A Region in the Seven-transmembrane Domain of the Human Ca$^{2+}$ Receptor Critical for Response to Ca$^{2+}$

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Of 12 naturally occurring, activating mutations in the seven-transmembrane (7TM) domain of the human Ca$^{2+}$ receptor (CaR) identified previously in subjects with autosomal dominant hypocalcemia (ADH), five appear at the junction of TM helices 6 and 7 between residue Ile$^{819}$ and Glu$^{837}$. After identifying a sixth activating mutation in this region, V836L, in an ADH patient, we studied the remaining residues in this region to determine whether they are potential sites for activating mutations. Alanine-scanning mutagenesis revealed five additional residues in this region that when substituted by alanine led to CaR activation. We also found that, whereas E837A did not activate the receptor, E837D and E837K mutations did. Thus, region Ile$^{819}$–Glu$^{837}$ of the 7TM domain represents a “hot spot” for naturally occurring, activating mutations of the receptor, and most of the residues in this region apparently maintain the 7TM domain in its inactive configuration. Unique among the residues in this region, Pro$^{823}$, which is highly conserved in family 3 of the G protein-coupled receptors, when mutated to either alanine or glycine, despite good expression severely impaired CaR activation by Ca$^{2+}$. Both the P823A mutation and NPS 2143, a negative allosteric modulator that acts on the 7TM through a critical interaction with Glu$^{857}$, blocked activation of the CaR by various ADH mutations. These results suggest that the 7TM domain region Ile$^{819}$–Glu$^{837}$ plays a key role in CaR activation by Ca$^{2+}$. The implications of our finding that NPS 2143 corrects the molecular defect of ADH mutations for treatment of this disease are also discussed.

The extracellular Ca$^{2+}$ receptor (CaR) is a member of family 3 of the G protein-coupled receptor (GPCR) superfamily (1, 2). Family 3 members are typically characterized by a large extracellular amino terminus comprised of Venus’s flytrap (VFT)-like and cysteine-rich domains, in addition to the signature seven-transmembrane (7TM) domain of all GPCRs. The three-dimensional structure of the VFT domain of the rat metabotropic glutamate type 1 receptor showed that it is an intermolecular disulfide-linked dimer (3). Agonist binding to the cleft of the VFT leads to VFT closure and a 70° rotation of one monomer relative to the other about an axis perpendicular to the dimer interface. How this agonist-induced conformational change in the VFT domain is transmitted to the 7TM domain to cause CaR activation is a key unanswered question.

Naturally occurring CaR mutations identified in subjects with familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia (ADH), in addition to validating the physiologic importance of the CaR in extracellular Ca$^{2+}$ homeostasis (4), provide unique insights into CaR structure and function (2). Missense, activating mutations identified in ADH could help identify regions of the CaR particularly important for receptor activation. More than 30 such activating mutations have been identified to date in subjects with ADH (5), and these are non-randomly distributed (Fig. 1). Ten ADH mutations cluster between residues 116–131 in a part of the VFT domain, loop 2, which is the site of intermolecular disulfide-linked dimerization (6). The corresponding loop in the glutamate type 1 receptor three-dimensional structure represents a “switch region” in that it is disordered in the active, agonist-bound form of the VFT, but in the inactive form residues equivalent to 117–123 of the CaR become α-helical. We suggested that ADH loop2 mutations cause activation of the CaR by facilitating the agonist-induced conformational change in the VFT domain (2).

