca\(^{2+}\) binding pocket within the VFTM of various class III GPCRs with different affinities, such that the range of Ca\(^{2+}\) concentrations resulting in modulation of receptor activity would be distinct for each receptor. Such a range of Ca\(^{2+}\) concentrations is likely to be observed under pathophysiological conditions in specific tissues. Indeed, extracellular Ca\(^{2+}\) is lowered after activation of glutamate receptors in brain or following epileptic seizures (47, 48), whereas high extracellular Ca\(^{2+}\) concentrations occur in the microenvironment of bone cells. Most interestingly, the presence of an extracellular cation-sensing receptor in osteoblasts with distinct cation specificity has been identified on CaSR (+/−) osteoblasts (49, 50).

The molecular positioning of Glu-297 within the cleft of the two lobes that form the VFTM is in agreement with results obtained by the analysis of mutated receptors. Mutation of this residue in patients with ADH has not been described previously. The E297D mutation would favor the stabilization by Ca\(^{2+}\) of VFTM in the “closed” configuration necessary for receptor activation, as indicated by the lower desolvation energy for the mutated receptor compared with that of WT receptor. In contrast, the E297K mutant associated with FHH, the Lys-297 ammonium side chain would keep the VFTMs closed by occupying the Ca\(^{2+}\) binding area, thus preventing Ca\(^{2+}\) binding. However, its interaction energy with neighboring amino acids is not sufficient to induce receptor activation.

In conclusion, we propose that the Ca\(^{2+}\)-binding site in the CaSR involves a set of polar residues directly involved in Ca\(^{2+}\) coordination (Ser-170, Asp-190, Gin-193, Ser-296, and Glu-297), and an additional set of residues that contributes to complete the coordination sphere of the cation (Phe-270, Tyr-218, and Ser-147). However, our model based on the x-ray structure of mGluR1 should be strengthened by the x-ray structure of the CaSR VFTM that has yet to be obtained. The in vitro demonstration in this and previous studies (21, 23) that these residues are important for the CaSR activation together with the clinical observation that the E297D and E297K mutations are responsible for ADH and FHH, respectively, are in agreement with the model. The presence of a calcium recognition site at the level of the transmembrane domains (51, 52) and of a proline residue (Pro-823) located within the putative transmembrane domain 6 playing a critical role for response to Ca\(^{2+}\) (53) suggests that, besides the VFTM, other determinants located in the seven-transmembrane domain participate in the Ca\(^{2+}\) mode of action. Our data support the physiological relevance of the VFTMs in the activation of receptors stimulated by the binding of small ligands in their extracellular domain. They are also consistent with the presence of a conserved Ca\(^{2+}\) binding pocket within class III GPCR VFTM, and thereby raise the possibility that changes in extracellular Ca\(^{2+}\) may modulate a wide range of biological responses associated with these receptors.

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