In the present work, we identified a novel ADH mutation, V836L, in the 7TM domain and noted that it is located in another region of clustered ADH mutations, residues 819–837, at the junction of TM6 and TM7 proximal to the extracellular surface of the plasma membrane (Fig. 1). Moreover, we had previously identified Glu$^{857}$ in TM7 as a residue critical for action of positive allosteric modulators of the CaR (7). This suggested to us that the TM6/TM7 junction may also represent a switch region that is critically involved in the activation mechanism of the CaR. To begin to evaluate this possibility, we performed alanine-scanning mutagenesis of residues in this region. We identified multiple additional TM6/TM7 residues that lead to receptor activation upon substitution of alanine and a unique mutation in TM6, P823A, that blocks CaR activation, mimicking the effect of a negative allosteric modulator.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the hCaR—The full-length human CaR (hCaR) cDNA cloned in the pCR3.1 expression vector was described previously (8). Site-directed mutagenesis was performed using the QuikChangeTM site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) according to the manufacturer’s instructions. Parental hCaR cDNA in pCR3.1 vector was amplified using pfu Turbo DNA polymerase...
FIG. 1. Schematic diagram showing amino acid sequence of the hCaR with boundaries of transmembrane helices predicted recently by Petrel et al. (18) based on alignment with rhodopsin rather than those predicted earlier by Garrett et al. (27) previously used by us. The location of signal peptide, N-linked glycosylation sites, and the sequence of synthetic polypeptide used to raise monoclonal antibody ADD is indicated. All cysteines are shown in black background. The beginning and end of the VFT domain and the four loops in lobe 1 of the VFT are indicated. Naturally occurring activating mutations identified previously in the hCaR and the V836L mutation reported herein as well as the inactivating V817I mutation (boxed) are indicated. Glu837, shown to be involved in binding of the allosteric modulators NPS R-568 and NPS 2143, and Pro823, reported herein to be critical for the function of the receptor, are shown in bold print. The two regions with clustering ADH mutations, residues 116–131 and residues 819–837, are shaded.
with mutagenic oligonucleotide primers (sequences available on request) for 16 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the parental DNA with DpnI for 1 h, the amplified DNA with incorporated nucleotide substitution was transformed into Escherichia coli (DH-5α strain). The sequence of mutant receptors was confirmed by automated DNA sequencing using a dhodamine Terminator cycle sequencing kit and ABI PRISM-373A DNA sequencer (PE Applied Biosystems, Foster City, CA).

**Transient Transfection of Wild Type and Mutant Receptors in HEK-293 Cells—** Transfections were performed using 12 μg of plasmid DNA for each transfection in a 75-cm² flask of HEK-293 cells. DNA was diluted in serum-free DMEM (BioFluids, Inc., Rockville, MD) mixed with diluted Lipofectamine (Invitrogen), and the mixture was incubated at room temperature for 30 min. The DNA-Lipofectamine complex was further diluted in 6 ml of serum-free DMEM and was added to 80% confluent HEK-293 cells plated in 75-cm² flasks. After 5 h of incubation, 15 ml of complete DMEM containing 10% fetal bovine serum (BioFluids, Inc.) was added. 24 h after transfection, transfected cells were split and cultured in complete DMEM.

**Phosphoinositide (PI) Hydrolysis Assay—** PI hydrolysis assay has been described previously (8). Briefly, 24 h after transfection, transfected cells from a confluent 75-cm² flask were split. Typically one-eighth of cells were plated in one well in a 6-well plate, and whole cell lysate was prepared 48 h post-transfection for Western blot assay. The remaining cells were plated in two 12-well plates in complete DMEM medium containing 3.0 μCi/ml of ³H-labeled myoinositol (PerkinElmer Life Sciences) and cultured for another 24 h. Culture medium was replaced by 1 × PI buffer (60 mM NaCl, 2.5 mM KCl, 2.8 mM glucose, 0.2 mM MgCl₂, 10 mM LiCl in 12.5 mM PIPES, pH 7.2) and incubated for 1 h at 37 °C. After removal of PI buffer, cells were incubated for an additional 30 min with an ECL (Amersham Biosciences). Confluent cells in 6-well plates were rinsed with ice-cold phosphate-buffered saline and scraped on ice in lysis buffer containing 20 ml Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and freshly added protease inhibitors mixture (Roche Applied Science). For immunoblotting of full-length receptors, 50 μg of protein per lane reduced with β-mercaptoethanol (5%) was separated on 5% SDS-PAGE gel. The proteins on the gel were electroblotted onto nitrocellulose membrane and incubated with 0.1 μg/ml of protein A-purified mouse monoclonal anti-hCaR antibody ADD (raised against a synthetic peptide corresponding to residues 214–235 of hCaR protein). Subsequently, the membrane was incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Amer sham Biosciences) at a dilution of 1:2,000. The hCaR protein was detected with an ECL (Amer sham Biosciences).

**RESULTS**

**Expression and Characterization of Naturally Occurring, Activating CaR Mutations Identified in the TM6/TM7 Junction Region—** We identified a novel heterozygous germline, nonsense mutation V836L, in a subject with ADH. To assess the functional significance of this and five previously reported ADH mutations located in this region of the CaR (12–16), we measured PI hydrolysis as a function of extracellular Ca²⁺ concentration and CaR expression by immunoblot in HEK-293 cells transfected with WT and mutant CaR cDNAs. WT and all mutant receptors showed comparable expression patterns on immunoblot (Fig. 2, right panel) consisting of a doublet shown previously (8) to correspond to an incompletely processed, high mannose intracellular form of the CaR (lower band at ~130 kDa) and a fully glycosylated, cell surface-expressed form (upper band at ~150 kDa). The V836L mutation led to increased sensitivity of the CaR to Ca²⁺ activation (EC₅₀ = 1.11 mM).
compared with WT (EC50 = 3.08 mM), as did to varying degrees the five other ADH mutations, although as seen previously for other activating mutations identified in ADH, maximal activity at higher concentrations of Ca2+ was often lower than that of WT CaR (Fig. 2, left panel, and Table I).

Expression and Characterization of Alanine-scanning Mutations of CaR Residues in the TM6/TM7 Junction Region—To evaluate the importance of other residues in the TM6/TM7 junction region in determining sensitivity of CaR to activation by Ca2+, we systematically mutated individual residues from 819–837 (apart from the six identified as sites of ADH mutations) to alanine (Ala826 was mutated to Ser) and tested their expression and activity. Five of these residues when mutated to alanine, Ile819, Ile822, Tyr825, Gly830 and Lys831, caused increased CaR sensitivity to Ca2+ (Fig. 3, left panel, and Table I). Alanine substitution for other residues in this region (Ser827, Tyr829, Val833, Ser834) and serine substitution for Ala826 caused no significant change in Ca2+ sensitivity, whereas alanine substitution for Thr828 caused a slight reduction in sensitivity.

**Table I**

| Mutation     | EC50 (mM) |
|--------------|-----------|
| WT hCaR      | 3.08 ± 0.07 |
| Naturally occurring activating mutation |            |
| S820F        | 1.25 ± 0.15 |
| F821L        | 1.42 ± 0.16 |
| A824S        | 2.22 ± 0.11 |
| F832S        | 1.45 ± 0.08 |
| A835T        | 1.74 ± 0.12 |
| V836L        | 1.11 ± 0.03 |
| Artificial activating mutation |            |
| I819A        | 2.71 ± 0.08 |
| I822A        | 2.08 ± 0.04 |
| Y825A        | 2.37 ± 0.06 |
| G830A        | 2.23 ± 0.13 |
| K831A        | 1.92 ± 0.04 |
| E837D        | 1.28 ± 0.05 |
| E837K        | 2.04 ± 0.11 |

Data shown are means ± S.E. of three to six experiments.

Fig. 3. Concentration dependence for [Ca2+]o stimulation of PI hydrolysis (left) and immunoblot (right) of CaR in transiently transfected HEK-293 cells expressing WT hCaR, I819A, I822A, Y825A, G830A, K831A, E837D, and E837K mutant hCaRs. Methods and format for presentation of results are as in the legend to Fig. 2. Each value on a curve is the mean of duplicate determinations. Results shown are representative of three separate experiments.
FIG. 4. Concentration dependence for [Ca\textsuperscript{2+}]\textsubscript{o} stimulation of PI hydrolysis (left) and immunoblot (right) of CaR in transiently transfected HEK-293 cells expressing WT hCaR, P823A, and V817I mutant hCaRs. Methods and format for presentation of results are as in the legend to Fig. 2. Each value on a curve is the mean of duplicate determinations. Results shown are representative of three separate experiments.

FIG. 5. A, concentration dependence for [Ca\textsuperscript{2+}]\textsubscript{o} stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing WT hCaR and P823A mutant hCaR with or without treatment of 1 \textmu M NPS R-568. B–D, concentration dependence for [Ca\textsuperscript{2+}]\textsubscript{o} stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing WT hCaR, single mutants (L125P, V836L, and A843E), and double mutants (L125P/P823A, V836L/P823A, and A843E/P823A). Methods and format for presentation of results are as in the legend to Fig. 2. Each value on a curve is the mean of duplicate determinations. Results shown are representative of three separate experiments.
E837K mutations did activate the receptor. Although our studies found that whereas E837A was not activating, E837D and region could also be potential sites for activating mutation, as we fictiously to alanine. Moreover, the remaining residues in this tant was not affected by NPS 2143 (Fig. 7 

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FIG. 6. Concentration dependence for [Ca\(^{2+}\)], stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing WT hCaR, E837D, and E837K mutant CaRs with or without treatment of 1 \(\mu\)M NPS 2143 (A) or 1 \(\mu\)M NPS R-568 (B). Methods and format for presentation of results are as in the legend to Fig. 2. Each value on a curve is the mean of duplicate determinations. Results shown are representative of three separate experiments.

tion of altered receptor expression as measured by immunoblot (data not shown).

Effects of a Negative Allosteric Modulator on Ca\(^{2+}\) Response of WT and Mutant CaR—NPS 2143 is a negative allosteric modulator that reduces WT CaR sensitivity to Ca\(^{2+}\) (Fig. 6A) (17). Like the positive allosteric modulator NPS R-568, NPS 2143 critically depends on Glu\(^{837}\) for its action presumably because the central amine of both compounds binds to the negatively charged glutamate (18, 19). Indeed, mutation of Glu\(^{837}\) to Asp or Lys increased sensitivity to Ca\(^{2+}\) (Fig. 3A, left panel); but whereas E837D remained subject to both activation by NPS R-568 and inhibition by NPS 2143, E837K lost responsiveness to either allosteric modulator (Fig. 6). We tested the effect of NPS 2143 on a series of other activating mutations of the CaR to determine whether they were sensitive to inhibition by the negative allosteric modulator. VFT domain activating mutants such as L125P (Fig. 7A), N124K (Fig. 7B), and K29E (not shown), as well as additional 7TM domain activating mutations such as V836L (Fig. 7C) all were inhibited by NPS 2143. In marked contrast, the constitutively activating A843E mutant was not affected by NPS 2143 (Fig. 7D).

DISCUSSION

We (20) and others (5, reviewed in Ref. 2) have identified activating CaR mutations in subjects with ADH. Unlike loss of function CaR mutations identified in subjects with familial hypocalciuric hypercalcaemia, the majority of which cause either loss of protein expression or receptor trafficking to the cell surface, missense mutations causing ADH may provide insight into the mechanism of CaR activation. Including the novel V836L mutation we identified, the 7TM domain ADH mutations include one missense mutation each in TM1 and TM3, none in TM2 and TM4, two in TM5, and four each in TM6 and TM7. Of the latter, six cluster at the junction of TM6 and TM7 (Ile\(^{819}\)–Glu\(^{837}\)). In addition to these naturally occurring activating mutations, we showed that five other residues in this region increase sensitivity to activation by Ca\(^{2+}\) when mutated artifically to alanine. Moreover, the remaining residues in this region could also be potential sites for activating mutation, as we found that whereas E837A was not activating, E837D and E837K mutations did activate the receptor. Although our studies of course do not exclude a role for other parts of the 7TM domain, these results suggest that a small part of this domain, i.e. Ile\(^{819}\)–Glu\(^{837}\), involving the extracellular surface proximal portions of TM6 and TM7 may play a key role in CaR activation.

Current models for GPCR activation are based largely on the crystal structure of bovine rhodopsin (21), 11-cis-retinal is covalently bound to Lys\(^{296}\) in TM7 and is bracketed by residues in TM3 and TM6. 11-cis-retinal and a series of helix-helix contacts involving primarily TM1–4 keep the 7TM domain in an inactive conformation. How photon-mediated retinal isomerization promotes the active rhodopsin conformation has not been clearly defined, but a recent NMR study suggests that “rigid body” motion of TM6 is a key element in the activation mechanism (22). A proline highly conserved in family 1 GPCRs, Pro\(^{267}\) in TM6 (6.50 in Ballesteros numbering scheme (23)), acts as a flexible hinge for rotation of TM6.

Given the very limited sequence homology between the 7TM domains of family 1 versus family 3 GPCRs, one should be cautious in extrapolating from the rhodopsin three-dimensional structure to that of family 3 GPCRs. Nonetheless, it is interesting to speculate that a similar rotation of TM6 in the CaR acts as part of a “switch” mechanism upon receptor activation. A key role for Pro\(^{267}\) in such a TM6 rotation is suggested by the effects of the P823A mutation. Despite good expression of the mutant receptor, its ability to be activated by Ca\(^{2+}\) is drastically reduced. A positive allosteric modulator enhanced the Ca\(^{2+}\) response of the mutant receptor but not to WT levels. Further, the P823A mutation blocked or reduced the enhanced Ca\(^{2+}\) response of activating VFT or TM6 mutations. Pro\(^{267}\) is highly conserved in family 3 GPCRs but is in a slightly different location (6.53 rather than 6.50) than the proline in TM6 highly conserved in family1 GPCRs. The study of prolines and TM helix kinks in GPCRs by Yohannon et al. (24) suggested that Pro to Ala mutations often do not impair structure or function because of compensatory evolutionary changes in residues in other helices. This does not appear to be true for Pro\(^{267}\) in the CaR, and we suggest that mutation of this residue in other family 3 GPCRs would have similar consequences. The P823A mutation had qualitatively, if not quantitatively, similar effects as the CaR negative allosteric modulator, NPS 2143. Both blocked the effects of agonist and of activating CaR mutations but had little if any effect on the A843E mutation that uniquely among ADH mutations causes constitutive activation (25).
We and others recently found by alanine substitution that residue Glu\textsuperscript{837} is critical for the action of allosteric modulators of the CaR with a phenylalkylamine structure, such as NPS R-568 and NPS 2143 (7, 18, 19). It was speculated that a critical salt bridge might form between the negatively charged glutamate and the positively charged central amine in these compounds. We report here the study of E837D and E837K mutants providing direct evidence that the negative charge at residue 837 is crucial for the response of the CaR to both NPS R-568 and NPS 2143. Such responsiveness was abolished when Glu\textsuperscript{837} was substituted by either a neutral or positively charged residue.

How agonist-induced VFT closure and dimer rotation leads to activation of the 7TM domain of family 3 GPCRs remains unclear. Our results, however, lead us to speculate that the TM6/TM7 junction region (Ile\textsuperscript{819}–Glu\textsuperscript{837}) plays a critical role in maintaining the CaR in its inactive state. Mutation of many of the residues in this region, as well as the action of positive allosteric modulators binding to Glu\textsuperscript{837} (such as NPS R-568), reduces the inhibitory constraints in the 7TM domain, thus enhancing sensitivity to Ca\textsuperscript{2+} activation. In contrast, negative allosteric modulators which also bind to Glu\textsuperscript{837} (such as NPS 2143) and the P823A mutation stabilize the inactive conformation of the CaR perhaps by impeding rotation of part of TM6. We suggest that the relative insensitivity of the A843E mutant to inhibition by NPS 2143 or by superimposing the P823A mutation reflects the more “distal” location of this mutation in TM7. The A843E mutation may cause a structural change in the CaR intracellular domain that directly leads to constitutive G protein activation and is less susceptible to constraints caused by changes elsewhere in the 7TM domain.

A final point worth noting is the ability of NPS 2143 to inhibit the activity of all ADH mutations tested with the exception of A843E. ADH mutations, with the exception of the constitutively activating A843E, cause hypocalcemia and inappropriate inhibition of parathyroid hormone secretion and re-

**FIG. 7.** Concentration dependence for \([\text{Ca}^{2+}]_o\) stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing L125P (A), N124K (B), V836L (C), and A843E (D) mutant hCaRs with or without treatment of 1 μM NPS 2143. Methods and format for presentation of results are as in the legend to Fig. 2 except that the maximal response is L125P at 30 mM (A), N124K at 4 mM (B), V836L at 4 mM (C), and A843E at 30 mM (D) without NPS 2143. Each value on a curve is the mean of duplicate determinations. Results shown are representative of three separate experiments.
nal Ca\textsuperscript{2+} reabsorption by increasing the Ca\textsuperscript{2+} sensitivity of the CaR expressed in parathyroids and kidney. Conventional treatment of ADH using vitamin D and calcium supplements is unsatisfactory because of the resultant hypercalciuria leading to nephrolithiasis. Negative allosteric modulators such as NPS 2143 offer the possibility of more "physiologic" correction of the molecular defect in ADH by decreasing the sensitivity of the CaR to Ca\textsuperscript{2+}, enhancing PTH secretion and renal Ca\textsuperscript{2+} reabsorption. Negative allosteric CaR modulators are currently under study for possible use in treatment of osteoporosis (26), but further studies in animal models and eventually in humans may be warranted to evaluate such agents for a new therapeutic indication, correction of hypocalcemia in subjects with ADH.

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